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Separation from self explains failure of circulating T-cells to respond to the CD28 superagonist TGN1412

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Stimulatory or superagonistic (SA) CD28-specific monoclonal antibodies (mAbs) are potent polyclonal activators of regulatory T cells and have proven highly effective as treatment in a wide range of rodent models for autoimmune and inflammatory diseases. In these models, a preferential activation of regulatory T cells was observed by in vivo administration of CD28SA. In stark contrast, human volunteers receiving TGN1412, a humanized CD28-specific mAb, experienced a life-threatening cytokine release syndrome during the first-in-man trial. Preclinical tests employing human peripheral blood mononuclear cells (PBMC) failed to announce the rapid cytokine release measured in the human volunteers in response to TGN1412.

Our aim was to find an explanation of why standard PBMC assays failed to predict the unexpected TGN1412-induced "cytokine storm" observed in human volunteers. CD28 superagonists can activate T cells without T cell receptor (TCR) ligation. They do depend however on “tonic” TCR signals received by MHC scanning, signals that they amplify. PBMC do not receive these signals in the circulation. Short-term in vitro preculture of human PBMC at a high cell density (HDC) resulted in massive cytokine release during subsequent TGN1412 stimulation. Restoration of reactivity was cell-contact dependent, associated with TCR polarization and tyrosine-phosphorylation, and blocked by HLA-specific mAb. In HDC, both CD4 T cells and monocytes functionally mature in a mutually dependent fashion. CD4 memory T-cells proliferate upon TGN1412 stimulation, and were identified as the main source of pro-inflammatory cytokines. Importantly, responses to other T-cell activating agents were also enhanced if PBMC were first allowed to interact under tissue-like conditions.

A new in vitro protocol is provided that returns circulating T-cells to a tissue-like status where they respond to TGN1412 stimulation. This method might fill out the gap between in vitro and animal testing, representing a more reliable preclinical in vitro test for both activating and inhibitory immunomodulatory drugs.
Microglia emerge from erythromyeloid precursors via Pu.1- and Irf8-dependent pathways

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Microglia are crucial for immune responses in the brain. Although their origin from the yolk sac has been recognized for some time, their precise precursors and the transcription program that is used are not known. We found that murine microglia were derived from primitive c-kit⁺ erythromyeloid precursors that were detected in the yolk sac as early as 8 d post conception. These precursors developed into CD45⁺ c-kit⁺ CX₃CR₁⁺ immature (A1) cells and matured into CD45⁺ c-kit⁻ CX₃CR₁⁺ (A2) cells, as evidenced by the downregulation of CD31 and concomitant upregulation of F4/80 and macrophage colony stimulating factor receptor (MCSF-R). Proliferating A2 cells became microglia and invaded the developing brain using specific matrix metalloproteinases. Notably, microgliogenesis was not only dependent on the transcription factor Pu.1 (also known as Sfpi), but also required Irf8, which was vital for the development of the A2 population, whereas Myb, Id2, Batf3 and Klf4 were not required. Our data provide cellular and molecular insights into the origin and development of microglia.
Intestinal lymphoid follicles such as cryptopatches and isolated lymphoid follicles (ILFs) are lymphoid organs that develop postnatally under the influence of the intestinal microflora and other as of yet unidentified cues. ILFs are important places of T cell-independent IgA production which is a prerequisite for the protection of epithelial surfaces. In the small intestine, ILFs develop from cryptopatches which are organized structures of approximately 1000 cells located underneath the crypts of the small intestine. The cells forming cryptopatches are RORγt (retinoic acid orphan receptor γ t)-expressing innate lymphoid cells (ILCs) believed to have lymphoid tissue inducing (LTi) function. Furthermore, RORγt+ ILCs are an important source of interleukin-22, a cytokine essential for the defense against bacterial pathogens. In this study, I investigated the role of the aryl hydrocarbon receptor (AhR) for development and function of RORγt+ ILCs. Mice genetically lacking AhR had substantially reduced numbers of RORγt+ ILCs and failed to develop cryptopatches and ILFs. In contrast to Ahr-proficient mice, RORγt+ ILCs from mice lacking the AhR had very low levels of Kit expression. Kit is known to promote cell survival and proliferation in various cell types and Kit was recently reported to be required for the development and/or maintenance of intestinal RORγt+ ILCs. Interestingly, dietary AhR ligands were found to control the pool size of RORγt+ ILCs by maintaining the high Kit expression. In addition, consistent with the failure to expand the IL-22 producing RORγt+ ILCs, AhR-deficient mice were highly susceptible to Citrobacter rodentium infection, a mouse model of attaching and effacing infections such as those with enterohemorrhagic E. coli strains. Collectively, the data shows that AhR signals are indispensable for the expansion and/or maintenance of RORγt+ ILC with LTi function required for the postnatal formation of intestinal lymphoid follicles.
Thymus-autonomous T cell development

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T lymphocytes develop from progenitors continuously migrating from the bone marrow to the thymus. To address the fate of a thymus in the complete absence of developmentally competent bone marrow progenitors, we have transplanted normal wild type thymus into Rag2-/-gc-/-KitW/Wv mice. Compound mutant Rag2-/-gc-/-KitW/Wv mice lack competitive hematopoietic stem cells (HSC) and are devoid of T cell progenitors. Using this strain as recipient for wild type thymus grafts, we unexpectedly found that thymocytes persisted and T lymphocytes continued to be produced and exported for several months. This implies that the thymus contains cells that are capable of self-renewal. Moreover, sequencing of the expressed alpha and beta TCR loci in progenitor-deprived thymus grafts shows that the TCR repertoire is still diverse. Thymus transplantation into single mutants uncovered that gc-mediated signals play a key role in the competition between thymus-resident and bone marrow-derived progenitors. Taken together, our data indicates that the turnover of each generation of thymocytes is not only based on short life span but is also driven via expulsion of resident thymocytes by fresh progenitors seeding the thymus.
Lipid oxidation at the interface of innate and adaptive immunity

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Enhanced lipid oxidation by 12/15-lipoxygenase (12/15-LO) promotes the progression of chronic inflammatory diseases such as atherosclerosis. However, physiological implications of such enzymatic lipid oxidation processes have remained incompletely understood. Recent data from our laboratory highlight an important, but so far neglected role of 12/15-LO-mediated lipid oxidation during the regulation of the innate and adaptive immune response.

Our data show that 12/15-LO, which catalyzes the oxidation of free and esterified fatty acids, contributes to the generation of various lipid oxidation products including oxidized phospholipids and different classes of eicosanoids. These 12/15-LO-derived compounds act as bioactive lipid mediators and regulate leucocyte activation and migration. Thereby, 12/15-LO orchestrates the resolution of inflammatory processes as well as the clearance of apoptotic cells within inflammatory infiltrates. Furthermore, 12/15-LO is critically involved in the regulation of T-cell differentiation and the maintenance of tolerance to apoptotic cell-derived autoantigens.
IS 6
“Sein und Dasein” in lympho-hematopoietic differentiation.

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The development of B lymphoid lineage cells from the earliest embryonic progenitors to pluripotent hematopoietic stem cells, multipotent myeloid/lymphoid progenitors, common lymphoid progenitors and precursor B cells to immature and mature B lymphocytes is influenced and controlled by cell interactions of hematopoietic cells at given stages of development with other hematopoietic, and with non-hematopoietic cells, forming specialized microenvironments. In turn, these cell-to-cell interactions, mediated by chemokines and cytokines and their receptors, and by cell contacts, induce intracellular signalling reactions, which change the controls of expression of genes, leading to changes in proliferation, survival, differentiation and migration of these cells. Examples that illuminate these molecular and cellular programs and their flexibilities in the process of B cell development will be given.
Role of Interleukin-6 in Autoimmunity

S. Rose-John

Cytokine receptors exist in membrane bound and soluble form. The IL-6/soluble IL-6R complex stimulates target cells not stimulated by IL-6 alone, since they do not express the membrane bound IL-6R. We have named this process 'trans-signaling'. Soluble gp130 is the natural inhibitor of IL-6/soluble IL-6R complex responses. Recombinant soluble gp130 protein is a molecular tool to discriminate between gp130 responses vs membrane bound and soluble IL-6R responses. We used neutralizing monoclonal antibodies for global blockade of IL-6 signaling and the sgp130Fc protein for selective blockade of IL-6 trans-signaling in several animal models of human autoimmune and infectious diseases. Inhibition of IL-6 trans-signaling was beneficial in a sepsis model. Defense against bacterial infections rely on the membrane bound IL-6R. The extent of inflammation is controlled by the release of the soluble IL-6R, which is mediated by the protease ADAM17. Using the sgp130Fc protein or sgp130Fc transgenic mice we demonstrate in animal models of inflammatory bowel disease, peritonitis, rheumatoid arthritis, atherosclerosis pancreatitis, colon cancer, ovarian cancer and pancreatic cancer, that IL-6 trans-signaling via the soluble IL-6R is the crucial step in the development and the progression of the disease. Therefore, sgp130Fc is a novel therapeutic agent for the treatment of chronic inflammatory diseases and cancer and it currently undergoes phase I clinical trials as an anti-inflammatory drug.


Keywords: interleukin-6, soluble receptor, trans-signaling, ADAM17, inflammation
T cells migrate through the body and communicate with other hematopoietic or tissue-resident cells. In each new environment, T-cell surface receptors have to interact with ligands on other cells or the extracellular matrix. In addition, T cells must adapt to the surrounding micromilieu, for instance alterations in the redox milieu. Physiologically, a pro-oxidative milieu exists in the gut. Pathologically, it occurs during inflammation where activated granulocytes and macrophages release reactive oxygen species. In contrast, dendritic cells can provide a reducing milieu upon antigen presentation. We identified that the micromilieu controls the activity of T cells via fine tuning of actin dynamics. High flexibility of the entire cell body is a prerequisite for T cell-mediated immune surveillance. The actin cytoskeleton plays a central role for these shape changes. It provides a scaffold for protein clustering and signal transduction and serves as an engine that generates physical forces important for T cell activation and migration. Cofilin is an actin binding protein that depolymerizes and/or severs actin filaments. This dual function of cofilin makes it to one major regulator of actin dynamics. The activity of cofilin is spatio-temporally regulated. Phosphorylated cofilin is inactive and represents the dominating cofilin fraction in the cytoplasm of resting human T cells. In addition, a fraction of dephosphorylated cofilin is kept inactive at the plasma membrane by binding to PI(4,5)P2. Costimulation via the TCR/CD3 complex (signal 1) together with accessory receptors (signal 2) or triggering through the chemokine SDF1α induce Ras-dependent dephosphorylation and thereby activation of cofilin important for immune synapse formation, T cell activation and T cell migration in 3D-environments.

Only recently, it became evident that cofilin is highly sensitive for microenvironmental changes, particularly for alterations in the redox milieu. Cofilin is inactivated by oxidation provoking T cell hyporesponsiveness or necrotic-like programmed cell death. In contrast, in a reducing environment even PI(4,5)P2-bound cofilin becomes active leading to actin dynamics in the vicinity of the plasma membrane. This microenvironmental control of the actin reorganizing protein cofilin delivers a modulating signal for T cell-dependent immune reactions.
Human memory B cells (Note: DGfI Invited Speaker)

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There is strong indication that the human memory B cell compartment not only encompasses class-switched memory B cells, but also a large subset of IgM+IgD+CD27+ B cells. By performing V gene sequence analysis of sorted memory B cell subsets, we previously validated an origin of class-switched and IgM+IgD+CD27+ memory B cells from common germinal center B cell clones for several memory B cell clones. This analysis is now being extended by performing deep sequencing of rearranged IgV genes from human memory B cell subsets. To gain further insight into the distinct features and functions of IgM+IgD+ and class-switched memory B cells, we performed global gene expression profiling studies of such cells and revealed numerous differentially expressed genes. The role of these differences in gene expression for the specific immunological functions of the distinct memory B cell subsets is now being tested in vitro by functional assays.
IS 10

Functional relevance of the dynamics of T cell-dendritic cell interactions

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During clonal selection, direct interactions between antigen presenting dendritic cells (DCs) and naive T lymphocytes allow engagement of the TCR by its cognate ligand. Stable T cell-DC interactions correlate with the induction of effective T cell activation, expansion, effector functions and memory, while the induction of brief, unstable contacts result in T cell activation, poor expansion and no memory. Both the number of peptide/MHC (pMHC) complexes present and the affinity of the TCR for these complexes, together with adhesion between the two cells, play critical roles in the formation of stable conjugates, and thereby on the onset of effective immune responses. Regulatory T cells (Tregs) were shown in the past few years to control the stability of DC-T cell contacts in vivo. Using 2-photon dynamic imaging, we analyzed the mechanisms of control of the stability of the DC-T cell interactions by Tregs. We show that Tregs control of the dynamics of the initial DC-T cell contacts, and that the avidity of the TCR for the pMHC complex influences the capacity of Tregs to disrupt stable DC-T cell conjugates. In the absence of Tregs, CCL-3, -4 and -5 chemokines are overproduced by antigen presenting dendritic cells, leading to stabilization of conjugates with low avidity CD8+ T cells. The resulting enhanced activation and expansion of low avidity T cells reduces the overall avidity of primary CD8+ T cell responses. In mice infected with Listeria monocytogenes, selective inhibition of the low avidity T cells by Tregs is required for optimal high avidity T cell responses and for the establishment of effective memory.
Identification of a kidney-specific chemokine receptor - implications for glomerulonephritis therapy

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The chemokine receptor CX₃CR1 is widely used to identify dendritic cells (DCs) and macrophages, but few functional roles are known. We found that CX₃CR1-deficient mice possess markedly reduced DC numbers in the healthy and inflamed kidney, but not in other organs. This was possibly due to the strong expression of its ligand, CX₃CL1, in this organ. Crescentic glomerulonephritis, a DC-dependent aggressive type of kidney inflammation, was strongly attenuated in CX₃CR1-deficient mice. As an underlying reason, we found that kidney DCs in the kidney cortex processed antigen for the intrarenal stimulation of T helper cells, a function important for glomerulonephritis progression. In contrast, kidney medullary DCs were specialized at inducing innate immunity against bacterial pyelonephritis. CX₃CR1-deficiency had little effect on the immune defense against this infection, because medullary DCs were less CX₃CR1-dependent than cortical DCs, and because recruited neutrophils produced chemokines to compensate for the DC paucity. These findings identify CX₃CR1 as a potential therapeutic target in glomerulonephritis that may entail fewer adverse side-effects such as impairing anti-infectious defense or compromising DC functions in other organs.
The German Research Foundation (DFG), the main German third-party public funding organization geared towards basic research, offers a full set of funding opportunities to junior and senior scientists. An experienced program director will give an introduction to the major funding instruments available and elucidate on the framework conditions to obtain funding. Additionally, advice on criteria for successful proposals will be given. Participants will be advised on the writing of a promising application and about frequent mistakes that can be avoided. Insight on the current funding situation including recent funding rates will be given.
There’s more to publishing than you think - new policies at EJI

C. Livingstone

Wiley-VCH Verlag GmbH & Co. KGaA, Managing Editor EJI, Weinheim, Germany

Publishing continues to evolve with the open access movement, initiatives such as the San Francisco Declaration on Research Assessment and the desire of scientists to have greater involvement as highlighted by the quote “by scientists, for scientists” being examples of recent developments. The new policies introduced by the editorial team of the European Journal of Immunology (EJI), headed by Professor Andreas Radbruch, to address these issues will be discussed, offering you a chance to discover the benefits of publishing in EJI.
Evolution of pathogenic anti-dsDNA antibodies in SLE

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The generation of autoantibodies against nuclear antigens is considered to be the serologic hallmark of systemic lupus erythematosus (SLE). Anti-DNA antibodies play an important role in the pathogenesis of the disease. Anti-DNA antibodies arise by somatic mutation, as has been shown for several autoimmune mouse strains and also for SLE patients in our previous work. Importantly, we have shown that antibodies in which somatic mutations were reverted to germ-line V regions did not show any measurable autoreactivity, suggesting that anti-DNA autoantibodies develop from non-autoreactive B-cells by somatic hypermutation, presumably during the germinal center reaction. Therefore, self-tolerance checkpoints at the postmutational stage of B cell differentiation have to exist that normally prevent the induction of pathogenic anti-DNA, as the random nature of somatic hypermutation will inevitably and frequently create autoreactive B cells. The tolerance mechanisms that operate at the post-mutational stage are not known and might be the key for our understanding of the etiology of autoantibodies in systemic autoimmunity.

To analyze potential germinal-center tolerance mechanisms in the context of anti-DNA autoantibodies, we created a mouse model in which an antibody of a patient-derived anti-dsDNA hybridoma, in which all somatic mutations were reverted, is expressed as B cell receptor. As expected from our previous work, B cells expressing the revertant B-cell receptor are developing normally and show no evidence for tolerization. There is no evidence for receptor editing detectable. Upon immunization with a surrogate phage antigen the transgenic B cells form germinal centers and undergo somatic hypermutation. The three critical somatic hypermutations, which are necessary and sufficient for the acquisition of high affinity anti-dsDNA binding, were not observed in a large collection of sequences analyzed. In accordance with this, anti-DNA autoantibodies do not develop, even after repetitive immunizations and when FDCs were MFG-E8 deficient. These results strongly suggest a self-tolerance checkpoint by deletion of autoreactive clones during or after the germinal center reaction.
IS 15

Autoimmunity to posttranslational modifications

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In tissue-specific and MHC class II associated autoimmune diseases autoreactive lymphocytes are aberrantly activated. In several of these diseases, including type I diabetes and rheumatoid arthritis it is realized that the breakage of tolerance is to directed to neo-epitopes created by posttranslational modifications of proteins. It has also been realized that the autoimmunity is built up many years before the clinical onset of disease. It’s not pathogenic but predicts pathogenicity. I will discuss recent progress in rheumatoid arthritis and animal models of rheumatoid arthritis in which immune responses to several posttranslational modifications plays an important role. It includes recognition of citrullinated and glycosylation epitopes on proteins occurring systemically as well as in the joints.
Long-term single cell quantification: New tools for old questions in stem cell research

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Stem cell systems are highly complex and dynamic, and consist of large numbers of different cells expressing many molecules controlling their fates. Despite intensive research, many long-standing questions in stem cell research remain unsolved. One major reason is the fact that it is usually followed by analyzing the fate of populations of cells rather than individual cells at very few time points of an experiment, and without knowing their individual identities. Real-time tracking of individual cells in culture, tissues or whole organisms would be an extremely powerful approach to fully understand the developmental complexity of this stem cell driven regeneration. We are therefore developing culture and imaging systems to follow the fate of individual cells over long periods of time. New software is programmed, helping to record and display the divisional history, position, properties, interaction etc. of all individual cells in a culture over many generations. Our approaches also allow the continuous long term quantification of protein expression levels or activity in living cells. This novel kind of quantitative data of single cell behavior and molecule expression is used as the basis for the improved generation and falsification of models describing the molecular control of stem cell fates. I will discuss how we use these approaches to try to find answers for long standing questions in hematopoiesis and pluripotency research.
RNA interference and RNA binding proteins may have evolved as an ancient innate immune response to viral RNA. In eukaryotes these mechanisms have been developed into a powerful system of post-transcriptional gene regulation. The Roquin family proteins bind to characteristic stem-loop structures of mRNAs and interfere with their expression. The proteins play an essential role in the adaptive immune system and a point mutation of Roquin-1 in the mouse has been shown to lead to autoimmune disease and T cell lymphoma. Our recent work has established that Roquin-1 and Roquin-2 proteins serve redundant functions in the prevention of autoimmunity. The proteins control T cell biology through post-transcriptional regulation of ICOS and Ox40 costimulatory receptor mRNAs. These are well established targets among a number of other potential candidate mRNAs. Loss of Roquin-1 and Roquin-2 in the mouse leads to spontaneous T cell activation and follicular helper T cell differentiation. Our current work focuses on the molecular mechanism of Roquin-1/2 function and asks how these proteins are regulated.
A central function of our innate immune system is to sense microbial pathogens by the presence of their nucleic acid genomes or their transcriptional or replicative activity. In mammals, a receptor-based system is mainly responsible for the detection of “non-self” nucleic acids. In the past years tremendous progress has been made to identify host constituents that are required for this intricate task. With regard to the detection of RNA species, a picture is emerging that certain families of the toll-like receptor family (TLR-7, -8 and -13) and the RIG-I like helicases (RIG-I and MDA5) respond to microbial RNA molecules. Moreover, TLR9 detects microbial DNA within the endolysosomal compartment. At the same time, the presence of intracellular DNA can also trigger potent innate immune responses, yet the relevant players in this field are only now becoming clear. In this talk an update is given on our latest progress on intracellular DNA sensing mechanisms of the innate immune system and its role in health and disease.
Molecular adaptations of Th lymphocytes to chronic inflammation

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T helper (Th) lymphocytes can initiate and drive chronic inflammatory immunopathology. Obviously they escape physiological immunoregulation, persist and contribute to refractoriness of chronic inflammatory diseases to available therapies. To understand the molecular mechanisms underlying the transition of an acute, self-limiting T cell response to a chronic pathogenic effector/memory response, we have analysed the molecular adaptation of Th cells to repeated restimulation with (self)antigen. We have compared the global gene expression of Th cells of the Th1, Th2 and Th17 lineages, activated just once or repeatedly reactivated, before and after restimulation. Among the genes differentially expressed, the genes Twist1 and Hopx are upregulated selectively in proinflammatory Th1 cells upon repeated restimulation. Twist1 encodes an E-box binding transcription factor and is highly expressed in Th cells isolated from inflamed tissue of patients with rheumatic or gastrointestinal inflammation, and restimulated ex vivo. In Th cells, twist1 expression is induced by Stat4, NFAT and NFkB. Knock-down of Twist1 in Th1 cells of murine models of arthritis exacerbates chronic inflammation. Thus Twist1 limits immunopathology. But it also promotes the survival of Th1 cells, since it induces microRNA-148a, which regulates expression of Bim. Hopx expression in Th cells is dependent on T-bet, and it promotes the survival of Th1 cells. shRNA-mediated downregulation of Hopx in Th1 cells reduces their ability to persist in vivo, following adoptive transfer. Th1 cells lacking Hopx do not induce inflammation in experimental colitis or arthritis. Thus, Twist1 and Hopx, and the genes they control, qualify as markers of Th1 cells driving chronic inflammation, they adapt the Th cells to chronicity, regulate their function and persistence, and may serve to target therapies to pathogenic Th lymphocytes.
Immune cell signaling: the Tespa1 connection

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Tespa1 (thymocyte-expressed, positive selection-associated 1) is a newly identified adaptor protein that is expressed in T cells, B cells and mast cells. Our previous study revealed its critical role in mediating TCR signaling and thymocyte positive selection. Tespa1-deficient mice have fewer mature CD4+ and CD8+ SP cells in thymus, reflecting the impairment of T cell development in these mice. We further revealed that Tespa1 interacts with Lat signolosome components Grb2 and PLC-γ1 and is essential for the assembly of this signolosome and mediating of downstream signals. Tespa1 is also highly expressed in Mast cells. We thus further explored the role of Tespa1 in Mast cell activation. We found that disruption of Tespa1 causes mast cells to be hyperresponsive upon the stimulation of FceRI receptor, evidenced by enhanced degranulation, cytokine production, and their ability to mediate anaphylaxis. We also found that more Grb2 and PLC-γ1 were recruited to the LAT1 signolosome rather than LAT2 signolosome, suggesting Tespa1 might regulate the balance of LAT1 and LAT2 signolosome assembly and downstream distal signaling transduction in mast cells.
Dendritic cells (DC) are strategically positioned at epithelial borders to the environment including the lungs. Here, they are specialized to detect invading pathogens and initiate a protective immune response. At the same time, DC exert a pivotal regulatory function to prevent inappropriate immune activation against harmless foreign and self-antigens. Asthma is an allergic inflammatory disease predominantly mediated by T helper (Th) type-2 lymphocytes and characterized by bronchial constriction and airway infiltration with a plethora of inflammatory cells. This deleterious reaction results from a loss of tolerance towards innocuous environmental antigens (allergens) and DC are both necessary and sufficient to induce allergic asthma.

While we have a detailed understanding of the pro-inflammatory signals that drive immunogenic activation of DC, the factors that control their regulatory phenotype are less clearly defined. I will discuss our current work to unravel the role of β-catenin, the central component of the canonical Wnt signaling pathway, and the well-known immune-regulatory cytokine IL-10 to govern DC function in vivo, i.e. in the steady-state and, in particular, during a mouse model of allergic asthma.
Liver is an unique immune organ with predominant innate immunity and characterized with very strong immunotolerant features. Understanding the innate immune features and mechanisms of immune tolerance of the livers are helpful to explore the therapeutic approaches to liver diseases including viral hepatitis and hepatocellular carcinoma. One of the particular features of liver is its high content of NK cells, for which we found a big subpopulation of hepatic NK cells which was almost not existed in other immune organs. This liver-specific NK cells are CD3$^-$CD122$^+$DX5$^-$CD49a$^+$ and characterized with many other features than those from DX5$^+$CD49a$^-$ NK cells in liver, spleen, lung, thymus, lymph nodes, blood and bone marrow. Recently, a study group found hepatic NK cells had a potential with adaptive immunity in contact hypersensitivity (CHS) mice, but the phenotypic feature is not clear. Interestingly, we found this liver-specific NK cell was able to deliver the memory of adaptive immunity in CHS wild type mice or RAG2$^{-/-}$ mice. The mechanisms underlying the liver-specific NK cells-delivered adaptive immunity is studied.
Eosinophil and basophil responses to allergens and helminths

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Eosinophils and basrophils are generally associated with type 2 immune responses which unfold in response to allergens and infections with helminths. Both cell types are loaded with potent effector molecules and produce a large variety of lipid mediators, chemokines and cytokines including IL-4, IL-5 and IL-13 suggesting that they play an important role as modulators and effectors of type 2 immunity.

Our work on murine eosinophils revealed that they develop in distinct stages in the bone marrow and have a life span of about 36 hours which increases in response to infection with the helminth N. brasiliensis. Activated eosinophils were found in the lung but also in the lymph node where they accumulated in the subcapsular sinus and T cell zone. Eosinophils were actively recruited to the peritoneal cavity where they survived for several days. By selective deletion of IkB α in eosinophils we found that NFκB-regulated genes promote survival during N. brasiliensis infection. Eosinophil-deficient mice showed normal Th2 polarization and worm expulsion during primary and secondary infection.

We further developed a mouse strain that constitutively and selectively lacks basophils (Mcpt8Cre mice). Basophils appeared to be dispensable for Th2 polarization and IgE production in response to allergens or helminths. Passive IgE- or IgG1-mediated systemic anaphylaxis was also normal in Mcpt8Cre mice. However, IgE-mediated chronic allergic inflammation of the skin and protective immunity against secondary infection with gastrointestinal nematodes was impaired in Mcpt8Cre mice. This indicates that basophils can be beneficial for protection against helminths but also detrimental during chronic allergic reactions.
Linking cancer exomes to immunotherapy

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There is now very strong evidence that human cancers, and in particular melanoma, can be recognized and destroyed by T lymphocytes. As a first example, ipilimumab, an antibody that abrogates the function of the T cell checkpoint molecule CTLA4, has now been approved for the treatment of metastatic melanoma, and blockade of the PD1 - PD-L1 axis appears even more effective. As a second example, infusion of \textit{ex vivo} expanded tumor-infiltrating T cells has shown clear effects in phase I/II trials in a number of clinical centers. An important gap in our understanding of these therapies is however which antigenic determinants on cancer cells are the targets in T cell-mediated tumor destruction. Knowledge of such antigens would be of obvious value, as it would allow one to move towards approaches in which tumor-specific T cell activity is boosted in a more directed manner.

Towards this goal, we have first developed technologies for high-throughput analysis of T cell reactivity that are based on conditional MHC ligands that can be exchanged for epitopes of interest by light exposure. We have subsequently used these technologies to analyze patterns of reactivity in clinical samples, in particular in melanoma.

Here I will discuss how the combination of cancer exome sequencing and immunomonitoring may be used to identify T cell reactivity against the patient-specific neo-antigens that are formed by mutations. The ability to describe patient-specific tumor-reactive T cell responses in an accurate manner should form a first step towards the development of personalized cancer immunotherapy. Potential approaches for such personalized immunotherapy, as well as their possible relevance for other human cancers, will be discussed.
IS 25
Molecular and metabolic control of adaptive immunity by tumor-induced myelopoiesis

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The clinical inefficiency of cancer immunotherapy is in part due to the presence of an immunosuppressive network that favors tumor progression; in fact, by producing soluble molecules such as cytokines, interleukins and growth factors, tumors induce an alternative hematopoiesis, both at medullary and extra-medullary sites such as the spleen, which modifies the normal myeloid cell differentiation, pushing proliferation and expansion of cells with immunosuppressive function called myeloid-derived suppressor cells (MDSCs). The MDSC presence and frequency in blood of tumor patients is more and more often reported as a negative prognostic marker that correlates with the clinical outcome and response to therapy, comprising both conventional chemotherapy and immunotherapy. These cells use distinctive and redundant pathways to suppress the proliferation and function of antigen-stimulated T lymphocytes, including the metabolism of the semi essential amino acid L-arginine. Although MDSCs are heterogeneous, it appears that three main immunosuppressive cell subsets can be identified: granulocytic, monocytic and more immature cells, which might be able to originate the other two subpopulations. Interestingly, a linear differentiation pathway from more immature elements to monocyte and hence granulocytes seems to be peculiar of tumor-induced myelopoiesis. Our knowledge about the metabolic mechanisms used by MDSCs to restrain adaptive and innate immunity, the possibility to generate MDSCs by in vitro culture of bone marrow precursors, the definition of transcription factors and microRNAs regulating their in vivo expansion and maturation allow to envisage effective and innovative strategies to modify the tumor macro- and micro-environment and sustain an increased efficacy of cancer immunotherapy.
Intestinal Microbiota Dynamically Modulate the IgA Repertoire

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The gut microbiota affects susceptibility to infection and inflammatory diseases and shapes the development of the immune system. Conversely, the host immune system influences the intestinal microbiota through a multi-layered system of innate and adaptive immune responses, most prominently the production of secretory immunoglobulin A (SigA). SigA shields the gut epithelium from the microbiota and thereby preserves intestinal homeostasis and epithelial function. How the IgA system adapts to dynamic changes in the microbiota and how stable host-microbiota interaction are achieved remains unknown. Here we show in mice and humans that changes in the microbiota were accommodated in the IgA repertoire primarily at the level of somatic mutations and to a lesser extent by the recruitment of new B cell clones. IgA diversity increased upon mono-colonization of germ-free mice but a complex flora was required to generate highly diverse IgA repertoires. Thus, the IgA repertoire in the gut permanently adapts to closely match the intestinal microbiota and this ready-made set of B cell clones can also fuel the plasma cell population in mammary glands. We propose that a confined clonal architecture of the IgA repertoire may contribute to microbiota resilience and stabilize a symbiotic host-microbe relationship in the gut in mice and humans.
A new autoimmune hepatitis model in genetically predisposed individuals, initiated by environmental factors and driven by CD4+ T cells

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Autoimmune hepatitis (AIH) is a chronic autoimmune inflammation of the liver eventually leading to cirrhosis if left untreated. The disease is normally recognized during advanced stages of disease, therefore knowledge about onset and course of disease is very limited. 80-90% of patients can be treated with a life-long immunosuppression. Unfortunately there are strong drug related side effects and steroid-refractory patients. Therapeutic intervention is restricted to steroids with potentially severe side-effects. This explains the desperate need for a good preclinical animal model, reflecting main characteristics of AIH in patients.

The most important findings about AIH and patients with the disease can be grouped in clinical, histological and laboratory criteria. Beside sex and age, important genetic factors for predisposition autoimmunity are the MHC molecules. There is a strong link to HLA-A1, -B8, -DR3 and -DRB1 to AIH. Other polymorphisms of immunomolecules like CTLA-4, CD45 and TNF-a have also been described. Next to genetic predisposition, environmental factors have been postulated for their role in the development of AIH. But due to the temporal discrepancy between onset and recognition of the AIH, the knowledge is limited. So far, No environmental agent could be linked to the induction of AIH in a large number of patients.

Histological features for AIH in liver biopsies are lymphocytoplasmatic, periportal and intralobular infiltrates as well as interface hepatitis, while viral hepatitides and liver diseases like PBC and PSC have to be excluded. The hepatic inflammation is T-cell mediated sometimes described as TH1-triggered. Next to histopathology, diagnosis of AIH has to be ensured by laboratory criteria. Patients have elevated serum transferases, hypergammaglobulinemia and autoantibodies, e.g. AIH type II often shares autoantibodies directed against cytochrome p450 2D6 or formiminotransferase cyclodeaminase (FTCD).

There is a need for a model system to investigate the complex immunopathogenesis of this chronic disease and subsequently to develop new therapeutic interventions. Therefore, we developed a new model of experimental murine AIH (emAIH) by a self-limited adenoviral infection with the hepatic autoantigen FTCD.

After an initial transient hepatitis there was a chronic evolving AIH finally leading to portal and lobular fibrosis. We could show that the genetic predisposition provided by the NOD background was essential for creating a fertile field for the development of liver specific autoimmunity. However, a strong environmental trigger was additionally necessary to initiate the disease. Besides break of humoral tolerance, T cell tolerance against hepatic self-antigens was also broken and CD4+ T cells were identified as essential drivers of the disease. As emAIH was successfully treated with steroids in here, this model will be helpful to develop and test new therapeutic interventions.
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Wnt signaling during thymus development

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During their development in the cortex of the thymus T cells are probed for their ability to recognize self-MHC molecules, a process called positive selection. Subsequently, in the thymic medulla, T cells are negatively selected against strong self-reactivity to safeguard self-tolerance. Both selection processes depend on highly coordinated molecular events in developing T cells on the one hand and the stromal cell compartment on the other hand (1). A number of signalling pathways have been identified to be essential both for thymus organogenesis and T cell development. FoxN1, the transcription factor defective in nude mice, is essential for thymic epithelial cell specification (2;3). The regulation of FoxN1 in early TEC progenitors is poorly understood. Secreted Wnt glycoproteins, which act in an autocrine or paracrine fashion, have been reported to act upstream of FoxN1 (4). Wnt signals are transmitted through Frizzled (Fzd) receptors. The importance of balanced Wnt signaling in the thymus has been documented by a rapid thymic atrophy following over-expression of the Wnt inhibitor DKK1 (5) and a block in T cell development in mice, which exhibit over-active Wnt signalling (6).

Question:
What is the precise donor/acceptor cell relationship of Wnt signaling in the thymus?
Does the requirement for Wnt signaling change during TEC development?

Methods:
FACSSorting, qRT-PCR, RTOC, 3d culture system, mouse mutants

Results:
We first assessed the expression profile of different Wnt- and Frizzled family members in qPCR arrays. Newly identified TEC precursor subsets were compared to mature medullary TECs (mTECs) and to thymic dendritic cells (DCs). This analysis revealed in each case cell-type specific expression patterns for Fzd receptors and Wnt pathway components. Based on these results we will analyze the functional implications of differentially expressed key Wnt molecules. We will also apply specific pharmacological inhibitors to modulate Wnt signaling and investigate how this will affect distinct stages of TEC development in fetal- or re-aggregate thymus organ cultures. Furthermore the precise role of TECs in Wnt signalling in the thymus will be addressed in vivo using TEC-lineage specific Wnt mutant mice.

Conclusion:
Expression of Wnt components in the thymus is sharply demarcated between different thymic cell types and between different developmental stages of TECs. Future experiments will aim at assigning functional properties to differentially expressed Wnt components.

Reference:
3 Synoviocytes. Autonomous Drivers of Chronicity?

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Immunization with Glucose-6-phosphate isomerase (G6PI) induces arthritis in susceptible strains of mice. Depletion of regulatory T cells (Tregs) prior to immunization switches the usually acute, self-limiting course to a non-remitting, destructive arthritis. This provides a possibility to study molecular switches for the transition from acute, self-limiting to chronic, destructive arthritis within one mouse model.

To examine the role of fibroblast-like synoviocytes (FLS), which are known to modulate immune responses via the production of pro- and anti-inflammatory mediators, the phenotype and function of FLS from mice with either acute, self-limiting or non-remitting, destructive arthritis was studied.

FLS from DBA/1 mice that developed either the acute or the chronic form of arthritis were isolated from joints over a time course of 56 days. To investigate the phenotype of FLS ELISA studies as well as zymography have been performed. For the functional clarification of those cells the matrix-associated transepithelial resistance invasion (MATRIN) assay and a cartilage attachment assay have been used. Furthermore, FLS have been transferred in vivo into the knee joints of immunodeficient mice and the joints have been scored histologically.

FLS from Treg-depleted mice produced significantly more cytokines (e.g. Interleukin 6 (IL-6)) upon stimulation with other cytokines, growth factors and TLR ligands. This increased susceptibility to cytokine stimulation in chronic animals compared to acute ones is observable throughout the disease course (56 days). Furthermore, the secretion and activity of matrix metalloproteases (MMPs) was enhanced in the FLS from chronic mice compared to samples from acute ones. Additional functional differences include the collagen-destructive potential and the potential to attach and eventually invade wild type cartilage. Here, FLS from Treg-depleted chronic arthritic mice showed a higher invasive and destructive potential. Ultimately, FLS from Treg-depleted mice were able to destroy cartilage in immunodeficient mice.

Our results are compatible with the hypothesis that uninhibited inflammation in the early phase of Treg-depleted mice causes the acquisition of an autonomously aggressive phenotype of synoviocytes which contribute to the switch from acute to chronic arthritis even in the absence of late support from T and B lymphocytes.

Fig. 1: Clinical progression of G6PI-induced arthritis in DBA/1 (nondepleted) and DBA/1 DEREG (Treg-depleted) mice. Depletion of Tregs was achieved by i.p. injection of 0.5 µg diptheria toxin at day -2, -1, 4 and 5. All mice have been immunized with 400 µg rhG6PI in CFA by subcutaneous application at day 0. Animals were scored for clinical signs of arthritis (erythema, swelling, ankylosis). Error bars represent SD and significances have been calculated with the student’s t test (° P<0.05; ** P<0.01).
Interaction of CREM and CREB controls FoxP3 expression in regulatory T cells

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CREMα belongs to the superfamily of bZip transcription factors that contain a basic domain/leucine zipper (also comprising inducible cAMP response element repressor (ICER) and cAMP responsive element binding protein/activating transcription factor (CREB/ATF)). These proteins share the ability to bind an 8 bp palindromic DNA sequence (TGACGTCA), the so called CRE site. CREMα is overexpressed in T cells from patients with systemic lupus erythematosus (SLE), and CREMα promoter activity correlates with disease activity in these individuals. Studies in SLE patients also show decreased numbers or defective suppressive capacity of Tregs. Thus, we here aimed to investigate the role of CREMα for the development and functional properties of Tregs. By analyzing CREM⁻/⁻ and WT mice we found elevated levels of FoxP3 mRNA in CD4⁺CD25⁺ T cells of CREM⁻/⁻ mice. Furthermore, frequencies of splenic CD4⁺FoxP3⁺ cells were increased in CREM⁻/⁻ mice compared to WT mice, and naive CD4⁺ T cells from CREM⁻/⁻ mice showed an increased differentiation towards FoxP3⁺ cells when stimulated in vitro in the presence of TGF-b. We identified several putative CRE sites within the promoter and CNS regions of the FoxP3 gene locus. The Treg-specific demethylated region (TSDR), a CpG-rich region in the first intron (+4201 to +4500), contains a full CRE site which is highly conserved between men and mice. It was previously demonstrated that the TSDR possesses transcriptional enhancer activity in a methylation-dependent manner, and beyond others CREB has been identified to bind and mediate the transcriptional enhancer activity. Here, we could show that CREMα can bind to the TSDR and can mediate a significant down-regulation of its transcriptional enhancer activity. We therefore suggest that the interaction of CREMα and CREB in Tregs controls proper FoxP3 expression. Our studies might provide new insights into the regulation of FoxP3 expression and might be relevant for Treg function in autoimmune conditions like SLE.
Fast repopulation of microglia after ablation

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Questions:
Unlike other glia cells, microglia originate from the myeloid cell lineage and therefore often are considered as resident brain macrophages. They act in the first response to direct injury or peripheral insults. However, their exact contribution in brain homeostasis is not yet clear.

Using a novel system microglia were depleted in vivo. After optimizing depletion protocol, the role of microglia in health and disease was analyzed.

Methods:
We used the new Cre-expressing mouse strain CX₃CR¹CreER to express a diphtheria toxin receptor (DTR) specifically on microglia/macrophages. In these mice, administration of tamoxifen leads to Cre-mediated excision of a loxP flanked transcriptional STOP element in both macrophages and microglia, thus allowing for the expression of the DTR as well as an YFP reporter. Due to their fast turnover macrophages are replaced by unaffected precursors whereas microglia persist in the modified stage. These mice were used for in vivo and ex vivo analysis of microglia by FACS and histology.

Results:
Our optimized protocol with two tamoxifen injections at the age of 2 weeks resulted in 80-90% of reporter-positive microglia in adulthood, whereas all peripheral macrophages were reporter-negative. With additional three successive DT injections we could achieve a microglia ablation efficiency of 80%. Interestingly, histological as well as FACS analysis revealed 50% of microglia repopulation already 4 days after depletion. Two weeks after depletion microglia numbers were even higher compared to unaffected controls. On day 6, we found a CD45.2<sup>hi</sup> Ly6C+ population, indicating replenishment by peripheral monocytes. In BM chimera experiments, where peripheral cells were labeled with a congenic marker, we obtained striking evidence showing that almost all of the repopulating cells are of peripheral origin.

Conclusions:
After depletion microglia were rapidly repopulated, which emphasizes their critical role in brain homeostasis and function.
T cell-specific loss of Glycoprotein A Repetitions Predominant leads to diminished regulatory T cell function and identity

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Regulatory T cells (Tregs) are a crucial subset of T cells responsible for both the suppression of excessive activation of the immune system and the maintenance of self-tolerance. Diminished Treg function and the consequent loss of their immunosuppressive capacity is causal for the development of autoimmune diseases. Recently, Glycoprotein A Repetitions Predominant (GARP) was identified as a cell surface protein specifically expressed on both human and murine Tregs. It has been shown that GARP serves as an anchor for latent TGFβ by tethering the inactive cytokine to the cell surface. However, it still remains elusive which function GARP plays in Treg biology.

In order to elucidate the role of GARP in Treg function, we generated a T cell-specific Garp knockout mouse (Garp−/−) by breeding mice homozygous for a floxed Garp allele with mice homozygous for the Cd4Cre transgene. As expected, Garp−/− CD25+CD4+ Tregs showed neither extra- nor intra-cellular expression of GARP. Phenotypic analysis of the Garp−/− mice revealed a modest shift in the distribution of T cell subpopulations. While in the spleen the overall frequency of CD4+ cells was slightly reduced compared to wildtype (wt) mice, the number of Tregs was unchanged. Histological analyses of Garp−/− mice showed spontaneous lymphocyte infiltrations into multiple organs, such as the heart, the gut, the liver and the lung. In the serum, Garp−/− mice displayed elevated levels of autoantibodies, all together indicating the presence of a mild autoimmune phenotype. In line with these observations, a more severe course of acute colon inflammation was observed in the knockout mice after application of dextran sodium sulfate. These results suggest that the loss of GARP in Tregs results in an increased susceptibility for autoimmune diseases, which prompted us to specifically test the function of Garp−/− Treg cells. Analysis of the suppressive capacity of Garp−/− Tregs in vitro experiments showed no alteration compared to wt Tregs. However, co-transplantation assays with Garp−/− or wt Treg cells with effector T cells in wt mice hinted towards a diminished Garp−/− Treg function in vivo. Analysis of gene expression in Garp−/− Tregs revealed the expression of several genes, which are normally not active in wt T cells. This skewed gene expression profile correlated with our observation that protein expression of FoxP3, the master transcription factor of Tregs, was less stable in knockout Tregs compared to control Tregs during in vitro culture.

In summary, our data yield a first evidence for the importance of GARP for the function of Tregs. They demonstrate that T cell-specific GARP deficiency results in a mild spontaneous autoimmune phenotype and an increased susceptibility for autoimmune diseases. This phenomenon might be caused by lineage-inappropriate gene expression in Garp−/− Tregs and a subsequent loss of stable Treg identity. Profound knowledge of the role of GARP might contribute crucially to our understanding of Treg biology and be of major value for the identification of novel treatment strategies in autoimmune diseases.
Control of experimental autoimmune encephalomyelitis by targeted delivery of neural autoantigen to liver sinusoidal endothelial cells

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The lack of treatments that specifically suppress the immune response to disease-relevant self-antigens is a major obstacle for the efficient therapy of autoimmune diseases such as multiple sclerosis. Previously, we have shown that the liver favors the generation of autoantigen-specific CD4+ Foxp3+ regulatory T cells (Tregs) that could suppress autoimmune disease (Lüth S et al., J Clin Invest. 2008 Oct;118(10):3403-10). However, the cellular and molecular basis of hepatic Treg induction is still unclear.

Here we investigated the Treg-inducing mechanisms in the liver, and explored whether Treg-induction by liver cells could be harnessed for the suppression of autoimmune disease.

To assess the capacity of various liver cells to induce Treg from CD4+CD25-Foxp3- non-Tregs, we stimulated myelin basic protein (MBP)-specific non-Treg from tg4xf3x3-gfp mice on primary liver sinusoidal endothelial cells (LSECs), Kupffer cells (KCs), liver dendritic cells (DCs) or, as control, on splenic dendritic cells in the presence of MBP-peptide. Treg functionality was assessed in vivo in mouse models of multiple sclerosis. For the treatment of autoimmune disease, we developed a nanoparticle carrier system for delivery of autoantigen peptides to Treg-inducing liver cells in vivo.

We show that liver sinusoidal endothelial cells (LSECs) efficiently induce the TGF-β-dependent conversion of CD4+ Foxp3- non-Tregs into CD4+ Foxp3+ Tregs. To harness LSECs for Treg-based treatment of autoimmune disease, we engineered a nanoparticle carrier that facilitates selective and efficient delivery of autoantigen peptides to LSECs in vivo. In two independent mouse models of multiple sclerosis, administration of nanoparticles loaded with autoantigen peptides provided complete disease protection and effective therapy of established disease. Thus, LSECs are potent inducers of antigen-specific Tregs. The selective delivery of autoantigen peptides to LSECs by nanoparticles enables effective treatment of autoimmune disease.
Chemokine-directed T cell migration is important for the therapeutic effect of glucocorticoids in CNS autoimmune disease

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Acute relapses of patients suffering from Multiple Sclerosis (MS) are generally treated with high doses of glucocorticoids (GCs) but their exact mechanisms of action are still incompletely understood. Using experimental autoimmune encephalomyelitis (EAE) we had previously shown that peripheral T cells are the main target of this therapeutic regimen. However, analysis of three different strains of genetically engineered mice suggested that induction of T cell apoptosis was dispensable for therapeutic efficacy of GCs in the treatment of EAE. In search for other possible mechanisms we found that GCs exert profound and sometimes opposing effects on T cell migration along various chemokine gradients. Following GC treatment migration toward CCL19 was reduced whereas the migration toward CXCL12 was enhanced. The dimerization-deficient GC receptor that lacks both the capacity to induce apoptosis and the ability to activate gene expression was sufficient and necessary to mediate these effects. Inhibition of CXCR4 by Plerixafor administration to mice impaired the therapeutic effect of GCs as revealed by an aggravated disease course and more pronounced CNS infiltration. Moreover, the migration of human T cells from healthy donors toward CXCL12 in vitro was enhanced by GCs. Similarly, methylprednisolone pulse therapy in vivo strongly increased the capacity of peripheral blood T cells from MS patients of different subtypes to migrate toward CXCL12. We conclude that high-dose GC therapy exerts its beneficial effect in the treatment of EAE and MS at least in part by targeting the CXCL12/CXCR4 axis in T cells.
Characterization and therapeutic depletion of plasma cells in experimental autoimmune encephalomyelitis

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Multiple sclerosis (MS) is a chronic inflammatory demyelinating autoimmune disease of the central nervous system (CNS) and a leading cause of long lasting neurological disability in young adults. There is increasing evidence that B cells are key players in the pathogenesis of MS, and it is assumed that plasma cells play a pathogenic role by antibody production. Here, we analyze the role of plasma cells during neuroinflammatory processes in the CNS to evaluate therapeutic strategies targeting antibody-producing cells.

For our investigations we use the mouse model experimental autoimmune encephalomyelitis (EAE), characterized by clinical symptoms like plegia, ataxia, perivascular inflammation and demyelination. The mice develop disease after immunization with myelin oligodendrocyte glycoprotein (MOG), a protein situated on the outer lamellae of the myelin sheath.

We characterized histomorphologically the niches of cerebral plasma cells in the inflamed CNS-tissue of EAE mice during different timepoints of the disease. There are well-defined follicle-like structures consisting of B cells around vessels in the subarachnoidal space in the meninges during remission and in the chronic phase of EAE. The follicular structures are located close to PDGFRβ+ cells with processes forming a reticular matrix. While PDGFRβ+ cells are only found adjacent to vessels in healthy mice, we can detect an expansion of the PDGFRβ+ network into the parenchyma in EAE lesions. PDGFRβ+ cells in the lesions co-express ICAM. By two-photon microscopy of the brain stem we could found B cells located close to structures emitting a second harmonic generation (SHG) signal, which is only detectable during inflammation and which is known to guide T cell migration in the CNS.

In order to determine the lifetime of plasma cells in the chronically inflamed CNS we labeled the DNA of proliferating cells with EdU for 14 days. During this EdU-pulse, dividing cells incorporate the thymidine analogon which can later be used to identify long-lived cells. Up to seven weeks later we could still detect EdU+ long-lived plasma cells in the CNS, suggesting that the CNS provides survival niches for long-lived plasma cells.

Further, we depleted the plasma cells using the proteasome inhibitor Bortezomib during EAE. The depletion before onset of the disease leads to a significant milder disease course at the peak of disease. When treatment is started during the acute EAE phase we found an improved remission in the chronic phase.

Taken together, we characterized long-lived plasma cells in the CNS and we showed that cerebral plasma cells have a pathogenic effect during establishment of the disease in mice, suggesting new perspectives for treatment strategies of neuroinflammatory diseases especially in patients who do not respond to therapies targeting B and T cells.
Microglia produce IFNβ in the context of CNS autoimmunity and microbial stimulation and contribute to the clearance of myelin debris

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Introduction:
Multiple sclerosis (MS), the most common neurological disease in western countries, is a chronic autoimmune disorder of the CNS of unknown aetiology. Autoreactive myelin specific T cells destroy the neuronal myelin sheath leading finally to neuronal degeneration. The standard medication of relapsing remitting MS is recombinant IFNβ. This leads to a decrease in frequency of relapses and severity of disease, but the exact mode of action remains unknown. IFNβ is also protective in the MOG35-55 induced experimental autoimmune encephalomyelitis (EAE), the well-established mouse model for multiple sclerosis. Genetic deletion of IFNβ in mice leads to aggravation of disease symptoms in MOG35-55 induced EAE.

Objectives:
We want to define the localization of endogenous IFNβ expression at different time points after induction of EAE and the cell type responsible for its production. Functional analysis of IFNβ producing cells shall allow for the identification of IFNβ mediated effects. Furthermore we study IFNβ production in the CNS after stimulation with pathogen compounds known to be potent IFNβ inducers.

Material and Methods:
To determine the kinetics and localization of IFNβ expression we performed qRT-PCR analysis on lymphnodes, spleen, spinal cord and brain of MOG35-55 immunized mice. Using the IFNβ/YFP reporter mouse model (IFNβmob/mob) we visualized IFNβ producing cells on a single cell basis by flow cytometry on d17 of EAE induction and after intracerebroventricular poly(I:C) stimulation. The establishment of a primary adult microglial culture and organotypic slice cultures allowed for the analysis of the influence of IFNβ on the phagocytotic capacity in vitro and in situ using WT, IFNβmob/mob mice and IFNβ−/− mice.

Results:
Time course studies showed that IFNβ mRNA was expressed in the early phase of EAE in secondary lymphoid organs, while in the effector phase highest IFNβ levels were detected in the CNS, especially in the spinal cord. In IFNβmob/mob mice we identified resident microglia in the CNS as main producers of IFNβ at the peak of EAE. FACS sorting of intracerebral cell populations according to the expression of CD45 versus CD11b and the analysis of the respective IFNβ mRNA levels corroborated these findings. An adult primary microglial culture system was used for further functional analysis of IFNβ− producing microglia in vitro. Here stimulation with defined pathogen compounds confirmed their high ability to produce IFNβ. Functionally, poly(I:C) or IFNβ stimulation enhanced the capacity of adult microglia to phagocytose myelin. In organotypic slice cultures IFNβ/YFP-producing cells showed the morphology of activated microglia and were stained co-positive for the microglia marker Iba-1 in situ. IFNβ deficiency lead to inefficient removal of myelin products in vitro and in situ.

Conclusion:
Taken together, we show for the first time that activated microglia are the major endogenous producers of the protective IFNβ at the peak of EAE. Poly(I:C) stimulation induces IFNβ production in microglia, which promotes phagocytosis of myelin debris. This study provides an up to now unknown function of IFNβ in enhancing phagocytosis and possibly neuronal regeneration during EAE.
Impact and fate of distinct T cell subsets in the course of Experimental Autoimmune Encephalomyelitis

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Objective:
Autoimmune neuroinflammation resulting in neuronal damage and demyelination is supposed to be mainly driven by autoreactive T cells in the case of multiple sclerosis (MS) and its mouse model Experimental Autoimmune Encephalomyelitis (EAE). Former studies have shown the potency of distinct T cell subsets with specificity for myelin antigens to induce EAE (Jäger et al., 2009) and to interact directly with the neuronal compartment in vivo (Siffrin et al., 2010). Nevertheless the encephalitogenic potential of different T cell phenotypes and their fate during the disease progression remain to be controversial. Therefore we investigated the plasticity of distinct T cell subsets as well as their impact on the clinical outcome in the course of EAE.

Methods:
Passive EAE was induced in RAG1⁻/⁻ mice by adoptive transfer of chronically activated Th17 cells from MOG35-55 TCR transgenic 2d2 CD90.1⁺ mice. After seven days Th1, Th2, Th17, natural Treg or CD8⁺ T cells generated from CD90.2⁺ mice were co-transferred. The clinical outcome was monitored and validated by histological analyses. T cells were recovered from the CNS on the peak of the disease for detailed analysis of their phenotype and origin. In parallel T cell subsets were co-cultured with primary neuronal cultures to investigate changes in their cytokine profile after contact with neurons.

Results:
Transfer of Th1, Th2 and CD8⁺ cells slowed down disease progression while transfer of Th17 cells worsened the clinical outcome. A strong influence on the disease inducing Th17 subset by co-transferred Th1 and Th2 cells was observed resulting in changed cytokine profiles towards less inflammation promoting phenotypes. Th17 cells co-cultured with neurons showed higher expression levels of IL-17 as a direct response to immune-neuronal interactions in vitro.

Conclusions:
First results demonstrate a high encephalitogenic capacity of Th17 cells in comparison to other T cell subtypes. Co-transferred (non-Th17) T cells induced a less severe disease progression presumably by competing with Th17 cells on CNS entry sites and, as it could be demonstrated for Th1 and Th2 cells, promoting plasticity of the disease inducing Th17 cells. In vitro data suggest a direct crosstalk between Th17 cells and neurons resulting in an enhanced cytokine response and neuronal damage.
Inhibition of the immunoproteasome strongly ameliorates experimental autoimmune encephalomyelitis

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Objectives:
In this study, we investigated the therapeutic efficacy of an inhibitor of the immunoproteasome (ONX 0914) in two different mouse models of multiple sclerosis (MS).

Methods: Disease progression in ONX 0914 treated mice after active and passive induction of EAE (both in myelin oligodendrocyte glycoprotein (MOG)35-55 and myelin proteolipid protein (PLP)139-151-induced EAE) was analysed.

Results:
ONX 0914 attenuated disease progression after active and passive induction of EAE, both in MOG35-55 and PLP139-151-induced EAE. Isolation of lymphocytes from the brain or spinal cord revealed a strong reduction of cytokine-producing CD4+ cells in ONX 0914-treated mice. Additionally, ONX 0914 treatment prevented disease exacerbation in a relapsing-remitting model. An analysis of draining lymph nodes after induction of EAE revealed that the differentiation to Th17 or Th1 cells was strongly impaired in ONX 0914 treated mice. Blockade of the immunoproteasome prevented infiltration of immune cells into the brain and spinal cord and diminished initial Th1 and Th17 differentiation. T cell-derived GM-CSF sustains neuroinflammation via myeloid cells that infiltrated the CNS and IL-23 is necessary for the induction of EAE in mice via its role in the maintenance of Th17 cells. ONX 0914 reduced GM-CSF production of activated mouse T cells under GM-CSF polarizing and non-polarizing conditions and in human PBMC stimulated through the T cell receptor. Additionally, ONX 0914 blocked IL-23 production of human PBMCs and the differentiation of CD4+ cells to IFN-γ producing Th1 and IL-17 producing Th17 cells.

Conclusion:
These results strongly implicate the immunoproteasome in the etiology and symptomology of EAE and suggest immunoproteasome inhibitors as promising drug targets for the treatment of MS. This study provides a therapeutic rationale for targeting the immunoproteasome in autoimmune disorders.
Dynamic of auto-reactive T cell activation illuminated by fluorescent NFAT marker

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Introduction:
T cells recognizing myelin auto-antigens infiltrate into the central nervous system (CNS) to induce inflammatory autoimmune disease such as multiple sclerosis. On their way to the target organ they undergo complex sequential interactions with individual components of the vascular blood-brain-barrier (BBB) which may result in T cell activation.

Objectives and Method:
To determine the functional consequences of these interactions we introduced a truncated nuclear factor of activated T cells (NFAT) fused to GFP (ΔNFAT-GFP), as a fluorescent real-time activation indicator. In transfer model of experimental autoimmune encephalomyelitis we used ΔNFAT-GFP expressing T cells to by intravital 2-photon imaging follow their activation dynamic around the BBB.

Results:
Imaging documented the cytoplasmic-nuclear translocation of ΔNFAT-GFP, indicative of calcium-dependent activation of the T cells in the perivascular space, but not within the vascular lumen. The activation was related to T cell contacts with perivascular phagocytes, and their reduced motility, but did not imply long lasting T cell arrest. Individual activated T cells were able to sequentially visit other APCs and duration of interactions implies that they either maintain T cell activation level or escalate it continuously.

Conclusion:
Our data suggest that the presentation of local auto-antigen by perivascular APCs provides stimuli that guide autoimmune T cells, enabling them to attack the CNS.
Anti-CD4 treatment inhibits autoimmunity through the attenuation of co-stimulatory signals independent of Foxp3+ Tregs

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Introduction:
There has been considerable clinical interest in the role of non-depleting anti-CD4 antibodies in order to control detrimental inflammatory responses such as transplant rejection and autoimmunity. However, the mechanisms involved are surprisingly poorly understood.

Objectives:
Recent results have established the paradigm of a Foxp3+ Treg-dependent mechanism of action. However, this questions the effectiveness of anti-CD4 antibodies as a therapy for human autoimmune diseases because a dysfunction of Foxp3+ Tregs is implicated in their etiology. Moreover, CD4 blockade is expected to completely fail in scurfy mice or patients suffering from X-linked foxp3 mutations. The main objective was to define whether anti-CD4 treatment has tolerizing activity in the absence of Foxp3+ Tregs.

Materials & methods:
We utilized bacterial artificial chromosome (BAC)-transgenic DEREG mice and Foxp3-deficient scurfy mice to assess the T cell tolerizing activity of anti-CD4 treatment in the absence of Foxp3+ Tregs. Mixed lymphocyte reactions, Treg induction- and suppression assays were used for in vitro studies. Autoimmune inflammation of scurfy mice was assessed by histological analysis of affected organs and by the quantification of autoantibodies or inflammatory cytokines. Adoptive T cell transfer experiments were performed for mechanistic studies.

Results:
Anti-CD4 treatment inhibited CD4+ T cell proliferation and activation independent of Foxp3+ Tregs. In line with this finding, no prominent induction of Foxp3+ Tregs and no activation of the suppressive function of Tregs occurred during CD4 blockade. This surprising finding prompted us to perform in vivo studies with scurfy mice lacking Foxp3+ Tregs. Strikingly, autoimmunity was inhibited in scurfy mice by anti-CD4 therapy by a recessive T cell-intrinsic mechanism. This involved the defective upregulation of co-stimulatory receptors on tolerized T cells.

Conclusion:
We discovered a novel Foxp3+ Treg-independent mechanism of anti-CD4-induced tolerance. Our results imply that CD4 blockade is a powerful treatment for autoimmune diseases characterized by Treg dysfunctions. This may particularly apply to the human IPEX syndrome which closely resembles the autoimmune disease of sf mice.
Conditional deletion of the TGF-β inhibitor Smad7 in dendritic cells limits autoimmune CNS inflammation by induction of regulatory T cells

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Question:
Dendritic cells (DCs) as professional antigen presenting cells, play a vital role in the pathogenesis of multiple sclerosis (MS) and its animal model, experimental autoimmune encephalomyelitis (EAE). DCs secrete and respond to the anti-inflammatory cytokine transforming growth factor (TGF-β). We therefore sought to determine the role of Smad7, a negative regulator of TGF-β signalling, specifically in DCs during autoimmune neuroinflammation.

Methods:
We examined mice in which Smad7 promoter and Exon1 were flanked by loxp sites (Kleiter et al., Brain 2010). Upon crossing these mice to a DC specific CD11c Cre mouse line, we resulted in a mouse line devoid of Smad7 specifically in DCs. These mice were furthermore subjected to MOG35-55 induced EAE and disease progression was assessed.

Results:
Deletion of Smad7 specifically in DCs resulted in almost complete resistance to EAE. Following immunization of these mice, we observed two fold increase of T regulatory cell (Treg) induction in the periphery and accumulation in the CNS. Consequently, after Treg cell depletion, disease progression in the DC-specific Smad7 deficient mice was similar to controls. Analysis of dendritic cell subsets revealed an increase in the CD8α and CD103 positive DCs, which are known to have a strong capacity to induce Tregs and thus are implicated in tolerance induction.

Conclusion:
Taken together, our results indicate that Smad7 can regulate inflammatory cellular responses in vivo. Deletion of Smad7 in DCs promotes the generation of tolerogenic DC-subsets, which further promotes Treg cell generation, resulting in amelioration of EAE progression.
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Titration of autoimmunity in adult DEREG mice

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Introduction:
Foxp3+ regulatory T cells (Tregs) play a critical role at mediating tolerance to self-antigens. Bacterial artificial chromosome (BAC)-transgenic DEREG mice utilize foxp3 regulatory sequences to drive expression of diphtheria toxin (DT) receptor fused to eGFP. DEREG mice depleted of Foxp3+ Tregs by DT administration have been very valuable to investigate Treg functions in vivo. This relates to the circumstance that adult DEREG mice do not develop overt autoimmunity after Treg depletion. In contrast, neonatal DEREG mice and adult Foxp3-DTR knock-in mice develop fatal autoimmunity in the absence of Tregs. The basis for these discrepancies remains poorly defined. Thus, DEREG mice are unique model to investigate the factors triggering autoimmunity.

Objectives:
Acute DT administration deletes about 90-95% of Foxp3+ Tregs in DEREG mice whereas chronic DT treatment results in the outgrowth of eGFP Foxp3+ Tregs. Thus, a minor Treg population appears to be DT-resistant. However, eGFP Foxp3+ Tregs cannot be isolated due to their lack of transgene expression and because of CD25 upregulation on conventional T cells following Treg depletion. Our main objectives were to investigate the origin of eGFP Foxp3+ Tregs and their potential role at suppressing autoimmunity.

Materials and methods:
Helios- and neuropilin-1 expression were analysed by flow cytometry in order to establish a thymic versus peripheral origin of eGFP Foxp3+ Tregs. Additionally, genomic DNA was isolated from sorted eGFP Foxp3+ Tregs and the presence of genomic GFP and DTR transgenes was assessed by PCR. For the isolation of transgene-negative Tregs in chronically DT-treated DEREG mice, we crossed DEREG mice with Foxp3GFP knock-in mice. Autoimmunity was assessed macroscopically and by histopathological analyses of affected organs. Moreover, anti-mitochondrial autoantibodies were determined.

Results:
Helios- and neuropilin-1 stainings revealed that eGFP Foxp3+ Tregs derive predominantly from the thymus, as also seen for the bulk Foxp3+ Treg population in secondary lymphoid organs. Genomic DNA isolated from eGFP Foxp3+ Tregs still contained GFP and DTR transgenes, suggesting transgene inactivation at the transcriptional or translational level in a small fraction of Tregs. DEREG x Foxp3GFP/+ mice were established in order to isolate DT-resistant Tregs. Strikingly, adult DEREG x Foxp3GFP/+ rapidly developed autoimmunity following DT treatment, including uveitis, inflammation of several organs and the production of autoantibodies. Treg numbers in these mice were substantially reduced following chronic DT treatment when compared to WT and DEREG mice. In line with these findings, massive T cell activation occurred in DT-treated DEREG x Foxp3GFP/+ mice.

Conclusion:
Foxp3GFP mice were recently described to be a hypomorph due to disturbed Foxp3 interactions caused by the N-terminal GFP fusion. This causes a functional Treg insufficiency of Tregs in inflammatory environments. We established DEREG x Foxp3GFP/+ mice as a novel model of inducible autoimmunity. Because the timing and dosing of DT treatment can be tightly controlled, these mice allow the “titration” of autoimmunity. This provides important insights into the regulation of self-tolerance by Foxp3+ Tregs. Our results for the first time prove that a minor population of DT-resistant Foxp3+ Tregs are sufficient to protect adult DEREG mice from autoimmunity while tumour- and foreign-specific immunity is augmented.
The transcription factor Bcl-3 represses the function of regulatory T cells leading to the development of colitis

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The proto-oncogene Bcl-3 (B cell leukemia-3) is a member of the IkB family of NF-κB inhibitors and was originally identified as a component of a chromosomal rearrangement in some B cell lymphomas. In contrast to other IkB proteins, Bcl-3 associates with p50 and p52 homodimers and is predominantly a nuclear protein involved in regulating nuclear NF-κB activity.

To understand more about the effects of Bcl-3 on T cells, we have generated a new mouse model, where Bcl-3 is overexpressed specifically in T cells.

We could show that mice overexpressing Bcl-3 solely in T cells developed spontaneous colitis accompanied by a massive cell infiltration into the colon. Interestingly, by analyzing regulatory T cells we could demonstrate that these cells were not able to suppress an inflammatory response. Due to the suppressive dysfunction, Tregs of Bcl-3 overexpressing mice expressed less CD25, Foxp3 as well as less of the co-inhibitory molecule CTLA-4. Hence, the loss of suppression of Bcl-3 overexpressing Tregs might result in the development of severe spontaneous colitis. Our data emphasize a novel pathway in the maintenance of viability and suppressive function of Tregs in the gut that critically involves Bcl-3 as a negative regulator, qualifying this protein as an interesting pharmacological target for the treatment of IBD.
Autoimmune diseases frequently occur in humans but the pathomechanisms underlying the loss of self-tolerance are largely unknown. Cutaneous autoimmune disorders, such as lupus erythematosus (LE), compromise the integrity of the skin barrier and it has been shown that in particular epidermal Langerhans cells (LC), which constantly migrate from the skin to regional lymph nodes, contribute to the progression of inflammation. In regional lymph nodes co-stimulatory molecules of the TNF/TNF receptor family (like CD40 ligand and its receptor CD40) are important for the regulation of adaptive immune responses induced by LC. Accordingly, in a transgenic mouse model with epidermal overexpression of CD40L (K14-CD40L tg mice) the migration CD40/CD40L-stimulated LC from the skin to draining lymph nodes represents the first step in a cascade of immune reactions resulting in the breakdown of tolerance towards self and the induction of a systemic LE-like autoimmunity including dermatitis, nephritis and the presence of autoantibodies. In K14-CD40L tg mice autoreactive CD8+ T cells are crucial for the onset of disease since adoptive transfer of pathogenic CD8+ T cells from autoimmune-prone K14-CD40L tg mice induced disease in wildtype (wt) recipients. Autoreactive CD8+ T cells from tg mice are characterized by an up-regulated expression of IL-17. Since it has been shown that in CD4+ T cells the IL-17 expression can be controlled by the aryl hydrocarbon receptor (AhR) we investigated whether AhR might also be involved in the pathogenicity and homeostasis of IL-17-expressing CD8+ T cells. Therefore, K14-CD40L tg mice were crossed to AhR deficient animals. Surprisingly, the double mutants showed a delayed onset of disease and moreover, the severity of autoimmune dermatitis was significantly reduced in K14-CD40L x AhR-/- mice compared to K14-CD40L tg controls. Additionally, K14-CD40L x AhR-/- mice exhibited a rescued renal function as evidenced by the decreased protein excretion in the urine and the reduced immunoglobulin depositions (IgM as well as IgG) at the kidney tissue. Notably, in contrast to K14-CD40L tg controls activated B cells and autoantibodies were not detectable in the serum of double mutants suggesting an important role of the AhR during the development and progression of CD40L-induced systemic autoimmunity. To characterize the impact of AhR on the pathogenicity of autoreactive CD8+ T cells in more detail, the IL-17 and IL-22 expression as well as the expression/phosphorylation of ROR-γt, Runx1, STAT1 and STAT4, all transcription factors, which have been implicated in Tc17 development, were analyzed on gene expression and protein level in CD8+ T cells from K14-CD40L x AhR-/- double mutants. Interestingly, we observed a significantly decreased expression of IL-17 and IL-22 as well as a reduced expression/phosphorylation of the transcription factors in CD8+ T cells from K14-CD40L x AhR-/- mice compared to age- and sex-matched K14-CD40L tg animals. The down-regulated expression of pro-inflammatory cytokines and the decreased expression/phosphorylation of characteristic transcription factors in CD8+ T cells from double mutants resulted in a reduced migration of CD8+ T cells to lesional skin and thus, the amelioration of dermatitis. Together, these data indicate that the AhR might be involved in the pathogenicity of autoreactive CD8+ T cells in K14-CD40L tg mice.
Toso is critical for inflammation and autoimmune responses

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The ability to mount a strong immune response against pathogens is crucial for the survival of mammals. However, excessive and uncontrolled immune reactions can result in autoimmune diseases. Unravelling how the reactive versus tolerogenic state is controlled might lead to novel therapeutic strategies to treat autoimmune diseases. The surface receptor Toso/Faim3 has been linked to apoptosis, IgM-binding and to innate immune responses. In this study we used Toso-deficient mice to investigate an important function of Toso in tolerance and autoimmunity. We found that Toso⁻/⁻ mice are protected from experimental autoimmune encephalomyelitis (EAE), the mouse model for the human disease multiple sclerosis. Toso⁻/⁻ dendritic cells (DCs) induced significant lower levels of disease associated inflammatory T cell cytokines and were less sensitive to toll like receptor stimulation. In consistency with this, disease resistance could only be transferred by Toso⁻/⁻ DCs, indicating their tolerogenic potential. After EAE-induction Toso⁻/⁻ showed significant increased Treg numbers, which correlated with decreased encephalitogenic cellular infiltrates in the brain when compared with WT animals. Finally, blocking of Toso activity in vivo prevented progression of EAE at multiple stages of the disease. All together, our data identified Toso as a novel regulator of inflammatory autoimmune responses and as an attractive target for therapeutic intervention.
Immune complexes as 'adhesion molecules' for CD16 positive DCs and NK-cells

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Immune complex (IC) deposition in the vascular bed or the presence of anti-endothelial cell antibodies is a hallmark of several autoimmunity-driven inflammatory diseases like lupus erythematosus and vasculitis. Dysfunctional outcomes of the Fc receptor-mediated detection of these autoICs include IC-triggered inflammation and tissue damage. We applied a perfusion assay-based experimental setup coupled with time-lapse video microscopy to measure the arrest function of purified human blood leukocytes on small immobilized ICs. Under flow conditions mediating surface shear stress corresponding to that of human venous capillaries (>0.5 dynes/cm²) we observed a strong recruitment of Fc gamma R III (CD16) positive dendritic cells (slanDCs) and the subset of CD16 positive NK cells, two potent effectors of antibody-dependent cell-mediated cytotoxicity. Plasmacytoid DCs, CD1c⁺ DCs or Fc receptor negative control cells completely failed to adhere. Specific blocking of either CD16 or CD32 on slanDCs clearly demonstrated the dependence of the shear-resistant attachment on CD16a while Fc gamma R II (CD32) was largely dispensable. Consistent with these results, when we used mature slanDCs that have proteolytically downregulated CD16 no attachment could be observed. In additional experiments we investigated the attachment of slanDCs on human IgG subtypes and observed that especially human IgG3 strongly promoted the firm attachment of slanDCs, again in a largely CD16-dependent manner. To further corroborate our results we then used monolayers of human dermal microvascular endothelial cells. As expected we found enhanced recruitment of slanDCs on endothelial cells that were pre-incubated with IgG-anti-endothelial cell antibodies. Finally, immunohistochemical identification of slanDCs in tissue sections of lupus nephritis and allergic vasculitis lends further support to a CD16a/IC-mediated recruitment at the vascular interface. Collectively, our results show that CD16a mediates an efficient shear stress-resistant adhesion of circulating slanDCs and NK cells to immobilized ICs. These data provide evidence for a novel conduit of rapid FcR-dependent recruitment of immune cells in IC-mediated tissue inflammation.
Intra-renal IL-18 Triggers Systemic Lupus, while Caspase-1 Inhibition Ameliorates IL-18 Mediated Nephritis in MRL-Fas<sup>−/−</sup> mice.

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Several cytokines, including IFN-γ, IL-18, IL-12, and IL-23 have been implicated in the pathophysiology of autoimmune disease. IL-18, a potent inducer of IFN-γ, enhances Th1 responses that are thought to be synergistic and dependent on IL-12. We tested the hypothesis that intra-renal IL-18 mediates kidney and systemic disease in MRL-Fas<sup>−/−</sup> mice. By constructing IL-12p40/IL-23<sup>−/−</sup>MRL-Fas<sup>−/−</sup> mice and using an ex-vivo gene transfer to deliver IL-18 intra-renally, we determined that IL-18, independent of IL-12 and/or IL-23, incites kidney disease in MRL-Fas<sup>−/−</sup> mice. Moreover, we provide the novel finding that local intra-renal IL-18 mediates systemic disease (lung pathology, systemic auto-Abs). Thus, our data indicate that IL-18 is a potential therapeutic target for immune mediated kidney and systemic disease in MRL-Fas<sup>−/−</sup> mice. Using a caspase-1 inhibitor, that inhibits the release of active IL-18 and IL-1b, we successfully treated kidney (improved renal function, pathology) and systemic disease (skin lesions, lymphadenopathy, and splenomegaly) in MRL-Fas<sup>−/−</sup> mice, while administration of an IL-1 receptor antagonist did not influence disease progression. Probing further we found that inhibition of IL-18 activation results in an amelioration of lupusnephritis by a reduction of intra-renal infiltrating leukocytes (macrophages and T cells) and reduced activation of these leukocyte population. Moreover, Caspase-1 inhibition resulted in decreased INF-γ and IL-17 production, indicating an altered balance of Th17 and Th1 cell responses in this model. Taken together, our findings indicate that IL-18, independent of IL-1b, IL-12 and/or IL-23, is the major mediator of kidney and systemic disease MRL-Fas<sup>−/−</sup> mice. Therefore, caspase-1 inhibition is a potential therapeutic target for autoimmune disease in the MRL-Fas<sup>−/−</sup> mice.
Introduction:
Type I interferons (IFN) are among the first lines of defense of the innate immune system against viral infections. Their release is triggered by nucleic acid sensing pattern recognition receptors. Uncontrolled production of IFNs in response to endogenous nucleic acids accumulating due to inefficient degradation in extracellular and endosomal compartments was shown to cause autoimmunity. Interestingly, also defects of intracellular nucleases TREX1, RNase H2 and SAMHD1 cause the Lupus-like disease Aicardi-Goutières syndrome (AGS), which, like Lupus, is characterized by an overproduction of type I IFN in the absence of detectable viral infection. In fact mutations in the genes encoding for the nucleases TREX1 and SAMHD1 have been shown to cause the prototypic autoimmune disease Systemic lupus erythematosus (SLE). In addition, human SAMHD1 restricts retroviral replication by reducing cellular dNTP concentration. TREX1-deficient mice die of a type I IFN-dependent lupus-like condition which is characterized by multi organ inflammation and anti nuclear antibodies (ANA).

Objectives:
While TREX1-deficiency in the mouse has been extensively characterized in the past decade, no SAMHD1 deficient mice were available to study in vivo functions of the enzyme.

Materials & Methods
We generated SAMHD1-deficient mice and investigated spontaneous IFN production and retroviral replication.

Results:
We found out that mouse SAMHD1 degrades cellular dNTPs in various cell types. SAMHD1-deficient mice were more susceptible to infection with an HIV-reporter virus, demonstrating that murine SAMHD1 restricts retroviral replication. SAMHD1 deficiency led to a spontaneous transcriptional signature of type I IFN genes, which was dependent on the production of IFN-β and intact signaling via the interferon alpha receptor.

Conclusion:
Murine SAMHD1 functions to reduce cellular dNTP concentrations below a threshold needed for HIV reverse transcription. Importantly, like TREX1-deficient mice, these animals feature spontaneous production of type I IFN, similar to patients with mutations in SAMHD1. The mice will therefore be of importance for the identification of the pathogenic IFN-inducing nucleic acids accumulating in the absence of SAMHD1.
Low CD18 levels promote expansion of inflammatory γδ T cells collaborating with CD4+ T cells in murine chronic psoriasiform dermatitis

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Interleukin-17 (IL-17) is a critical factor in the pathogenesis of psoriasis and other inflammatory diseases. The impact of gd T cells, accounting for an important source of IL-17 in mouse models of imiquimod- and IL-23 induced acute skin inflammation, in human psoriasis is still unclear. Using the polygenic CD18hypo PL/J psoriasis mouse model spontaneously developing chronic psoriasiform dermatitis and arthritis due to reduced CD18/b2 integrin expression to 2-16% of wild-type levels, we herein investigated the influence of adhesion molecule expression on generation of inflammatory gd T cells and dissected the roles of IL-17 producing gd and CD4+ T cells in disease chronification. Severity of CD18hypo PL/J psoriasiform dermatitis correlated with a loss of skin-resident Vγ5+ T cells and concurrent skin infiltration with inflammatory IL-17+, IL-22+ and TNF-α+ gdTCRlow cells preceded by increases in Vγ4+ T cells in local lymph nodes. In vitro, reduced CD18 levels promoted expansion of inflammatory memory-type gd T cells in response to IL-7. Similar to IL-17 or IL-23/p19 depletion, injection of diseased CD18hypo PL/J mice with anti-gdTCR antibodies significantly reduced skin inflammation and largely eliminated pathological gd and CD4+ T cells. Moreover, CD18hypo PL/J gd T cells induced allogeneic CD4+ T cell responses more potently than CD18wt counterparts and upon adoptive transfer, triggered psoriasiform dermatitis in susceptible hosts. These results demonstrate a novel function of reduced CD18 levels in the generation of pathological skin-infiltrating gd T cells in the CD18hypo PL/J psoriasis mouse model that was confirmed by detection of enhanced fractions of CD18low gd T cells in psoriasis patients. This new role of CD18 in gd T cell biology may also have implications for other autoimmune diseases and cancer immunotherapy.
Mechanisms of type I IFNa-mediated protection against antigen-induced arthritis

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Mechanisms of type I IFN-mediated protection against antigen-induced arthritis

Type I interferons are pleiotropic cytokines which may both enhance and inhibit inflammation. If interferon-alpha (IFN-α) is present at immunisations with the antigen methylated bovine serum albumin (mBSA), then antigen-specific cellular immunity is skewed towards tolerance and induction of arthritis by intra-articular injection of mBSA is abolished (Ying et al., Europ. J Immunol 2011, 41 1687-95). Type I IFN could thus potentially be used to deviate autoimmune responses, but given the pro-inflammatory properties of type I IFN in other settings it is important to isolate its tolerogenic effect.

Question:
How does IFNa confer resistance to antigen-induced arthritis?

Method:
Arthritis was induced by intra-articular injection of mBSA in mice pre-immunized twice one week apart three weeks earlier with mBSA with or without 100-5000 U IFN-alpha. Four weeks after the first immunization, the inflammation in the knee is evaluated by histopathological analysis. During the course of arthritis antigen specific antibody of different isotypes was determined by ELISA and produced cytokines were determined by Luminex.

Results:
IFNa protected mice from arthritis in a dose-dependent manner, and clearly inhibited release of pro-inflammatory cytokines (IL-6, TNF, IL-12) in serum and prevented antigen-induced re-call responses (upon intra-articular injection) in vivo of IL-1β, IL-10, IL-12, TNF, IFNγ and IL-17. In contrast, IFNa clearly enhanced activation of TGF-beta, both initially and after intra-articular challenge with mBSA, which in the absence of IFNa was clearly inhibited at arthritis manifestation. Likewise, IFNa inhibited pro-inflammatory cytokine release upon antigen restimulation ex vivo and enhanced that of TGF-beta. In apparent contrast to the effect on cellular immunity, presence of IFNa at immunisations did little to affect antibody levels of IgG1, IgG2a, IgG2b, IgA and IgE.

Conclusion:
Presence of IFNa at immunisations skews the antigen-specific cellular response towards a less pro-inflammatory phenotype characterized by increased TGF-beta levels while keeping pro-arthritisic cytokines as TNF and IL-17 at bay. This may be the mechanism behind type I IFN-mediated protection against antigen-induced arthritis.
Secreted semaphorin 5A activates immune effector cells and is a biomarker for rheumatoid arthritis

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Semaphorins were initially described as axon pathfinders, but recently they showed the capacity to modulate the immune response. The aim of this study is to investigate the role of the multifunctional protein Semaphorin 5A (Sema5A) in the modulation of cellular immune responses and rheumatoid arthritis (RA).

Soluble recombinant Sema5A was used to assess its effect on the functions of primary T and NK cells isolated from the peripheral blood of healthy donors. Cell proliferation and expression of transcription factors was assessed by flow cytometry. Cytokine secretion was analysed using Luminex technology. Sera of 101 RA patients and control sera from healthy individuals or patients with non-RA rheumatic diseases were analysed for the presence of secreted Sema5A by ELISA and immunoblotting.

Soluble Sema5A strongly increased T- and NK-cell proliferation and induced the secretion of proinflammatory Th1/Th17 cytokines. Accordingly, Sema5A stimulation caused a significant upregulation of T-bet and RORγt levels in T cells. In addition, significantly elevated levels of secreted Sema5A were detected in the sera of RA patients compared to healthy controls. The highest Sema5A levels were observed in RA patients positive for the RA biomarker anti-cyclic citrullinated peptide (p<0.05, compared to systemic lupus erythematosus and Sjögren syndrome patients). Moreover, Sema5A levels significantly correlated with levels of the RA marker rheumatoid factor.

Soluble Sema5A is a potent activator of T and NK cells in vitro and high Sema5A serum levels seem to be specifically associated with RA. Taken together, the results indicate that Sema5A might contribute to the pathogenesis of RA through antigen-independent T- and NK-cell activation. Hence, Sema5A is a promising complementary biomarker for the diagnosis of RA.
Targeting dendritic cells with lentiviral vectors induces tolerance of effector T cells and protects mice from experimental autoimmune encephalomyelitis

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Multiple sclerosis (MS) is an autoimmune disorder characterized by self-reactive T cells that target myelin components of the central nervous system (CNS). The present treatment relies on delaying and ameliorating disease symptoms. The aim of this project was to induce permanent, antigen-specific tolerance of previously activated T cells involved in MS. The strategy includes the ex vivo modification of autologous hematopoietic stem cells (HSC) with lentiviral vectors that express myelin oligodendrocyte glycoprotein (MOG), an auto-antigen involved in experimental autoimmune encephalomyelitis (EAE), an animal model of MS which closely resembles the human disease. The MOG-transgene is under the control of a dendritic cell (DC)-specific promoter to transcriptionally target antigen expression to DCs. After re-infusion, the vector-transduced HSC will give rise to MOG-expressing DCs that, in a non-inflammatory condition, are expected to tolerize self-reactive T cells and, therefore, prevent/revert EAE. We demonstrated that all mice that had received HSC transduced with MOG-lentivirus vector were protected from EAE induction, while all mice that received HSC transduce with a control lentivirus vector developed EAE. In tolerized mice MOG-specific T cells were depleted and Foxp3+ regulatory T cells (Tregs) were generated, which appeared to be functional, as they were able to suppress the in vitro proliferation of MOG-specific T cells. Most importantly, we have demonstrated that tolerized mice did not develop any signs of autoimmune disease even when infused with pre-activated, MOG-specific effector T cells, which rapidly induced EAE in the control mice. The ability to revert the pathogenic MOG T cells highlights the potential of this strategist for a therapeutic model.
Sodium Chloride induces pathogenic Th17 cells and blocks suppressive activity of Foxp3+ Treg cells

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There has been a marked increase of autoimmune diseases in the Western world in recent decades. This increase could not be explained by genetic predisposition but must be triggered by changes in the environment. The balance between pro-inflammatory Th17 cells and immune suppressive Foxp3+ Treg cells plays a critical role for the development of autoimmune diseases. However, how changes in the environment could directly influence Th17 and Treg cells is not well established. Here we show that high-salt (sodium chloride; NaCl) concentrations boost the induction of Th17 cells with a highly pathogenic phenotype in vitro. Furthermore, mice fed a high-salt diet develop a severe form of experimental autoimmune encephalomyelitis (EAE), in line with augmented central nervous system infiltrating Th17 cells. Interestingly, high-salt conditions similarly affected Treg function. Increasing NaCl concentrations almost completely blocked the suppressive activity of Foxp3+ Tregs in vitro. Analogous to Th17 cells, high-salt conditions induced discrete changes in gene expression of Tregs. In line with these findings, a high-salt diet modulated human T cell function in a xeno-graft versus host disease (x-GvHD) model in vivo. Thus, increased dietary salt intake may represent an environmental risk factor for the development of autoimmune diseases by affecting both the induction of pathogenic Th17 cells and through modulation of Treg function.
Primary Sclerosing Cholangitis – Laboratory Findings

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Objectives:
Primary sclerosing cholangitis (PSC) is a chronic, autoimmune, cholestatic liver disease characterized by inflammation and fibrosis of both intrahepatic and extrahepatic bile ducts, leading gradually to cirrhosis, liver failure and cancer. Most patients are young, with a median age at onset of 30-40 years. In contrast to most autoimmune conditions, 2/3 of the PSC patients are male. The aim of this study is the presentation of the laboratory findings of a 40 year old male patient.

Materials and Methods:
Hepatic enzyme levels (AST, ALT, ALP, LDH, 5-NT and γ-GT), total and direct bilirubin, serum proteins, serum albumin, CRP, RF, IgG, IgA, IgM, C₃ and C₄ levels were measured by the OLYMPUS AU640 fully automated analyzer. The presence of ANA, AMA, ASMA and ANCA were detected by indirect immunofluorescence on Hep-2 cells, mouse stomach/kidney tissue slides and ethanol/formalin-fixed neutrophils respectively (INNOVA Diagnostics Inc, San Diego CA). ASCA IgA and IgG were detected by indirect immunofluorescence with the commercially available detection kit by Euroimmun, Germany. Complete blood count was evaluated on the Sysmex XT 4000i automated hematology analyzer and D-dimer on Siemens BCS XP hemostasis System.

Results:
AST 89.2, ALT 173.1, γ-GT 139.3, LDH 461.5, 5-NT 35.5 and ALP 242.8 U/L. Total bilirubin 17.3 mg/dl, direct bilirubin 9.2 mg/dl, serum proteins 6.31 g/dl, albumin 3.27 g/dl, CRP 180.3, RF 85.5, IgG 1256.9, IgA 409.1, IgM 310.2, C₃ 31.9 and C₄ 8.7 mg/dl. ANA, AMA, ASMA and ASCA negative, p-ANCA positive with pattern localized to the periphery of the nucleus. ESR 1st hour 56mm, hematocrit 35.0%, D-dimer 10.6 mg/L.

Conclusions:
The results are in accordance with PSC diagnostic criteria: cholestatic liver biochemistry, ESR 1st hour >50 mm, elevated CRP. Hyperbilirubinemia suggests advanced disease. Increased D-dimer is in accordance with liver failure, which is characterized by multiple alterations in the hemostatic system. p-ANCA are detected in up to 88% of patients with PSC and they are a useful diagnostic marker in conjunction with other standard diagnostic tests. Moreover, their presence draws attention to colon involvement. More than 80% of patients with PSC have ulcerative colitis (UC) and there is a high degree of specificity of p-ANCA for both disorders. In our study ASCA were not observed. ASCA can be used as an aid in distinguishing UC from Crohn’s disease and their presence correlates with small bowel involvement.
Anti-neutrophil cytoplasmic antibodies—Prevalence in Greek patients

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Objectives:
Anti-neutrophil cytoplasmic antibodies (ANCA) are IgG autoantibodies, directed against cytoplasmatic components of neutrophilic granulocytes. Two distinct ANCA forms can be observed: cytoplasmic (cANCA), directed against proteinase-3 (PR3) and perinuclear (pANCA), directed against myeloperoxidase (MPO). ANCA are strongly associated with the primary systemic vasculitides, which include Churg-Strauss syndrome, Wegener’s granulomatosis, microscopic polyangiitis, as well as their localised forms. They have also been reported in a variety of clinical conditions, such as infections, malignancies, connective tissue diseases, renal diseases, other types of vasculitis and gastrointestinal disorders. The aim of our study was to evaluate the prevalence of ANCA in Greek patients during a three year period of time.

Materials and Methods:
We evaluated the sera of 2200 patients, 1215 (55.3%) men and 985 (44.7%) women, aged between 25 and 75 years. Sera were submitted to the Immunology Department, with a test request for ANCA from different hospital departments (nephrology 2.3%, neurology 10.9%, gastroenterology 8.1%, internal medicine 24.3%, pneumonology 6.4%, rheumatology 5.5%, cardiology 5.9%, while 34.2% of the samples were derived from the outpatient clinics). ANCA were tested by indirect immunofluorescence (IFA) on formalin-fixed human neutrophil substrate slides (NOVA Lite® IFA ANCA kit by Inova Diagnostics Inc., San Diego, CA). All positive results were evaluated for MPO and PR-3 reactivity by a sensitive immunodot assay (ANCA Dot, GA GENERIC ASSAYS GmbH Dahlewitz, Germany).

Results:
46 (2.09%) patients, 26 men and 20 women were ANCA positive. Among these samples, 31 (67.4%) presented p-ANCA pattern staining, 11 (23.9%) samples c-ANCA and 4 (8.7%) samples atypical ANCA pattern staining. All p-ANCA samples were anti-MPO positive and all c-ANCA were anti-PR3 positive. ANCA-associated vasculitides represented only 6 (13.0%) of positive results. The majority of positive sera were derived from patients with other disorders as follows: 8 samples (17.4%) from patients with connective tissue disorders (SLE, Sjögren’s syndrome and rheumatoid arthritis) 3 samples (6.5%) with monoclonal gammopathy, 8 (17.4%) with renal disease, 4 (8.7%) with malignancies, 3 (6.5%) with infections, 3 (6.5%) with gastrointestinal disorders and 11 (23.9%) with other vasculitides such as Adamantiades-Behcet’s disease, leukocytoclastic vasculitis, polyarteritis nodosa and Takayasu’s arteritis.

Conclusions:
Low prevalence (2.09%) of ANCA positive samples was observed. The p-ANCA staining pattern predominated among the positive patients. Connective tissue diseases, renal disease and non ANCA associated vasculitides predominated among ANCA positive patients, followed by other conditions including infections, malignancies and gastrointestinal disorders.
Expression of the chemokine receptors CXCR6 and CX3CR1 on CD8+ T-cells in diabetic RIP-LCMV-GP mice

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In Type 1 Diabetes (T1D) self-destructing lymphocytes are attracted to the islets of Langerhans by proinflammatory chemokines. The IFNγ inducible transmembrane chemokines CXCL16 and CX3CL1 with their receptors CXCR6 and CX3CR1 and their sheddase ADAM 10 could be novel targets for T1D therapy. Here we investigated the expression of CXCL16/CXCR6, CX3CL1/CX3CR1, and ADAM10 in the pathogenesis of T1D in a mouse model.

We used the well-established RIP-LCMV-GP model of T1D. As a target autoantigen in the β-cells, RIP-LCMV-GP mice express the glycoprotein (GP) of the lymphocytic choriomeningitis virus (LCMV) under control of the rat insulin promoter (RIP). Such mice develop T1D within two to three weeks after infection with LCMV.

Histologic studies of pancreata from RIP-LCMV-GP mice collected at different times after LCMV infection revealed an enhanced CX3CL1/CX3CR1 expression. CX3CR1 was predominantly found in infiltrates of CD4+ and CD8+ T cells. In contrast, although CXCL16 was expressed in the pancreas, no infiltrating CXCR6+ cells were
Opposite role for the chemokines CCL5 (RANTES) and CXCL3 (IP-10) in the development of autoimmune liver disease in the CYP2D6 mouse model

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The etiology of autoimmune hepatitis (AIH) is poorly understood although the major autoantigen, cytochrome P450 2D6 (CYP2D6), has been identified and immunodominant epitopes mapped. We generated a mouse model for AIH using the natural human autoantigen CYP2D6 as a triggering molecule. Wildtype C57BL/6 mice are infected with an adenovirus expressing the human CYP2D6 (Ad-2D6) in order to break self-tolerance to the mouse CYP2D6 homologues (molecular mimicry). Such mice display persistent features characteristic for liver damage associated with AIH (fibrosis, ‘fused’ lobules, cellular infiltrations and necrosis). Ad-2D6-infected mice generated anti-CYP2D6 antibodies recognizing the identical immunodominant epitope as LKM-1 antibodies from AIH patients and mount a robust CYP2D6-specific T cell response.

Here we investigated the role of chemokines during the pathogenesis of AIH in the CYP2D6 mouse model and found that among others the chemokines CCL5 (RANTES) and CXCL10 (IP-10) were strongly induced after Ad-2D6 infection. Interestingly, CXCL10 has been found previously to be increased in the serum of AIH patients. We therefore infected CCL5-/-, CXCL10-/- and wildtype C57BL/6 mice with Ad-2D6. CXCL10 deficient mice showed a reduced acute virus-induced liver damage (AST/ALT levels) and a diminished chronic autoimmune hepatitis. Cellular infiltrations were reduced and the frequency of CYP2D6-specific T cells was lower than in wildtype mice. In contrast, CCL5 deficient mice displayed similar serum aminotransferase levels than wildtype mice. In addition, chronic autoimmune hepatitis was slightly enhanced in CCL5 deficient mice. Our data indicate an opposite effect of the early expressed chemokines CCL5 and CXCL10 on the acute virus-induced damage to hepatocytes as well as on the chronic autoimmune hepatitis.
Induction of hepatic fibrosis by an adenovirus encoding the human liver autoantigen cytochrome P450 2D6

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Autoimmune hepatitis is an autoimmune liver disease whose cause is currently unknown. We recently developed an animal model which resembles AIH in that antigen-specific B- and T-cell responses were observed. To trigger the disease, an adenovirus (Ad) encoding the human Cytochrome P450IID6 (hCYP2D6), the major autoantigen in AIH type 2, was used. Importantly, infection with Ad-2D6 initiated autoimmune processes which caused chronic inflammation and fibrogenesis in the liver. Fibrosis was characterized by increased extracellular matrix deposition in and underneath the capsule whereas the liver parenchyma was less affected. Concurrently, we observed at the same locations elevated expression of alpha-smooth muscle actin, a marker for fibrogenic cell subpopulations, such as capsular fibroblasts or hepatic stellate cells (HSCs). Cell isolation experiments revealed an increased frequency of activated HSCs. Interestingly, intraperitoneal - but not intravenous injection of Ad-2D6 resulted in subcapsular fibrosis, suggesting that the infection route determines fibrosis location. We observed that intraperitoneal infection caused the accumulation of CD11b+ Ly6C+ F4/80+ inflammatory monocytes in the peritoneum. Since these cells expressed collagen I and TGF beta, they may play a central role in the specific location of Ad-2D6-induced fibrosis.

Conclusion:
Infection of wildtype FVB mice with Ad-2D6 triggers an autoimmune liver disease which results in permanent hepatic fibrosis. This model should shed light on autoimmune processes triggering fibrogenesis in the liver.
The role of different CD4 T-cell subsets in Experimental Autoimmune Glomerulonephritis

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CD4 T cells are considered as central mediators of anti-glomerular basement membrane glomerulonephritis (GN), and Th1 and Th17 cells have been implicated in induction and progression of renal disease. However, the exact role of these T-cell subsets in GN is still unclear. Therefore, we determined the role of Th1 and Th17 cells in the mouse model of Experimental Autoimmune Glomerulonephritis (EAG), which is characterized by autoimmunity against collagen fragments in the glomerular basement membrane. DBA/1 mice were immunized with the non-collagenous domain 1 of human α3IV collagen (α3IV-NC1). Spleen cells from immunized mice were stimulated with α3IV-NC1 and analyzed by intracellular cytokine staining. We detected IFN-γ and IL-17A in CD4 T cells indicating the generation of α3IV-NC1-specific Th1 and Th17 cells, respectively. To analyze the impact of Th1 and Th17 cells, DBA/1 mice deficient in IFN-γ receptor or in IL-17A and IL-23p19 were immunized with α3IV-NC1 and development and severity of EAG were determined. Wildtype mice and mice deficient in IFN-γR, IL-17A or IL-23p19 developed a similar degree of proteinuria and of clinical disease. All mouse strains also mounted a strong antibody response to α3IV-NC1 with a similar IgG isotype profile. However, histological analysis revealed less severe renal damage with reduced crescent formation in gene-deficient mice. In conclusion, our results indicate that immunization of mice induces α3IV-NC1-specific Th1 and Th17. Absence of Th1 or Th17 cells does not impair formation of specific IgG and proteinuria, however, both T-cell subsets contribute to renal damage.
A new generation of MHC II multimer reagents allows efficient enumeration of antigen-specific CD4+ T cells by flow cytometry

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Sensitive and reliable monitoring of cellular immune responses is important in immunological research, vaccine and immunotherapeutic development. Flow cytometric analysis using fluorescent MHC class I multimers is now a well established tool for enumeration and phenotypic characterization of antigen-specific CD8+ T cells. In contrast, the use of MHC class II multimers to detect antigen-specific CD4+ T cells is still very sparse, due to technical difficulties in producing high-quality MHC class II multimers. Peptide-MHC (pMHC) class II complexes typically have low affinity for their cognate TCRs, increasing the difficulties to detect and enumerate antigen-specific CD4+ T cells involved in e.g. autoimmune or inflammatory disease using conventional MHC multimers such as Tetramers.

Furthermore, efficient loading of peptide into the MHC class II complexes has been difficult. This is particularly problematic in the context of conventional MHC multimers because of their low valency, i.e. the fact that these only carry a few MHC complexes per reagent.

Here it is shown how the higher-valency MHC II Dextramer reagents can overcome these problems, thus enabling reliable detection of antigen-specific CD4+ T cells. It is shown how the increased resolution of the Dextramers allows accurate enumeration of antigen-specific CD4+ T cells that cannot even be detected by conventional multimers.
Pro-inflammatory Progranulin Antibodies occur in a wide spectrum of Autoimmune Diseases and are associated with alternative Progranulin conversion

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Introduction:
Autoantibodies play a major role in ANCA-associated vasculitis (AAV) as well as in non-ANCA-associated primary vasculitides as demonstrated by beneficial effects of B-cell depletion therapy. In order to identify new disease specific or pathogenically relevant autoantibodies in systemic vasculitides, we screened protein macroarrays with sera from patients with ANCA-associated and ANCA-negative primary systemic vasculitides.

Methods:
Screening of patients' sera for unknown autoantibodies was performed with protein macroarrays of human cDNA expression libraries. Identified candidate antigens were verified by ELISA. Verified candidate antigens were analysed by Sanger sequencing and Western-blotting for immunogenic mutations or secondary modifications. Progranulin serum levels were determined using a commercially available ELISA-kit (Adipogen). Functional effects of progranulin-antibodies were tested in cytotoxicity assays (EZ4U) by administration of TNF-α and serum with or without progranulin antibodies to cultured WEHI-S cells.

Results:
Autoantibodies against progranulin, a secreted and direct inhibitor of TNF-α receptors 1&2 (Tang et al., 2011) were frequently identified with relevant titres in primary vasculitides. In detail, progranulin antibodies were found during the course of disease in giant cell arteritis / polymyalgia rheumatica (14/65), Takayasu’s arteritis (4/13), classical panarteritis nodosa (4/10), Behcet’s disease (2/8), in granulomatosis with polyangiitis (31/81), Churg-Strauss syndrome (7/31) and in microscopic polyangiitis (6/17). In extended screenings progranulin-antibodies were also frequently detected in autoimmune connective tissue disorders, in rheumatoid and psoriatic arthritis and in inflammatory bowel disorders. In contrast progranulin-antibodies were detected only rarely in healthy controls (1/97), in patients with obesity (0/40), in residents of nursing homes (1/48), and not at all in patients with cutaneously limited psoriasis (0/100), in patients with sepsis (0/22) and in patients with melanoma (0/98). Sanger sequencing revealed no differences in the progranulin gene between antibody-positive or -negative patients. However, we observed in western-bLOTS of PBMCs and LCLs of all progranulin-antibody positive patients an exclusive alternative conversion/cleavage pattern of progranulin, showing an additionally and larger N-terminal fragment (55kDa instead of 45 kDa) and a shorter C-terminal fragment (25 kDa instead of 35 kDa). Moreover, a significant association of progranulin antibodies with active disease states in granulomatosis with polyangiitis suggests a pro-inflammatory activity of progranulin antibodies. This was supported by a neutralizing effect of progranulin antibodies on the levels of circulating progranulin. Moreover, functional assays revealed that progranulin antibody containing sera render WEHI-S cells far more sensitive to effects of TNF-α than sera of matched controls, providing evidence for the suspected pro-inflammatory effect of progranulin antibodies.

Conclusion:
Progranulin antibodies occur in a wide spectrum of autoimmune diseases and have a pro-inflammatory effect by neutralizing the physiological TNF-blocker progranulin. Alternative conversion of progranulin was exclusively observed in seropositive patients and might contribute to the break-down of self-tolerance to progranulin.
Effector CD8+ T cells recognize cross-reactive peptides in non-inflamed CNS but do not lead to autoimmunity in vivo

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T lymphocytes are critically involved in the initiation of CNS autoimmunity. In Multiple Sclerosis both CD4+ and CD8+ T cells are present in demyelinating lesions. Recent reports underline the importance of CD4+ T cells, however the role of CD8+ T cells is not clear. We followed here the question if CD8+ T cells are capable of initiating CNS autoimmunity or induce CNS damage. We used a T cell-acute hippocampal brain slice co-culture model and in vivo animal models to monitor immune-CNS interactions by two-photon laser scanning microscopy. Cross-reactivity of the OVA-specific transgenic T cell receptor on CD8+ T cells (OT-1) with a myelin antigen (MOG40-54) was exploited to monitor CNS-specific medium-avidity CNS-specific CD8+ T cells in CNS tissue ex vivo and in vivo. When challenged in vivo by adoptive transfer into lymphopenic Rag1-/- mice, in active EAE and passive EAE, we detected a clear-cut antigen recognition behavior of CD8+ T cells in EAE, although this did not lead to any clinical effect on disease course. Our findings suggest that CD8+T cells recognize antigenic peptides on CNS cells in vitro and in vivo in the CNS under inflammatory conditions, but this recognition is not sufficient to induce damage in a clinically significant manner.
CNS tissue response to cytokines and inflammation

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Introduction:
A well known inflammatory, autoimmune disease of the central nervous system (CNS) is multiple sclerosis (MS), where cells of the immune system invade the CNS, causing demyelination and neurodegeneration and leading to paralysis. The most common animal model of MS is experimental autoimmune encephalomyelitis (EAE), which can be induced by immunization of mice with myelin antigens. Not much is known about the response of neurons to the inflammatory triggers, although damage to them is the main initiator for paralysis in MS patients and EAE, because of the difficulty to analyze these cells. The CNS is composed of a pack arrangement of neurons and other cell types that makes it very difficult to separate and analyze them for gene expression on single cell level.

Objectives:
The aim of this project is to isolate neurons from adult mice afflicted with EAE and further analyze mRNA and micro-RNA (miRNA) expression levels as compared to RNA isolated from control animals. To this end, different experimental protocols will be established. Isolated neuronal mRNA and miRNA of naïve mice and mice with EAE will be sequenced by Next Generation Sequencing (NGS) to better understand how neurons respond to damage induced by inflammation. This study has implications for MS research and other inflammatory disease of the CNS. In the current innovative application we plan to establish two methods of RNA isolation from neurons.

Methods:
For the first approach, we will use transgenic mice generated by Frank Kirchhoff from Homburg. In these mice eYFP is expressed specifically in neurons under the human Thy1 promoter (Thy1-eYFP). Using of these reporter mice will allow us to isolate and sort neurons with high purity without fixation and staining steps based on eYFP expression, which is critical to validate the RNA quality.

In the second approach, we plan to immunoprecipitate mRNA with a modified Ribosomal Protein L22 including a HA-tag, using the RiboTag mice. After Crossing the RiboTag mice to the neuron-specific CaMKII-Cre mice, HA-tagged ribosomes will be specifically expressed in neurons.

Similar to the isolation of mRNA in the second approach, for isolating neuronal miRNA we will use the miRAP system, published last year. This system makes use of an MYC-tagged Argonaute protein AGO2. Epitope tagging of AGO2 protein allows direct purification of miRNAs bound to the AGO2, using antibodies against the engineered MYC-tag. Using a knockin mouse that can express miRAP following Cre-loxP mediated recombination, we will be able to express miRAP in neurons by crossing these mice with neuron-specific CaMKII-Cre mice.

Isolated RNA from neurons or from immunoprecipitation material of the RiboTag or miRAP mice will be further analyzed by deep sequencing using NGS technology in collaboration with the sequencing core facility of the Johannes-Gutenberg-University of Mainz.

Conclusion:
The final goal of this study is to develop new methods to analyze gene expression pattern of neurons that are attacked by pro-inflammatory cells and mediators during EAE. Understanding how neurons respond during autoimmune disease should allow designing better therapies that will protect these cells under such pathogenic conditions. This work has implications not only for patients of multiple sclerosis, but also for other neurological diseases, including Alzheimer’s disease and stroke.
Neuroectoderm-derived A20 is critical for restricting autoimmune encephalomyelitis in mice

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Question:
A20/TNFAIP3 is an ubiquitin-editing protein that is essential for the negative regulation of the NF-κB pathway. Tnfaip3−/− mice succumb to unfettered inflammation in several organs and targeted deletion of A20 in B cells, dendritic cells, or myeloid cells predisposes mice to autoimmunity. Polymorphisms and mutations in the A20 genomic locus are also associated with several human autoimmune diseases including multiple sclerosis. To study the contribution of A20 to the autoimmune disease experimental autoimmune encephalomyelitis (EAE), mice with A20 specifically deleted in neuroectoderm (astrocytes, neurons, and oligodendrocytes) was generated and investigated for EAE.

Methods:
Neuroectoderm-specific A20 knockout mice were generated by utilizing the Cre/loxP system under control of the Nestin promoter. A20 was efficiently and specifically deleted in the CNS of the Nestin-Cre A20fl/fl (KO) mice, while its expression was not affected in A20fl/fl (control) mice. EAE was induced by immunization with myelin oligodendrocyte glycoprotein (35-55) peptide. At day 15 and 22 post immunization, spinal cord was isolated for histological examination of demyelination and axonal damage, FACS analysis of inflammatory infiltration, and quantitative RT-PCR analysis of proinflammatory gene expression.

Results:
Nestin-Cre A20fl/fl mice developed clinically a more severe EAE than control mice. Histological examination revealed that demyelination and axonal damage was more prominent in the spinal cord of Nestin-Cre A20fl/fl mice than that in control mice at both day 15 and 22 post immunization. FACS analysis showed that there were more infiltrating CD4 T cells in the spinal cord of Nestin-Cre A20fl/fl mice. Consistent with the results of histology and FACS, higher levels of TNF-α, IFN-γ, IL-17, and GM-CSF were also detected in the spinal cord of Nestin-Cre A20fl/fl mice, indicating a stronger inflammation.

Conclusions:
Our results demonstrate that neuroectoderm-derived A20 is critical for restricting the development of EAE.
Defective cell death signalling along the Bcl-2 regulated apoptosis pathway compromises Treg cell development and function in mice

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Defects in cell death caused by overexpression of anti-apoptotic Bcl-2 or loss of pro-apoptotic Bim facilitates the appearance of autoimmunity in mice and is also associated with autoimmune diseases in humans. As the elimination of autoreactive lymphocytes in the thymus (central tolerance) is not complete, peripheral mechanisms involving regulatory T (Treg) cells keep these autoreactive cells in check (peripheral tolerance). Despite the well-established role of Bcl-2 family proteins in shaping the immune system and their frequent deregulation in autoimmune pathologies, it is poorly understood how these proteins affect Treg cell development and function. Here we compared the relative expression of a panel of 40 apoptosis-associated genes in Treg vs. conventional CD4 T cells. In the thymus, Treg cells expressed higher levels of Mcl-1, Bcl-xl, A1, Noxa and Puma whereas Bim expression was reduced compared to SP4 thymocytes. In contrast, in secondary lymphoid organs Treg cells displayed only increased amounts of Bcl-xl, A1 and Noxa. In addition, Treg cells displayed different susceptibility to apoptosis induced by cytokine deprivation, glucocorticoids, DNA damage, HDAC inhibitor and Fas/FasL ligation. Physiological significance of key changes was validated using gene-modified mice lacking or overexpressing pro- or anti-apoptotic Bcl-2 family members. We define a key role for the Bim/Bcl-2 axis in Treg cell development, homeostasis and function but exclude a role for apoptosis induction in responder T cells as relevant suppression mechanism. Notably, only lack of the pro-apoptotic BH3-only protein Bim or Bcl-2 overexpression led to accumulation of Treg cells while loss of pro-apoptotic Bad, Bmf, Puma or Noxa had no effect. Both Bim-deficient and Bcl-2 overexpressing Treg cells displayed reduced levels of key Treg cell markers compared to WT Treg cells. Remarkably, apoptosis resistant Treg cells showed reduced suppressive capacity both in vitro and in an in vivo model of T cell-driven colitis, posing a caveat for the use of such long-lived cells in possible therapeutic settings.
INTERFERON GAMMA PROTECTS MICE FROM CNS AUTOIMMUNITY ALSO IN THE ABSENCE OF IL-17A AND IL-17F

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Question:
The role of interferon (IFN)-gamma-producing T helper (Th) 1 and interleukin (IL)-17-expressing Th17 lymphocytes in multiple sclerosis (MS) and its animal model, experimental autoimmune encephalomyelitis (EAE) is still subject to intense investigations. Here we sought to decipher the specific involvement of these Th cell subsets during manifestation and the clinical phase of EAE. Special emphasis was put on IL-17 as effector cytokine and its cellular targets in the CNS context.

Methods:
We used a double-knockout mouse lacking both IL-17A and IL-17F (IL-17AFKO). Additionally, this mouse was crossed to the routinely used IFN-gamma deficient mouse, resulting in IL-17AFKO/IFNgKO triple-knockout animals. Mice were subjected to EAE induction with monitoring of the disease course and subsequent flow cytometric and histological analysis of CNS infiltrates.

Results:
As demonstrated before, following active MOG peptide immunization, IFNgKO mice developed an exacerbated EAE disease course compared to wild type C57BL/6 controls. In contrast, IL-17AFKO animals failed to induce prominent disease which was accompanied by a dramatic reduction of CNS-infiltrating immune cells. This phenotype could be partially reversed in triple-knockout animals as these mice showed a delayed onset of EAE with severity of disease reaching the level of wild type controls. Essentially, these results were confirmed in a model of passive EAE where in vitro-restimulated mutant T cells were adoptively transferred into wild type recipients. Transfer of IFNgKO as well as triple-knockout cells resulted in disease of comparable levels whereas IL-17AFKO cells failed to induce any signs of EAE. In addition, pilot experiments with manipulation of the gut microbiota revealed an essential connection between the commensal flora and the education of T cells. In fact, alterations of this system could partially reverse EAE resistance in our IL-17AFKO mice.

Conclusions:
Our results argue for a non-redundant role of IL-17(AF) for the induction of experimental neurodegenerative disease. Moreover, the beneficial role of IFN-gamma in EAE seems to be independent of the action of IL-17. Finally, our results clearly point towards T cells as carriers of these effects, which can be altered by interaction with the host microflora.
Dietary saturated fatty acids promote differentiation of Th1 and Th17 cells and aggravate neuroinflammation

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Question:
Multiple sclerosis (MS) is an autoimmune disease of the central nervous system (CNS) involving pathogenic Th1 and Th17 cells. While the genetic basis of this disease has been widely elucidated, environmental factors must ultimately drive the rapid increase of MS prevalence seen in the past half century. Epidemiological studies document a positive correlation between the incidence of MS and the intake of excess calories and saturated animal fat, but it is not clear if there is a causal relationship between intake of saturated fat and MS pathomechanisms.

Methods:
To explore possible mechanisms by which dietary fat could influence MS pathology, we investigated the influence of two different high fat diets (HFDs) in murine experimental autoimmune encephalomyelitis (EAE), an animal model of MS.

Results:
Feeding mice a HFD containing saturated mid-chain fatty acids or saturated long chain fatty acids consistently exacerbated clinical symptoms of EAE (mid-chain HFD; n = 4 HFD vs. 5 control, p < 0.05; long chain HFD, n = 4 HFD vs 6 control p < 0.05). Aggravated EAE under HFD containing mid-chain fatty acids was correlated with an increased ratio of activated (CD4\textsuperscript{+}CD62L\textsuperscript{low}) to naïve (CD4\textsuperscript{+}CD62L\textsuperscript{high}) T cells in the spleen (n = 3 p.g. p < 0.05) and increased numbers of Th17 cells in the CNS (n = 3 p.g. p < 0.05). In addition, we tested the effects of the saturated fatty acid laurate (C12/0) on T cell development \textit{in vitro}. Laurate (500 µM) enhanced the differentiation of Th17 (n = 3, p < 0.01) and Th1 (n = 6, p < 0.001) cells, while it diminished regulatory T cell development (n = 6, p < 0.05).

Conclusions:
Our findings suggest that dietary saturated fats may directly influence pathomechanisms underlying autoimmune neuroinflammation, corroborating the hypothesis that nutritional habits associated with a “Western” lifestyle contribute to MS prevalence and severity.
Interferon-beta efficacy is dependent on Indolamine-2,3-Dioxygenase in experimental autoimmune encephalomyelitis

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Question:
Interferon-beta (IFN-beta) is a standard treatment for relapsing-remitting multiple sclerosis, reducing relapse rates by one third and delaying progression of disability. However, the molecular mechanisms mediating its therapeutic efficacy remain obscure and we are lacking biomarkers which could reliably distinguish between responders and non-responders. Recent studies revealed that Th1- but not Th17-mediated experimental autoimmune encephalomyelitis (EAE) responds to IFN-beta treatment. Other reports could show that myeloid cells are the main responders to IFN-beta while T cell profiles do not change.

Degradation of the essential amino acid tryptophan by Indolamine-2,3-Dioxygenase (IDO) is a well-established immunosuppressive pathway important in several immunological settings like the fetal allograft, tumor immunology or autoimmunity. It is tightly regulated during inflammation, highly expressed in activated dendritic cells, and elicits its immunosuppressive effects by degradation of tryptophan and by production of its immunosuppressive metabolites, in particular kynurenine. The main inducers of IDO are type I and II interferons.

Methods and Results:
We treated murine EAE with IFN-beta. In contrast to wildtype (WT) mice, IDO-deficient (IDO\textsuperscript{-/-}) mice did not respond to IFN-beta treatment, indicating that the efficacy of IFN-beta is dependent on IDO. Chimeric mice, WT mice which were bone marrow transplanted with IDO\textsuperscript{-/-} haematopoietic stem cells, also showed a reduced response to IFN-beta, suggesting a role of IDO predominantly in leukocytes. qPCR analysis of sorted leukocytes cells from IFN-beta treated mice showed increased IDO expression in dendritic cells. We created a reporter mouse bearing GFP and luciferase in the IDO locus to detect IDO expressing cells upon IFN-beta treatment. Of note, flow cytometry analysis of encephalitogenic CD4\textsuperscript{+} T cells did not reveal any alteration in T cell subsets by IFN-beta treatment.

Conclusions:
We show that IFN-beta efficacy is mediated by IDO. This is most likely caused by induction of IDO in dendritic cells. Understanding the molecular mechanisms responsible for IFN-beta efficacy will guide the development of IFN-beta biomarkers and possibly novel therapeutic approaches.
Analysing the role of E2F7 and E2F8 in peripheral T cells and during T cell development

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Question:
Thymocytes undergo several important steps during their development towards mature T cells. Because of the random nature of the TCR rearrangement negative selection has to ensure tolerance through elimination of self-reactive thymocytes. We want to identify the master regulator that sets the machinery for negative selection in place.

Methods
We identified E2F7 and E2F8 as potential candidates by transcriptomic analysis. The expression levels of both genes during T cell development were verified by RT-PCR. Because the double deficiency is embryonic lethal (1) we crossed loxP-flanked alleles of E2F7 and E2F8 mice with Lck-cre and CD4-cre mice and analysed the phenotype of thymocytes and T cells by flow cytometry as well as apoptosis and proliferation.

Results:
E2F7 and E2F8 belong to the E2F family that is known to be important for differentiation, apoptosis and function as transcriptional repressors important for the control of cell proliferation (1). Astonishingly, we could show that the population sizes of thymocyte subsets, CD4+ and CD8+ T cells, naïve T-cells, T regs and TCRγδ T cells are not altered compared to control mice. Furthermore, we investigated B cells, macrophages, dendritic cells, NK and NK T cells, again without finding any differences in conditional double knockouts compared to controls. In addition, no differences in proliferation and apoptosis could be detected.

Conclusion:
Although E2F7 and E2F8 are highly expressed during the DP stage of T cell development compared to other stages those transcription factors seem not to play an important role at this stage of development and within mature T cells.

(1) Jing Li, Cong Ran, Edward Li, Faye Gordon, Grant Comstock, Hasan Siddiqui, Whitney Cleghorn, Hui-zi Chen, Karl Kornacker, Shusil K. Pandit, Mehrbod Khanizadeh, Michael Weinstein, Gustavo Leone, Alain de Bruin; Synergistic Function of E2F7 and E2F8 is Essential for Cell Survival and Embryonic Development; Dev Cell. 2008 January
Recent studies show that NF-kB signaling is involved in T and B cell development in mice. Knock-out mouse models confirm the importance of NF-kB in commitment and maturation of lymphocytes. Here we focus on T cell development in thymus of mice that lack the inhibitory p100 subunit, but still express the p52 subunit of NF-kB. p100-deficient mice (p100−/−) are characterized by increased alternative NF-kB signaling due to the constitutive presence of p52-RelB heterodimers in the nucleus. p100−/− mice develop premature thymic atrophy that is accompanied by a severe loss of double-positive (DP) CD4+CD8+ T cell precursors. Since it has been reported that p100-deficient mice have high corticosteroid levels which might result in massive DP cell death, we analyzed the status of DP thymocytes in p100−/− mice with defective glucocorticoid signalling. Therefore, p100−/− mice were interbred to GRdim mice, which carry a missense mutation in the dimerization domain of the glucocorticoid receptor. The GRdim mutation prevented the severe apoptosis of DP T cell precursors and restored the CD4+CD8+ precursor profile (FACS analysis) of the p100−/− mice. We conclude that the thymocyte hypoplasia in p100−/− mice was due to increased systemic stress mediated by increased glucocorticoid levels. To analyze the function of a constitutively active alternative NF-kB signaling in the hematopoietic compartment we generated bone marrow chimeric mice (p100−/− → WT). In these chimeras the homeostasis of abT cells, gd T cells and dendritic cells was not altered. The thymic invariant natural killer T (iNKT) cells were mildly blocked in the transition of the maturation status from the NK1.1− to NK1.1+ stage. In contrast, functional analysis of the of p100-deficient abT cells revealed a slightly increased proliferation in steady state. Consistent with this finding, the T cells produced increased levels of the mitogenic cytokine IL-2. In addition, they showed a reduced production of the Th2 cytokine IL-4. To investigate a function of a constitutively active alternative NF-kB signaling in thymic stromal cells we transplanted p100−/− fetal thymi under the kidney capsule of wild type host mice. Initial analysis of these p100−/− thymus grafts mice did not reveal differences in the abT cell or gdT cell development. Surprisingly, iNKT cell showed a similar mild block in the NK1.1− to NK1.1+ maturation as observed in the p100−/− → WT bone marrow chimeras. These results indicate that the constitutive activation of the alternative NF-kB pathway in the hematopoietic compartment results in mild changes of the abT cell homeostasis and function. A constitutively active alternative NFkB signaling pathway in the thymic stroma as well in iNKT cells per se inhibits their maturation into NK1.1+ iNKT cells.
Anti-inflammatory effects of NRI-1006 in the CIA mouse model of rheumatoid arthritis

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The purpose of our study was to investigate whether the immune modulator NRI-1006 has an impact on clinical signs of experimentally induced rheumatoid arthritis in DBA/1 mice.

Female DBA/1 mice were sensitized with a collagen II emulsion on day 0 and boosted on day 21. The animals were scored from day 17 for clinical signs of collagen induced arthritis (CIA). If the evaluated score exceeded a value of 5, CIA was considered to be established. From the day of CIA onset animals were treated with NRI-1006 or saline in different doses and treatment regimens. Scoring was done for a total of 21 days after CIA onset. Animals which developed CIA within the first 35 days after the immunization were included into the study.

Control animals as well as mice treated with NRI-1006 exhibited CIA and showed an increasing score within the observation period, whereas animals treated with the compound showed a reduction of clinical symptoms. 21 days after CIA onset, animals were sacrificed and samples were preserved for further investigations like cytokine measurements, histology and mRNA expression analyses.

In conclusion, NRI-1006 had a reducing effect on the severity of CIA symptoms in mice and may therefore present a novel therapeutic approach for patients suffering from rheumatoid arthritis and other inflammatory autoimmune diseases.
Induction of a MOG-specific immune response with subclinical inflammation in mice with a reconstituted human immune system

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Multiple sclerosis (MS) is a chronic inflammatory disease of the central nervous system (CNS), characterized by demyelination and loss of neurological function, local infiltration of T cells and macrophages.

Introduction:
Experimental autoimmune encephalomyelitis (EAE) serves as animal model for MS and can be induced in susceptible mice by the induction of myelin specific immune responses following active immunization against the latter antigens. Animal EAE models and MS show many similarities, but especially the recent years demonstrated important discrepancy of the model and the human disease. One disadvantage of the EAE model is the disparity of the mouse vs. the human immune system precluding pre-clinically testing of human-specific biologicals.

Objective:
For bridging the gap between mouse models and human clinical trials we envisaged the development of an EAE model in humanized mice, namely mice that contain a human immune system.

Methods:
For that purpose we humanized immunodeficient NOD/SCID/gc⁻/⁻ (NSG) mice with human peripheral blood mononuclear cells (PBMCs) of adult healthy donors, which permit the engraftment and expansion of human immune cells in vivo. These mice were then immunized with a combination of myelin-antigen pulsed dendritic cells (DCs) and active immunization in CFA with the latter antigens. Control mice were immunized with CFA and no antigen or with CFA and OVA.

Results:
We found engraftment of human B and T cells in the spleen of all humanized mice. CNS infiltration and human anti-myelin antibodies in the serum were found only in mice immunized with the combination of myelin-antigen. However no human monocytes were found in any organ of these mice. Notably, T-cells recovered from animals immunized with myelin-antigen showed specific proliferation and proinflammatory cytokine production such as IL-6, TNF-α, IL-17A, GM-CSF and IFN-γ when stimulated in vitro with myelin antigens. A non-specific infiltration or inflammatory reaction due to Graft-versus-Host disease was ruled out by histological scoring of gut, lung, skin and liver.

Conclusion:
In summary, we show that recombinant rat MOG protein 1-125 (rMOG) can be used as myelin-specific antigen in the humanized mouse model, and their use can lead to myelin-specific autoimmune responses in humanized mice. To improve this humanized mouse model we need to overcome the stage of subclinical inflammation which we believe is due to the lack of human monocytes in these mice. With a humanized mouse model, that develops full clinical EAE, autoantigens and possible MS-therapies targeting the human immune system could be tested in vivo.
Mutations in mitochondrial DNA (mtDNA) are thought to contribute to autoimmunity as mitochondria play a central role in most cellular processes including, activation, proliferation, oxidative stress and apoptosis. The most plausible mechanism is the increased production of reactive oxygen species (ROS) and its downstream effects. The mitochondrial proteins involved in ROS production are encoded by both the mitochondrial and nuclear genomes. However, due to the lack of any appropriate models, the direct evidence of the association between single mtDNA mutations and autoimmune skin blistering diseases (AIBD) is still to be confirmed. Our group has recently generated a series of conplastic strains carrying distinct mtDNA mutations. Using these conplastic strains, we are studying the role of mtDNA mutations in AIBD. We locally induced tissue damage in the skin by transferring rabbit anti-murine type VII collagen into ears (local EBA model) of a series of conplastic mice and observed varied disease susceptibility in mice with different mtDNA mutation. Conplastic mouse strains carrying a mutation in the ATP8 gene in complex V showed significantly reduced disease severity compared to the reference strain (p<0.005). The similar phenomenon was observed in conplastic mice carrying one mutation in the NDS gene in complex I and one in the COX3 gene in complex IV (p<0.014). We are currently further investigating to identify the underlying cellular and molecular pathways that are affected by the mtDNA mutations.
Towards Understanding the Pathogenesis of Granulomatosis with Polyangiitis: The Role of Neutrophil Extracellular Traps

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Granulomatosis with polyangiitis (GPA, Wegener’s granulomatosis) belongs to the antineutrophil cytoplasmic antibody (ANCA)-associated vasculitides and is frequently accompanied by the presence of autoantibodies against proteinase 3, a serine-protease located in the granules of neutrophil cells but is also detectable in neutrophil extracellular traps (NETs). NETosis, the ejection of DNA fibers armed with antimicrobial enzymes, is a physiological defence mechanism against invading pathogens but has also been linked to autoimmune diseases, e.g. systemic lupus erythematosus (SLE).

The aim of the study was to analyse the role of NETting neutrophils in the pathogenesis of GPA, especially their interplay with mononuclear cells (MNCs): We investigated the effect of NETs on proliferation of T- and B-cells (FACS), production of PR3-ANCA (ELISA and ELISpot), and secretion of the cytokines IL-4, IL-10, IL-17A and IFN-γ (ELISA) using isolated MNCs from PR3-ANCA-positive GPA patients (n=5) and healthy donors (n=5).

We could show that NETs of patients and healthy controls induce proliferation of CD4⁺ T-cells and CD19⁺ B-cells and maturation of B-cells. Production of IgG-PR3-ANCA was not detectable using ELISA or ELISpot technique. Furthermore, we detected a significant increase of IL-17A secretion after stimulating MNCs with NETs. A significant difference between MNCs from GPA patients and healthy controls was not detectable.
Grb2 regulates B cell maturation, B cell memory responses and inhibits B cell Ca\textsuperscript{2+} signalling.

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Growth factor receptor bound protein 2 (Grb2) is a small ubiquitously expressed adapter protein. It contains one central SH2 domain that is flanked by two SH3 domains and has no catalytic activity. In growth factor receptor signalling Grb2 activates Ras and MAP kinases (Buday and Downward, 1993; Egan et al, 1993). Whether Grb2 regulates MAP kinase activity in BCR signalling is controversial. It has been further described that Grb2 can inhibit BCR-induced Ca\textsuperscript{2+} signalling in B cell lines (Stork et al, 2004). Grb2 also binds to a multitude of other signalling molecules including PI3K, CD19 and BCAP (Neumann et al, 2009). To determine the physiological role of Grb2 in B cells, we generated a B cell specific Grb2-deficient mouse strain. We observed a strongly reduced number of mature follicular B cells in the periphery either due to a differentiation block or due to decreased survival. This phenotype cannot be rescued by crossing the Grb2-deficient mice with animals transgenic for the survival factor Bcl2, indicating that the reduced numbers of mature B cells are due to a Grb2-dependent differentiation block. Several important signalling pathways were altered in Grb2-deficient mice. These mice showed enhanced BCR induced Ca\textsuperscript{2+} signalling, modified MAPK activation patterns and also strongly impaired Akt and Foxo activation, suggesting a defect in PI3K signalling. Grb2 is described as important for Fc\textgamma{R2b} signalling (Neumann et al, 2011). Unexpectedly the reduction of Ca\textsuperscript{2+} signalling by crosslinking the BCR and the Fc\textgamma{R2b} was similar in Grb2-deficient mice to wild type mice. Interestingly, Grb2-deficient mice showed an impaired germinal center formation and had an impaired IgG and B cell memory response. This was detected after hCMV virus-like particle immunization and memory B cell transfer. A recently discovered Grb2 binding motif of the IgG tail is likely responsible for this phenotype (Engels et al, 2009). Summing up, Grb2-signalling is crucial for lymphocyte differentiation processes, as well as for the control of secondary humoral immune responses.

The genome of vertebrates contains endogenous retroviruses (ERVs) that are largely non-functional relics of ancestral germline infection by exogenous retroviruses. However, in some mouse strains ERVs are actively involved in disease. Here we report that nucleic acid recognizing Toll-like receptors (TLR) 3, -7 and -9 are essential for the control of ERVs. Loss of TLR7 function causes spontaneous retroviral viremia that coincides with the absence of ERV-specific antibodies. Natural antibodies against ERVs seem to be essential for the protection against ERVs. In summary, our study suggests that in addition to their role in innate immunity against exogenous pathogens, nucleic acid recognizing TLRs contribute to the immune control of activated ERVs and ERV-induced tumors by inducing protective antibodies. We generated monoclonal Ab from the natural occurring anti-ERV immune response and characterized also the anti-ERV response of sterile wild type mice. Implications for the function of B cells against ERVs will be discussed.
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Swiprosin-1/EFhd2 limits autoantibodies, the germinal center reaction and the IgE response

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Question:
Activated B cells become selected in germinal centers by regulation of their apoptosis and survival. The Ca²⁺ binding adaptor protein EFhd2 (also named Swiprosin-1) is able to promote apoptosis in activated B cells. EFhd2 might therefore limit humoral immunity by repressing the germinal center reaction and, thereby, prevent autoimmune or hypersensitivity reactions.

Method:
To test this hypothesis, we established EFhd2−/− mice.

Results:
B and T cell development as well as basal immunoglobulin levels were normal but EFhd2−/− mice developed anti-nuclear IgG antibodies spontaneously. In accordance with these findings, our preliminary data from ongoing experiments suggest that EFhd2 message is down-regulated in peripheral blood monocytes of humans suffering from systemic lupus erythematoses. In addition, Th2 inducing T dependent immunizations elicited higher antigen-specific antibody titers. Accordingly, infection with the hookworm Nippostrongylus brasiliensis induced strongly elevated IgE amounts and enhanced numbers of IgE⁺ plasma cells in EFhd2−/− mice in a B cell intrinsic manner. Moreover, EFhd2−/− mice developed enhanced germinal center reactions after Nippostrongylus brasiliensis infection and sheep red blood cell immunization. This propensity to hyperactivation was reflected by elevation of Igε transcripts after BCR and CD40 stimulation of EFhd2−/− B cells.

Conclusions:
We conclude that EFhd2 is a limiter of the germinal center output, with implications for autoimmunity, parasite biology and type I hypersensitivity reactions. Our data obtained with human samples data enforce our working hypothesis of EFhd2 as a disease-relevant negative regulator of humoral immunity.
A loss-of-function approach identifies the miR-15 family as a central regulator of early B cell proliferation, survival and differentiation

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**Question:**
MicroRNAs (miRNAs) are \textit{~}22-nucleotide noncoding RNAs that mediate post-transcriptional silencing of a predicted 60\% of protein-coding genes in mammals. Although miRNAs are prominently expressed in cells of the immune system, only a few have been conclusively studied with respect to their function. This project aims to provide a conclusive roadmap for the role of all miRNAs throughout early B cell development.

**Methods:**
A common experimental setup to investigate the function of a microRNA is a “gain-of-function” approach, in which the microRNA of interest is overexpressed to analyze its impact on a given process. However, a major drawback of this technique is that microRNAs, when expressed significantly above endogenous levels, may target genes that are not affected under physiological conditions. To circumvent this problem, we have generated a retroviral microRNA-knockdown library encompassing the majority of the microRNA families expressed throughout lymphocyte development. MicroRNA knock-down is mediated by so-called sponges, mRNAs that possess multiple binding sites for a certain microRNA family in their 3' UTR region. MiRNA sponges function as competitive inhibitors that sequester all specific microRNA/RISC complexes, thereby derepressing the endogenous target genes. With such a “loss-of-function” approach the physiological role of endogenous microRNAs can be elucidated.

**Results:**
Using this library in pre-B cells as a model system, we have characterized all microRNAs expressed in early B cells development according to their impact on processes such as proliferation, apoptosis and differentiation. In doing so, we show that functional knockdown of the known tumor-suppressive miR-15 family, comprising miR-15a/b, miR-16-1/2, miR-195 and miR-497, has only slight effects under cellular steady-state conditions. Upon withdrawal of the growth factor IL-7, however, loss of miR-15 function almost completely inhibits pre-B to immature B cell differentiation, partially protects from apoptosis and enables prolonged proliferation. In correspondence with that, knockdown of the miR-15 family confers a competitive advantage in suboptimal IL-7 concentrations, indicating that levels of miR-15 determine the sensitivity of cells towards growth factor receptor signaling.

Interestingly, IL-7 withdrawal seems to increase the activity of endogenous miR-15 family members in pre-B cells. Together with the loss-of-function data, this suggests that miR-15 functions as a central element in a positive feedback loop that reinforces cell-cycle arrest at low growth factor concentrations, which is considered a prerequisite for Rag1/2-mediated light chain gene recombination and differentiation.

**Conclusions:**
Together, our data identify the miR-15 family as an essential factor of early B cell development by regulating proliferation, differentiation and cellular fitness. This suggests that aberrant expression of the miR-15 family might not only contribute to cancer, but may also play a role in immune diseases. However, ongoing work is needed to elucidate how the miR-15 family is integrated into the regulatory network at the pre-B cell stage, especially in terms of its transcriptional regulation and relevant target genes.
A main focus of our research in the last years was the role of BCR expression as well as BCR signaling in the maintenance of mature murine B cells. The BCR is composed of the heavy chain (HC), light chain (LC) and the signaling molecules Ig-a and Ig-b.

Switching-off Ig-a expression in mature B cells results in loss of the surface BCR. To delete Ig-a we are using the cmb-1 mouse model, in which the tamoxifen-inducible Cre (mbt-CreER\textsuperscript{T2}) as well as an Ig-a/GFP\textsuperscript{inv} cassette are expressed under the control of the Ig-a encoding mb-1 locus promoter. Application of tamoxifen results in the inversion of the Ig-a/GFP\textsuperscript{inv} cassette and in expression of GFP in Ig-a and BCR deficient B cells allowing us to easily trace the fate of these cells by flow cytometry. Although BCR-deficient B cells have been described to be short-lived, Ig-a knockout (Ig-a KO) B cells were detected for up to 200 days in tamoxifen-treated mice. In accordance with the literature and in contrast to mb-1 deletion, conditional deletion of the HC gene with mbt-CreER\textsuperscript{T2} led to rapid elimination of mature B cells within 20 days. This might be due not only to lack of the survival signal but also to the induction of apoptosis via the unfolded protein response (UPR) detecting unassembled BCR components in the endoplasmic reticulum (ER). Mature B cells derived from the HC deleted mice show signs of UPR activation documented by the splicing of xbp-1 and phosphorylation of PERK. These two branches of the UPR are responsible for maintaining cellular homeostasis and consequently survival however under overwhelming conditions UPR signaling can lead to apoptosis. Surprisingly, deletion of Ig-a or Syk in mature murine B cells lacking the HC abrogated the UPR. In the meantime we are investigating the mechanism leading to elimination of HC-deleted mature B cells and the role of BCR signalling machinery in inducing UPR.
The role of the deubiquitinating enzymes CYLD and A20 in B-cell maintenance and maturation

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The NFκB-pathway plays a central role in development and survival of lymphocytes and controls the immune response to invading pathogens. In addition it was shown that it is a mechanistic link between inflammation and cancer and therefore its regulation is extremely important. CYLD and A20 are both negative regulators of the NFκB pathway by removing activating lysine-63 linked ubiquitin chains from numerous NFκB signaling molecules. Furthermore A20 is able to target those for proteasomal degradation by editing lysine-48 linked ubiquitin chains. Although both deubiquitinating enzymes share some of their target proteins, little is known about their capability to compensate for each other in controlling B cell maturation and maintenance, regulating NFκB-pathway activity, or preventing cancer development.

Therefore we crossed CYLD^{fl/fl} mice to mice that lack A20 expression in B cells (A20^{fl/fl}CD19Cre) creating a mouse strain deficient for FL-CYLD, and A20 in B cells. In addition these B cells over-express sCYLD, a naturally occurring splice variant of FL-CYLD, lacking the binding domains for TRAF2 and NEMO. These mice exhibit an expansion of the B cell compartment in the peripheral lymphatic organs as well as an increase in germinal center B cell formation. B cells isolated from these mice show prolonged survival and increased proliferation after α-IgM and α-CD40 stimulation compared to CD19Cre control mice. Interestingly, in contrast to the B cell specific knockout of A20, which showed a significant reduction of marginal zone cells and a shift to the B2 B cell compartment in the peritoneal cavity, B cell specific double KO has an increased marginal zone B cell population and an increased B1a B cell population in the peritoneal cavity. In addition, these double KO mice with the overexpression of sCYLD exhibit a significantly elevated B1a cell population in all secondary lymphatic organs, together with a dramatic increase of CD5 positive B cells in the blood. This CD5 positive B cell population in the blood even increases over time up to 96% in aged mice.

These data indicate a role of CYLD or respectively sCYLD downstream of A20. Furthermore, the abundant appearance of B1 cells in lymphatic organs and blood, coupled with an increased expression of CD5 and CD23 on B cells, enhanced B cell receptor (BCR) signaling and survival might point to chronic lymphocytic leukemia (CLL) development.
Early B cell development requires balanced expression of miR-191

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B cell development is a tightly regulated process yielding cells capable of producing highly diverse antibodies by random rearrangement of the B cell receptor gene loci. MicroRNAs (miRs) are small non-coding RNAs which negatively influence gene expression patterns on a post-transcriptional level. Several miRs have been reported to influence and regulate hematopoiesis. However, little is known about specific microRNAs influencing lineage fate and development in the B cell lineage. We found that miR-191 is upregulated during B cell development.

In order to understand the role of miR-191 in controlling B cell development, a gain-of-function approach was applied by reconstituting irradiated mice with retrovirally transduced bone marrow. Over-expression of miR-191 led to an accumulation of preB1 cells at the expense of later developmental stages. Employing various in vitro differentiation protocols, we found that proB/preB1 cells exhibit a developmental block, suggesting difficulties during V-J rearrangement. This is supported by gene expression analysis which revealed a diminished expression of the rearrangement genes RAG1 and RAG2 during heavy chain rearrangement. Subsequently, we observed an impaired expansion potential of in vitro cultured preB cells which can be linked to the preBCR/IL-7R signaling axis. Furthermore, abundant miR-191 levels increased apoptosis levels in immature B cells upon IgM cross-linking conditions, mimicking negative selection.

Overall, our data suggest that modulation of miR-191 expression is required to maintain proper B cell development.
BH3-only proteins Bim and Bmf mediate B cell death upon BAFF depletion

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Q: BAFF is a prosurvival cytokine of the TNF family critical for B cell development. Signalling via the BAFF-Receptor secures survival beyond the T1 stage of development presumably via non-canonical NF-kB, AKT and/or SYK mediated induction of pro-survival Bcl-2 family proteins including Bcl2, Bcl-x or A1/Bfl1. Consistently, overexpression of Bcl-2 or Bcl-xl can partially restore B cell numbers in TACI-Ig transgenic or BAFF-R-/- mice. These prosurvival molecules are neutralized by BH3-only proteins, members of the same family, to trigger mitochondrial Bax/Bak dependent apoptosis when BAFF is lacking. The nature of BH3-only proteins activated by the lack of BAFF remains undefined.

M: Using B cells derived from BAFF-depleted TACI-Ig transgenic mice we explored changes in the expression levels of individual Bcl-2 family proteins. In addition, we monitored mice lacking individual BH3-only proteins for resistance to BAFF depletion upon injection of recombinant TACI-ig. These studies identified Bim and Bmf as possible mediators of B cell death in BAFF deprived B cells forming a rational for genetic rescue experiments.

R: B cells lacking Bim and/or Bmf showed partial resistance to cytokine-depletion and showed impaired responsiveness to BAFF ex vivo. Loss of Bim or Bmf partially rescued B cell deficiency and function in TACI-Ig transgenic mice and combined loss of both BH3-only proteins led to a complete rescue of B cell development and function in the absence of BAFF. Consistently, lack of Bim and Bmf led to B cell pathology in aged mice, leading to premature lethality.

C: We conclude that the BH3-only proteins Bim and Bmf are the key targets of BAFF mediated survival signalling in B cells.
B cell specific overexpression of Akt1 impairs CSR and BCR signalling

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Introduction:
The serine/threonine kinase Akt controls not only cellular metabolism, cell survival and proliferation but is also an important regulator for peripheral B-cell maturation especially representing a critical checkpoint in phases of proliferation and differentiation.

Material and Methods:
In this study we investigated the role of constitutive Akt 1 activation in B cell development and maturation by using a mouse strain allowing for the conditional expression of an N-terminally myristoylated Akt (ROSA-AKT-C) in cell types that express the Cre recombinase. The myristoylation signal recruits AKT-C to the plasma membrane where AKT becomes constantly phospho-activated. This mouse was crossed to CD19 Cre mice leading to the B cell specific overexpression of AKT-C (AktBOE).

Objectives:
We want to investigate in more detail the role of Akt1 in B cell development and maturation.

Results:
AktBOE mice displayed splenomegaly compared to control mice as a result of increased absolute numbers of B cells but also T cells, neutrophils, monocytes and mature macrophages. Interestingly, the majority of splenic B cells were IgM positive. Further, these B cells do not express CD23, which could be in addition confirmed by RT-PCR. The investigation of immunoglobulin titers by ELISA revealed significant reduced serum levels of IgM, IgG1, IgG2a, IgG3 and IgA. By in vitro class-switch analysis we detected a defected class switch to IgG1 of splenic AKTBOE B cells compared to controls. Furthermore, in vitro proliferation of splenic B cells, labeled with violet cell trace revealed a proliferation defect upon anti-IgM stimulation. So it seems that the overexpression of Akt1 influences the BCR signalling.

Conclusion:
Thus, the B cell specific overexpression of Akt1 underlines its importance in activation of the BCR, B cell maturation and class-switch recombination.

References
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A novel, feeder cell-free in vitro system for the development of human B cells from CD34-positive hematopoietic stem cells.

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B-lymphocytes develop in the bone marrow (BM) from hematopoietic and lymphocyte precursor cells. These early BM stages of B cell development are precisely regulated by the progressive rearrangement of immunoglobulin heavy (IgH) and light (IgL) chain gene loci, by lineage and stage-specific transcription factors and cycles of proliferation. There exist considerable differences in B-lymphopoiesis between mice and men including age-dependence, expression of cell surface markers, cytokines inducing growth and differentiation, the role of the pre-B cell receptor (pre-BCR) and of associated signaling molecules. Because B lymphopoiesis plays an important role in therapeutic approaches like BM transplantation or immunosuppression and because human BM as source for all B cell precursors is rare and difficult to access, in vivo and vitro model systems suited to address the biology of human B lymphopoiesis by direct, functional studies would be extremely useful.

Starting from CD34\textsuperscript{+} cells isolated from cord blood (CB) and from BM, we here report the analysis of early stages of human B cell development using humanized mice and a novel, feeder-free in vitro system. We provide strong evidence that both models precisely represent the characteristic stages of BM dependent B cell development up to the stage of surface IgM-expressing immature B cells. The in vitro system differs to the in vivo humanized mouse model as it bears the advantage of a simple design combined with direct and easy experimental accessibility. The combination of both models provides the opportunity to study factors, therapeutic protocols, compounds and mutations affecting the development of human B cells.

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Naïve cognate B2-cells as a direct target of HIV-derived lentiviral particles \textit{in vitro}.

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**Question:**
Lentiviral particles (LP) are considered as possible HIV vaccines. However, little is known about the direct impact of LP on antigen-specific B cells.

**Methods:**
Hen Egg Lysozyme (HEL) was incorporated into LP (HEL-LP) derived from HIV to study their effect on HEL-specific B cell receptor-transgenic B-cells (HEL\textsuperscript{+} B-cells) \textit{in vitro}. We focused on activation and differentiation of naïve B-cells after incubation with cognate and Ag-mismatched LP in the absence of CD4\textsuperscript{+} T-cell help. Using Ag-specific intracellular staining we could directly show both activation of transcription factors and phenotypical changes in B-cells after the contact with cognate LP in vitro.

**Results:**
We demonstrated the preferential binding of HEL-LP to HEL\textsuperscript{+} B-cells and their internalization. HEL-LP were able to effectively cross-link B-cell receptors and mediate the loss of surface CD62L. In the absence of CD4\textsuperscript{+} T-cells activation events induced by LP in cognate naïve B-cells included increased expression of activation and co-stimulatory molecules and enhanced cell proliferation as well. Additionally, the B-cell phenotype shift towards germinal center pattern with further differentiation into memory and IgG- and IgA-producing cells was observed. The CD4\textsuperscript{+} T-cell independent activation may be due to the LP-mediated induction of CD40L production by the cognate B-cells.

**Conclusions:**
Thus, even in the absence of CD4\textsuperscript{+} T-cells, LP provide strong direct activation of antigen-matched naïve B-cells, that should be taken into consideration while interpreting vaccination experiments.
B cell-intrinsic STAT6 controls the germinal center response in type 2 immunity

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Introduction:
Infection with helminth parasites induces a strong Th2 response resulting in the secretion of the cytokines IL-4 and IL-13. These cytokines promote isotype switching in B cells to IgG1 and IgE which contribute to the expulsion of intestinal parasites. On the other hand, in predisposed individuals, recognition of specific common environmental antigens also results in a strong type 2 immune response with IgE being produced against the allergen. Signaling through the IL-4 and IL-13 receptors leads to phosphorylation of the signal transducer and activator of transcription 6 (Stat6) and activation of IL-4 and IL-13 target genes. It has been reported that the main IL-4 secreting T cell population found in the germinal centers (GC) are follicular helper T cells (TFH), the cells specialized in providing help to B cells. However, it was also found that Stat6 in B cells outside of GC become phosphorylated too. It remains unknown how and when IL-4 and IL-13 produced by T cells help B cells to mount a primary immune response.

Objectives:
The aims of this work were to define the roles of IL-4/IL-13 in type 2 and type 1 humoral responses and to understand when secretion of IL-4/IL-13 is required for optimal B cell responses.

Materials & methods:
To induce a type 2 immune response, mice with a selective deletion of IL-4 and IL-13 in CD4+ T cells (CD4Cre x IL-4/IL-13F/F), IL-4/IL-13-/- and wt mice were infected with the helminth Nippostrongylus brasiliensis s.c or were injected via i.p with sheep red blood cells (SRBC) or with OVA-alum. For induction of Th1 immune response, mice were infected intravenously with the lymphocytic choriomeningitis virus (LCMV) or i.p with the mouse cytomegalovirus (MCMV).

Results:
We found a dramatic reduction in the numbers of GC B cells when investigating the type 2 immune response against the helminth Nippostrongylus brasiliensis, SRBC or OVA-alum in IL-4/IL-13-/- mice. Light zone B cells were specially affected. Mice with a selective deletion of IL-4 and IL-13 in CD4+ T cells (CD4Cre x IL-4/IL-13F/F) also showed a strong reduction of the GC B cell numbers and impaired IgE and IgG1 switching. Interestingly, the number of TFH cells remained unchanged in these mice indicating that TFH cells differentiate independently of both autologous and heterologous IL-4/IL-13 during a Th2-associated immune response. Follicular dendritic cells were not reduced in the absence of IL-4 and IL-13, suggesting that the antigen presentation to B cells is unaltered. After infection with LCMV or MCMV, GC in the lymph nodes of wild type mice did not differ from that of IL-4/IL-13-/- mice. Stat6 deficient B cells did not form GC in mixed bone marrow (BM) chimeras of STAT6-deficient and wt mice. TFH upregulate CCR5 in order to enter B cell follicles and GC. BM chimeras generated with BM from IL-4/IL-13-/- and CCR5-deficient mice showed no impairment in GC nor in IgE or IgG1switching.

Conclusion:
Secretion of IL-4/IL-13 from T cells is necessary for GC reaction in type 2 immune responses but not for GC that form in response to virus infections. STAT6-deficient/ wt mixed BM chimera revealed that IL-4/IL-13 regulated genes in the B cell are necessary for proper GC formation. Furthermore, secretion of IL-4/IL-13 outside of the germinal center is sufficient for mounting a robust GC reaction. Our future studies will focus on the IL-4/IL-13-induced genes in B cells that are required for the GC reaction.
The immunoglobulin tail tyrosine (ITT) in the BCR of IgG-switched memory B cells boosts their reactivation and increases antibody production

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Introduction:
Activated B cells undergo class switch recombination in germinal centers, which alters the isotype not only of the secreted antibody but also of the membrane-bound B cell antigen receptor (BCR). While membrane bound (m)IgM and mIgD in the BCRs of naïve B cells have a cytoplasmic domain of only three amino acids (aa) and rely therefore on Igα and Igβ to transduce signals, BCRs of the mIgG and mIgE isotype have a cytoplasmic domain of 28aa with a conserved tyrosine-based signaling motif, which could signal in addition to Igα and Igβ. Previously, we have shown that this immunoglobulin tail tyrosine (ITT) is phosphorylated upon BCR crosslinking and recruits then the ubiquitous cytoplasmic adaptor protein growth factor receptor-bound 2 (Grb2). Recruitment of Grb2 into the signalosome of mIgG- and mIgE-containing BCRs amplifies BCR-proximal signaling events including phosphorylation of SLP65 and subsequent Ca²⁺ mobilization.

Objectives:
Aim of this study was to determine the role of the ITT in B cell responses in vivo.

Materials & methods:
We generated a mouse strain, in which the ITT of mIgG1 is inactivated by a tyrosine to phenylalanine (YF) substitution. These animals were then immunized to analyze their IgG1 titers during the primary and secondary immune responses.

Results:
Immunization of mIgG1-YF mice with T-dependent antigens led to substantially reduced primary and secondary IgG1 antibody responses and a lower frequency of IgG1-secreting plasma cells compared with wild type mice. Germinal center reactions seemed normal and memory cell were formed at a similar frequency. In heterozygous animals, cells expressing YF-mutant mIgG1 had a strong competitive disadvantage in entering the plasma cell compartment compared with cells expressing wild type mIgG1. Finally, we transferred antigen-specific memory B cells into immunodeficient recipients that were then challenged with virus-like particles. Again, mIgG1-YF expressing cells responded poorly and yielded only reduced IgG1 titers.

Conclusion:
Our results demonstrate that ITT signaling is essential for the vigorous production of IgG antibodies, especially in secondary immune responses, which protect the animal from reinfection upon antigen reencounter.
B cell activation/plasma cell differentiation initiates an IL-10 response mediating a negative feedback limiting neutrophil-mediated inflammation

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Introduction:
B cell activation eventually results in plasma cell formation and the production of antibodies necessary for immune protection, but which can also cause immune complex initiated inflammation and tissue destruction. Activated B cells and particularly plasma cells can also produce considerable quantities of IL-10, a pleiotropic cytokine exhibiting prominent immunosuppressive capacities.

Objectives:
Here, we test the impact of B cell activation on the initiation of an IL-10 response and its consequence for immune suppression during a plasma cell response.

Materials and methods:
Specific activation of B cells was achieved by injection of goat anti mouse IgD crosslinking the B cell receptor. Controls used were goat serum and PBS. IL-10 production was determined in IL-10 reporter mice (VertX). Inhibition of IL-10 mediated effects was achieved by injection of blocking IL-10 receptor antibodies. Effect of FoxP3 expression was determined in FoxP3 reporter mice and followed by FoxP3 staining. IL-10 and IFN-gamma production by T cells was quantified by flow cytometric analysis of intracellular cytokine staining. Neutrophil migration was tested by using boyden chamber assays in vitro. Impact of a plasma cell response on neutrophil migration was determined by quantification of infiltrating neutrophils into the peritoneum after injection of C5a. Impact of a massive plasma cell response and IL-10 on a neutrophil mediated inflammation was tested in a model for an autoimmune dermatosis.

Results:
Injection of goat anti mouse IgD lead to a massive plasma cell response and the production of IL-10 mainly in plasma cells/plasma cell precursors and FoxP3+ CD4+ T cells, which was absent from both control groups (PBS and goat serum), indicating that the primary activation of B cells results in a massive IL-10 response. Despite the massive production of IL-10, the injection of goat anti mouse IgD did neither suppress G6PI-induced arthritis nor EAE, diseases which might be primarily induced by inflammatory T cells. However, antibody/immune complex initiated and neutrophil mediated skin inflammation was efficiently blocked in an IL-10 dependent manner. In vivo B cell activation inhibited neutrophil influx into the peritoneum after C5a injection. In vitro studies showed that IL-10 directly blocked neutrophil migration towards C5a, the anaphilatoxin mediating neutrophil infiltration to sites of complement activation.

Conclusion:
B cell activation and plasma cell formation induces an IL-10 mediated negative feedback suppressing neutrophil mediated inflammation. This mechanism might not be relevant during most immune reactions where neutrophil activity is required for achieving optimal protective responses. However, massive plasma cell responses observed during severe infections and autoimmune diseases can lead to unwanted immune complex mediated inflammation and tissue destruction, which is counter-balanced and limited by a so far unknown IL-10 mediated negative feedback mechanism initiated during B cell activation/plasma cell differentiation.
Clonal composition and functions of human mature CD5⁺ B cells

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Introduction:
Human CD5⁺ B cells represent a heterogeneous population. The CD5 marker is found on mature but also on transitional B cells and may be transiently expressed on activated B cells. CD5⁺ B cells are discussed as the normal counterpart of chronic lymphocytic leukemia (CLL). However, mature CD5⁺ B cells are - despite of their abundance in peripheral blood (PB) and lymphoid tissue - largely unexplored.

Objectives:
We aim to characterize mature CD5⁺ B cells in young healthy adults to obtain comprehensive insight into their immunological functions and understand the cellular source for malignant transformation in CLL.

Methods:
We will perform an in-depth analysis of the clonal composition of and clonal expansions among human mature CD5⁺ B cells from PB and peritoneum by deep sequencing of immunoglobulin V gene rearrangements. We also plan to study the specific gene expression profiles of these CD5⁺ B cell subsets. This may help to identify specific functional features of human CD5⁺ B cells, to be validated in in vitro assays. In a further experimental approach, we will analyze human mature CD5⁺ B cells for selected properties of murine CD5⁺ (B1a) B cells to reveal potential similarities or differences to this unique B cell lineage in mice.

Results:
We previously found indication that mature CD5⁺ B cells are oligoclonally expanded in young healthy donors and display a gene expression pattern distinct from conventional (CD5⁻) B cell subsets. Moreover, transcriptome analysis suggests a strong similarity between CD5⁺ B cells and CLL.

Conclusion: We obtained preliminary indication for large oligoclonal expansions among human mature CD5⁺ B cells and that mature CD5⁺ B cells have a distinct gene expression pattern, pointing to specific immunological functions.
The adaptor protein Sly2 modulates B cell functionality

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The adaptor protein Sly2 (SH3 Lymphocyte protein 2), also known as HACS1, SAMSN1 and NASH1 is primarily expressed in immune tissues, but is also found in heart, brain and placenta. Its expression was reported to be induced following B cell activating stimuli. The analyses of Hacs1−/− mice revealed an immunoinhibitory role for Sly2 in adaptive immune responses, however, so far a concrete mechanism how Sly2 regulates immune responses remained unknown. We generated Sly2−/− (KO), which are similar to Hacs1−/− mice, and tg-Sly2 (TG) mouse models, which overexpress Sly2 in B and T cells, to study the immunologic role of Sly2 in more detail. We could proof that Sly2 plays a crucial role in B cell spreading, since splenic B cells, that were isolated of Sly2 transgenic mice, showed severe defects in cell spreading. According to our additional findings, that Sly2 also interacts with the nucleation-promoting factor cortactin, we suggest Sly2 as an important player in the regulation of actin dynamics and B cell spreading in vivo. Moreover, we found reduced B-1 cell numbers and serum IgM levels in TG mice and increased B-1 cell numbers and IgM levels in KO mice under basal conditions, indicating a specific function for Sly2 related to B-1 cells. Accordingly, IgM responses following Pneumovax® 23 vaccination were significantly altered. Together, we show a potential mechanism how Sly2 impairs B cell functionality in vivo and provide additional proof for Sly2 as an inhibitor of B-1 cell mediated immunity.
miRNA processor DGCR8 is essential for B cell activation and germinal center formation

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MicroRNAs (miRNAs) are small noncoding RNAs that regulate gene expression at the posttranslational level. Primary miRNA transcripts are trimmed by a nucelar microprocessor complex consisting of Drosha and DGCR8 and the RNAse DICER. To analyze the role of miRNAs during B cell activation and germinal center formation we crossed DGCR8⁵/² animals with Aidca⁻/⁻ deleter mice. Upon immunization with TNP-KLH (keyhole limpet hemocyanin) or sheep red blood cells we found a significant reduction in the number of germinal centers (GC) as revealed by PNA staining using fluorescence microscopy and flow cytometry. A more detailed analysis revealed that DGCR8-deficient mice have significant lower numbers of GL7⁺/CD95⁺ GC B cells. Moreover antigen-specific serum IgG amounts are drastically diminished. Hence, we conclude that DGCR8 and consequently miRNAs are critical regulators in B cell activation and efficient germinal center formation. Specific miRNAs crucial for B cell activation can now be identified by restoring DGCR8-deficient B cells with retrovirally transduced miRNAs.
Indirect class switch recombination to IgE dominates the IgE repertoire in vivo.

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Introduction:
The immune system enables animals to respond to a large number of antigens. The adaptive immune system is composed of T and B cells which constitute the cellular and humoral arm of the immune response, respectively. B cells generate and secrete immunoglobulins which provide important protective functions against pathogens but are also involved in immune responses against self-antigens and allergens. The germinal center reaction plays a central role for the humoral immune response as B cells interact with T follicular helper cells and follicular dendritic cells in the GC to form Ig-class switched antibodies with high affinity.

Objectives:
High levels of IgG1 and IgE are expressed in mice after class switch recombination in Th2-associated immune responses like helminths infections or allergic inflammation. Class switch recombination to IgE can occur by direct switching from IgM or indirectly by a sequential switch reaction from IgM to IgG1 and finally to IgE. We wanted to determine the relative contributions of direct and indirect IgE switching in vivo using the Nippostrongylus brasiliensis infection model.

Material & Methods:
Mice were infected with Nippostrongylus brasiliensis L3 larvae subcutaneously. At day 12 after infection serum and draining lymph nodes were taken for analysis. Serum IgG1 and IgE were determined by ELISA. FACS analyses were used to determine the content of certain cell populations. RNA was isolated from samples of draining lymph nodes to perform deep sequencing analysis in order to determine the overlap between the IgG1 and IgE repertoires.

Results:
To determine whether IgE is switched directly or indirectly via IgG1 in Nippostrongylus brasiliensis infection we used infected fluorescent IgG1-reporter mice (Cg¹Cre/Cre ROSA26loxP-stop-loxP-YFP/) and found that about 60% of IgE+ plasma cells expressed YFP indicating that they were derived from IgG1-expressing precursors. Furthermore we could show that IgG1 and IgE responses were decreased in mice where IgG1 expressing B cells were deleted by expression of diphtheria toxin (Cg¹Cre/Cre ROSA26loxP-stop-loxP-DTA/+/). We further analyzed the IgG1 and IgE repertoires by deep sequencing using the FLX titanium 454 platform. We could found a striking overlap between IgG1 and IgE sequences within each individual mouse but not between different mice.

Conclusion:
Given the large overlap between IgG1 and IgE repertoires these data demonstrate that the indirect pathway for IgE switching is indeed a major pathway for generation of IgE antibodies in vivo and highlights the role of the germinal center reaction where the IgG1 switching and affinity maturation takes place before the final switch to IgE occurs.
Investigating the role of Bcl2a1/A1/BFL-1 in leukocyte development.

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Introduction:
Programmed cell death plays an important role in maintaining homeostasis in the immune system. Bcl2 family proteins control mitochondrial-induced apoptosis by either promoting or preventing membrane integrity and cytochrome c release into the cytosol leading to caspases activation. The interplay between proapoptotic and antiapoptotic Bcl2 family proteins controls the mitochondrial apoptotic pathway during B lymphopoiesis in the bone marrow and B cell maturation in spleen. Deregression of B cell selection processes by impairment of Bcl-2 regulated cell death can cause immunodeficiency, autoimmunity or contribute to malignant transformation.

Objectives:
Murine A1 protein is an anti-apoptotic member of the Bcl2 family encoded in four genes in mice, A1-a, A1-b, A1-c, A1-d, with A1-c representing a processed pseudogene. A1 is mostly expressed during embryogenesis and in the hematopoietic system in adult mice. Activation of the BCR in B cells or the TCR in T cells is associated with increased levels of Bcl2a1, which suggests a cytoprotective function that is essential for the activation and survival of lymphocytes.

In transgenic Eµ-A1-a mice overexpressing A1-a protein the accumulation of pro-B cells and proportional decrease of class-switched mature B cells in the periphery has been observed. However, A1 is upregulated in mature B cells in periphery and during differentiation from transitional (IgMhigh) to mature follicular (IgMlow) B cells in the spleen. It was shown that constitutive A1 knockdown of all A1 isoforms in mice leads to a decrease in the percentage of mature follicular B cells and impairment of proliferation upon mitogenic stimulation. Whether these phenomena are due to impaired differentiation or increased B cell death remains unclear at present.

Materials & Methods:
To understand the role of A1 in hematopoietic system under physiological and pathological conditions we utilize Tet-regulated RNAi targeting all A1 isoforms in vivo. These double-transgenic animals harbor the miR30-embedded shRNA targeting A1 under control of the Tet-CMV™ promoter (referred as TREA1 mice) and the reverse Tet-transactivator (rtTA) placed under control of the CAG promoter (referred to as DTr mice). Administration of doxycycline drives the expression of a mi-shRNA targeting A1 leading to a significant knockdown of A1a, A1b and A1d mRNA. The knockdown efficiency was evaluated at the mRNA in developing as well as mature leukocytes.

Results:
FACS analysis of cells derived from primary and secondary lymphatic organs from DTr mice kept on doxycycline for 17 days revealed a variety of phenotypic abnormalities. We observed that inducible A1 KD show reduced percentages of total B cells in bone marrow, peripheral blood and lymph nodes mainly due to loss of mature (recirculating) B cells in bone marrow, lymph node and spleen. Most other cell types were not affected upon acute depletion of A1.

Conclusion:
We conclude that A1 is essential for the survival of mature follicular B cells, but dispensable for their development.
The Ca\textsuperscript{2+} binding adaptor protein EFhd1/Swiprosin2 needs to be down-regulated by the pre-B cell receptor to ensure efficient B cell development and homing

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The pre-B cell receptor (pre-BCR) regulates proliferation and differentiation of pre-B cells by changing their transcriptome. One of the genes that become down-regulated by the pre-BCR is efhd1. EFhd1/Swiprosin-2 and EFhd2/Swiprosin-1 are two conserved calcium-binding proteins with EF-hands that share 65\% sequence identity and protein-interaction domains. We have shown that in contrast to EFhd1, EFhd2 is expressed throughout all B cell stages. EFhd2 is involved in negative regulation of the germinal center reaction, helminth induced IgE and autoantibody production. EFhd1, on the other hand, has been implicated in carcinogenesis and cell proliferation. Its function in B lymphocytes, however, remains elusive.

To assess the functional impact of the pre-BCR induced down-regulation of efhd1, we ectopically expressed EFhd1 in freshly isolated pro-B cells. Furthermore, we generated transgenic (efhd1tg) mice that express EFhd1 under control of the VH promoter and Eµ enhancer also beyond the pre-B cell stage. To assess the function of EFhd1 in pro-B cells, where EFhd1 is expressed naturally, we are in the process of generating conditional EFhd1\textsuperscript{-/-} mice.

Ectopic expression of EFhd1 in pro-B cells by retroviral transduction slows down the decrease in surface c-kit expression, indicating a slower differentiation of pro-B into pre-B cells. efhd1tg mice (6-20 weeks old) show a trend to reduced B cells after the pre-B cell check point in the bone marrow. Mixed chimeras of wild-type and efhd1tg bone marrow reveal that wild type B lymphocytes have a significant advantage over efhd1tg B cells after the pre-B cell checkpoint. Surprisingly, however, although immature B cell numbers were reduced by ectopic EFhd1 expression, mature B cells in the spleen were significantly increased. In contrast, recirculating efhd1tg B cells were reduced in the bone marrow.

In conclusion, our experiments show that EFhd1 needs to be down-regulated by the pre-BCR to ensure efficient pre-B cell differentiation, and homing of recirculating B cells into the bone marrow. We are currently investigating whether these effects can be linked to the recently described interaction of EFhd1 with estrogen receptor alpha.
**Analysis of chemokine receptor expression and prognostic factors in B-CLL: interplay of CCR7 with CD38 with functional consequences on migration and apoptosis**

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**Introduction:**
B-cell chronic lymphocytic leukemia (B-CLL) is characterized by the progressive accumulation of distinct monoclonal B lymphocytes in blood and in secondary lymphoid organs (SLO). Chemokine receptors have been reported to promote homing of B-CLL cells into SLO which support their survival. Three chemokine receptors are mainly involved in the migration, localisation and maturation of B cells, namely CCR7 and its two ligands CCL19 and CCL21, CXCR4 and its ligand CXCL12 and CXCR5 and its ligand CXCL13. B-CLL patients with a low expression level of CD38 have been shown to have a better prognosis than the ones with a high level. CD38 expression has been associated to a more activated state with a higher reactivity to different stimuli including IFN and BCR stimulation.

**Objectives:**
Characterization of chemokine receptor expression and the responsiveness of B-CLL cells to chemokine stimulation according to known prognostic factors as CD38 and evaluation of the consequences of CD38 inhibition on chemokine signalling.

**Patients & methods:**
B-cells of 60 patients suffering from B-CLL were analysed and divided into two groups according to their CD38 expression status. We then investigated the ability of the cells to respond to chemokine stimulation in chemotaxis and apoptosis assays. In addition, we supported our results using the B-CLL cell line MEC-1 treated with CD38 agonists or antagonists.

**Results:**
B-CLL cells showed an increased level of chemokine receptors expression when compared to B-cells from healthy controls, however this expression was not changed during the progression of the disease (RAI status) and not associated to an increase in chemotactic ability. In the patients showing a low expression of CD38, chemotaxis towards the CCR7 ligands CCL19 and CCL21 allow to discriminate further between patients with a more aggressive disease or good prognosis. This observation was not linked to the level of expression of CCR7. Blocking CD38 in B-CLL cell lines resulted in increased migration ability toward different chemokines suggesting a role of CD38 in migration regulation. In addition, blocking CD38 enhanced the cellular apoptosis and this was reverted by treatment of the cells with CCL19 and CXCL13 but not CCL21 and CXCL12, suggesting a complex regulation interplay between CD38 and Chemokine receptors activation pathways.

**Conclusion:**
Our results suggest a complex interplay between chemokine receptors and CD38 on B-CLL leading to the regulation of cell localisation and viability. High CD38 expression is consistently associated with lower chemotaxis, however discrepancies between chemokines appeared in apoptosis regulation. Understanding these complex regulation processes might help to further discriminate between patients with good prognosis and those with a more aggressive disease, and the development of new therapeutic concepts.
Crosstalk between chemokine receptor and BCR signaling

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Introduction:
Chemokines are important for the normal function of all lymphocytes by guiding them to specific environments where they can undergo development and maturation. Among the most important chemokine receptors for B cells is CXCR4, which mediates homing of progenitor- and end stage B cells to the bone marrow. CCR7 and CXCR5 are responsible for the entry of later stage B cells into - and positioning within secondary lymphoid tissues. In B-cell chronic lymphocytic leukemia (B-CLL), all those receptors are severely dysregulated in comparison to B cells from healthy donors and may be associated with the survival of the tumor cells by guiding them to protective niches and activating pro-survival pathways. This is well known for CXCR4, which has been shown to engage signaling molecules of the BCR pathway like Syk and Lyn to mediate tumor-microenvironment interactions. In addition, antigen independent BCR signaling is a hallmark of CLL and has also been associated with tumor cell survival and growth.

Objectives:
We investigated the influence of BCR activation status on the expression of chemokine receptors on B cells as well as the function of those receptors. Furthermore, we looked for downstream signaling pathways on which chemokine receptor and BCR signaling may converge, comparing the effect of different chemokines and receptors in different B cell lines as well as B-CLL cells.

Materials & methods:
Six B cell lines representing different developmental stages, as well as primary B cells from healthy donors and CLL patients were analyzed. Surface expression levels of different chemokine receptors were detected by flow cytometry, and intracellular signaling events were evaluated by western blot. Functional activation was monitored by chemotaxis assays.

Results:
Within hours after BCR ligation, the surface expression of various chemokine receptors decreases, suggesting an unspecific internalisation of the receptors. After 24h of BCR stimulation by α-IgM, CXCR4 is downregulated while CCR7 and CXCR5 tend to be upregulated in different B-cell lines. Independent of receptor expression, BCR activation modulates some of the cellular answers to chemokine stimulation, such as MAPK activation and chemotaxis. These modulations change with the length of BCR engagement, and are unique for each chemokine receptor. For some of those answers, α-IgM and α-IgD stimulation show different and even controversial consequences. Whereas it is already known that CXCR4 stimulation leads to the activation of kinases of the BCR signaling pathway like Syk and Lyn, we show that they are also regulated by CCR7 and CXCR5. However the time course is different for each chemokine receptor, and in contrast to the other receptors, stimulation of CCR7 with its ligand CCL19 even decreases constitutive Lyn activation in CLL patient samples.

Conclusion:
Our results show that while all chemokine receptors share some common downstream pathways, they are differentially influenced by the activation state of a B cell. In the context of CLL, CXCR4 has been well established as a means of protection for the tumor cell. The role of the other chemokine receptors in B-CLL however is not as clear, and our results indicate that CCR7 stimulation in some cases even evokes effects contrary to those of CXCR4.
Imiquimod and resiquimod efficiently improve the immunostimulatory properties of human 6-sulfo LacNAc dendritic cells

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Activation of Toll-like receptors (TLR) by engineered agonists has been shown to induce antitumor reactions. In this context, imiquimod and resiquimod represent small-molecule agonists at TLR7/8, which emerged as attractive candidates for tumor immunotherapy by inducing proinflammatory immune responses. For example, the topical application of imiquimod was approved for the treatment of superficial basal cell carcinoma. In addition, the efficiency of imiquimod as a vaccine adjuvant has been investigated in clinical trials enrolling patients with prostate cancer and melanoma. The profound antitumor activity of imiquimod and resiquimod might depend on the efficient activation of TLR7/8-expressing immune cells. Therefore, we evaluated their impact on dendritic cells (DCs), which play a crucial role for the induction and maintenance of innate and adaptive antitumor immune responses. Recently, we defined 6-sulfo LacNAc (slan) DCs (formerly termed M-DC8+ DCs) as a major subpopulation of proinflammatory human blood DCs (Immunity 2002;17:289-301, Immunity 2006;24:767-777), which express TLR7/8. SlanDCs are characterized as principal producers of various proinflammatory cytokines. Furthermore, they efficiently activate CD4+ T lymphocytes and natural killer (NK) cells.

When analyzing the impact of imiquimod and resiquimod on the immunostimulatory properties of slanDCs, we demonstrated that both TLR7/8 agonists significantly enhance the production of the proinflammatory cytokines TNF-alpha, interleukin (IL)-1beta and IL-6. Notably, only resiquimod was able to induce the secretion of IL-12 by slanDCs. Functional data revealed that both TLR7/8 agonists improve the capacity of slanDCs to directly lyse tumor cells. In contrast to imiquimod, resiquimod markedly support the potential of slanDCs to activate NK cells and promote their interferon-gamma secretion as well as their tumor-directed cytotoxicity. In addition, resiquimod-activated slanDCs efficiently stimulate the proliferation of CD4+ and CD8+ T lymphocytes. These results provide evidence that imiquimod and resiquimod trigger various immunostimulatory properties of slanDCs, which may contribute to the antitumor effects mediated by these molecules.
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Tolerogenic human IL-10-modulated mature CD83$^{\text{high}}$CCR7$^{\text{high}}$HLA-DR$^{\text{high}}$ and immature CD83$^{\text{low}}$CCR7$^{\text{negative}}$HLA-DR$^{\text{low}}$ DC subpopulations as inducers of regulatory T cells

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Human IL-10-modulated, tolerogenic dendritic cells (IL-10DC), which are capable to induce anergic regulatory CD4$^+$ T cells (iTregs), consists of two subpopulations, mature CD83$^{\text{high}}$CCR7$^{\text{high}}$HLA-DR$^{\text{high}}$ and immature CD83$^{\text{low}}$CCR7$^{\text{negative}}$HLA-DR$^{\text{low}}$. Here, we investigated both IL-10DC subsets with regard to their phenotype and tolerogenic capacity in detail. As compared to fully mature DC (mDC) and the CD83$^{\text{high}}$ IL-10DC subset, the CD83$^{\text{low}}$ IL-10DC subpopulation exhibited a significantly diminished expression of CD80, CD86, ICOS-L, and CD40. In contrast, on both IL-10DC subsets we observed accompanied a slight (CD83$^{\text{low}}$ IL-10DC) and significantly (CD83$^{\text{high}}$ IL-10DC) upregulation of the (co-)inhibitory molecules PD-L2, ILT3, and ILT4, demonstrating significant differences in expression of costimulatory and inhibitory molecules between the two IL-10DC subsets. Notably, primary stimulation of naïve CD4$^{\text{positive}}$CD25$^{\text{low}}$CD45RA$^{\text{positive}}$ T cells and restimulation experiments demonstrated that both IL-10 DC subpopulations, regardless of their maturation state, induced anergic CD4$^+$ T cells as evaluated by a significantly reduced T cell proliferation and diminished Th1 and Th2 responses. In addition, both iTreg subpopulations showed regulatory activities and significantly suppressed the activation of responder T cells. The suppressive activity of both IL-10 DC subsets was found after various stimuli (syngeneic mDC, anti-CD3/anti-CD28mAb, PBMC/anti-CD3mAb) and was accompanied by loss of their anergic state during suppressor experiments. In conclusion, mature CD83$^{\text{high}}$CCR7$^{\text{high}}$HLA-DR$^{\text{high}}$ and immature CD83$^{\text{low}}$CCR7$^{\text{negative}}$HLA-DR$^{\text{low}}$ IL-10DC display properties of tolerogenic human DC, in particular of inducers of iTregs, which may be used as targets for the development of novel therapeutic approaches for allergies, autoimmune disease or transplant rejections.
Transcriptional targeting of mature DCs by the newly characterized human CD83 promoter - prospects for new DC vaccination strategies

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Question:
Since CD83 is one of the best-known surface marker for fully mature dendritic cells (DCs), its cell type- and maturation-specific regulation makes the CD83 promoter an interesting tool for the transcriptional in vivo targeting of mature DCs directly in tumor patients. However, the molecular mechanisms leading to its specific transcription were completely unknown.

Methods:
To determine the mechanisms regulating the cell type- and stadium-specific human CD83 expression, ChIP-on-chip, biocomputational, gene reporter, EMSA- and ChIP-analyses were performed. Transcriptional targeting of the DC cell line XS52 as well as of human mature DCs was analyzed by Western blot and ELISA.

Results:
Studies revealed a transcriptional activation complex formed by a 3D folding process involving three distinct DNA regions. Such a tripartite folding process has not been reported for any other gene so far. By ChIP-on-chip microarray we could identify a highly transcriptional active region within the human CD83 gene locus, particularly in mature DCs. Following deletion mutagenesis and gene reporter assays, we could characterize a short enhancer region of 185 bp. Importantly this regulatory element was demonstrated to be only active in mature DCs but not in immature tolerogenic DCs, or other cells expressing CD83, such as subsets of activated B- and T cells. Further biocomputational analyses identified an additional upstream regulatory element (URE) 85 bp upstream of the minimal promoter region which was shown to be the missing link to regulate cell-type-, stadium- and species-specific human CD83 expression (Fig.1). Moreover, these analyses indicated a complex framework of IRF- and NFκB-transcription factor binding sites (TFBSs) to be involved in the exact arrangement of this tripartite structure in DCs. In the following we verified the binding of transcription factors of the IRF- and NFκB-family to the CD83 promoter-complex by EMSA as well as by ChIP and assessed functionality by co-transfection- and mutation analyses. Finally, this highly complementary tripartite promoter-enhancer complex was shown to efficiently drive therapeutic transgene expression of MelanA and IL-12p70 in the DC cell line XS52 as well as in human mature DCs.

Conclusions:
Taken together, this newly identified DC-specific CD83 promoter now allows not only for the genome-wide discovery and understanding of molecular mechanisms involved in transcriptional activation, but especially for the development of new in vivo targeting strategies for next generation DC-vaccination directly in patients suffering from malignant disorders.

Figure legend:
Model of the CD83 promoter-enhancer region including bioinformatically predicted transcription factor binding sites. All genomic sequences were obtained from the Eldorado database and analyzed using the Genomatix Suite. Transcription factor binding sites (TFBSs) were identified by MatInspector. Depiction of the three defined regions with the putative transcription factor binding sites regulating CD83 transcription: URE (NFκB-sites 1 and 2 [blue] and IRF-site 3 [orange]), MP-261 (NFκB-sites 3, 4 and 5 [blue]) and enhancer (IRF-sites 1 and 2 [orange]).The combination of URE and MP-261 in genomic configuration was termed P-510.
Novel role of CD11c⁺CD8⁻ DCs in induction of cytotoxic T cell activation.

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Dendritic cells are major initiators of immune responses, but are also responsible for the maintenance of peripheral T cell tolerance. A critical decision for induction of immune activation or immune tolerance is dependent on the activation status of DCs, which could be induced by the presence of diverse pathogens. For the recognition of pathogen associated molecular patterns (PAMPs) DCs express pattern recognition receptors (PRR), such as Toll-like receptors (TLR), or C-type lectin receptors (CLR). As we could demonstrate before, DC subpopulations are specialized regarding processing and presentation of captured antigens. Therefore, DCs tightly control and direct activation of different T cell responses. Here, we investigate how TLR-mediated activation of murine CD11c⁺CD8⁻ and CD11c⁺CD8⁺ DCs influences the expression of molecules involved in the MHC class I antigen processing machinery. We provide evidence that activation of CD11c⁺CD8⁻ DCs induces upregulation of Calreticulin, Calnexin, Erp1, Erp57, Tap1, Tap2, and Tapasin under certain immunostimulatory conditions in vivo. Importantly, delivery of targeted antigens in combination with certain TLR-ligands allowed cross-presentation and activation of CD8⁺ T cells by CD11c⁺CD8⁺ DCs in wildtype and BATF3⁻/⁻ mice in vivo. Our findings suggest that the DCs are flexible in counteracting infections and tumor development if they are appropriately stimulated.

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Mouse and human dendritic cells (DCs) are comprised of functionally specialized subsets, but precise interspecies correlation is currently incomplete. Here, we showed that murine lung and gut lamina propria CD11b+DC populations were comprised of two subsets: FLT3- and IRF4-dependent CD24+CD64+DCs and contaminating CSF-1R-dependent CD24-CD64+macrophages. Functionally, loss of CD24+CD11b+DCs abrogated CD4+T-mediated IL-17 production in steady state and after Aspergillus fumigatus challenge. Human CD1c+DCs, the equivalent of murine CD24+CD11b+DCs, also expressed IRF4, secreted IL-23 and promoted Th17 cell responses. Our data revealed heterogeneity in the mouse CD11b+DC compartment and identified mucosal tissues IRF4-expressing DCs specialized in instructing IL-17 responses in both mouse and human. The demonstration of mouse and human DC subsets specialized in driving IL-17 responses highlights the conservation of key immune functions across species and will facilitate the translation of mouse in vivo findings to advance DC-based clinical therapies.
Dendritic Cells

Multicolor confocal immunofluorescence microscopy of DCs in human tissue

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Dendritic cells (DCs) orchestrate the immune system and can be found in lymphoid as well as non-lymphoid tissues. Immature DCs constantly search the periphery for pathogens and, once activated through antigen uptake and co-stimulation, travel to secondary lymphoid organs. In human peripheral blood three DC subsets have been described using novel blood derived dendritic cell antigen (BDCA)-markers and the C-type lectin receptor 9a (Clec9a): BDCA1+CD11c+ myeloid mDC1, BDCA3+CD11c+Clec9a+ mDC2 DCs and BDCA2+CD123+ plasmacytoid pDCs. Here, we provide a closer look at human tissue DCs in different lymphoid organs (spleen, thymus and tonsils) from healthy donors. With our newly established protocol we are able to perform high resolution confocal immunofluorescence microscopy using six different colors simultaneously, thus allowing interactive studies of various cell populations. We demonstrate localization, cell surface expression, and interaction of the different DC subpopulations with other cells in human tissues. We found mDC1 and mDC2 cells in a higher frequency than pDCs in human spleen. In contrast, pDCs were the most prominent DC subpopulation in human thymus and tonsils. One additional BDCA2+CD123+ cell population could be characterized being present in human spleen and tonsils.

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Induction of regulatory T cells by modification of antigens with Sialic acids

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Sialic acids are often found on the outer surface of glycoproteins of the host immune and tissue cells and have specificity to interact with inhibitory Siglec receptors. It is for this reason hypothesized that sialic acids may have a regulatory role in homeostasis or pathogen mediated immune modulation. We here show that uptake of sialylated antigens by dendritic cells (DCs) affects their T cell priming function. Identifying the mechanism involved we found splenic DCs loaded with Ovalbumin modified with sialic acids (Sia-OVA) to strongly promote the differentiation of OVA-responsive naive CD4+ T cells into FoxP3+ CD4+ regulatory T cells (Treg), while preventing induction of IFN-γ+ CD4+ cells. Sialylated antigens impose a tolerogenic function in DCs via SiglecE- as Treg induction is arrested in SiglecE−/− DCs. In vivo, injection of Sia-OVA led to enhanced Treg numbers and even prevented effector T cell generation. Moreover, the tolerogenic effect of Sia-OVA was still apparent under inflammatory conditions in vitro. Our data demonstrate the potency of antigen modification with sialic acids to treat detrimental immune reactions.
Dendritic Cells

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Danger signal-dependent activation of dendritic cells by factor VIII

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Question:
Haemophilia A is a genetic disorder characterized by impaired blood clotting, which may lead to life-threatening bleeding of patients. Though haemophilia A is incurable, it can be controlled through regular infusions of the deficient clotting factor VIII (FVIII). Plasma-derived and recombinant FVIII products are available for treatment. However, about 25% of patients develop so called inhibitors, which are anti-drug-antibodies (ADAs) resulting in a less or even ineffective treatment. Mechanisms leading to immunogenicity of FVIII products in patients are not yet fully understood. Also, whether the immunogenicity of recombinant vs. plasma-derived FVIII products might be different, is still a subject of controversial discussion. Since it was observed clinically that inhibitor risk is enhanced by events like surgery or infection, we first assessed whether danger signals can contribute to an increased activation of innate immune cells and thereby enhance immunogenicity of FVIII products. Subsequently, we compared different FVIII products for their danger signal-dependent immunostimulatory capacity.

Methods:
Dendritic cells build a bridge between innate and adaptive immunity by activating CD4⁺ T cells, which in turn interact with B cells leading to antibody responses. Thus, we decided to employ an assay using human monocyte-derived dendritic cells (DC). These DC were incubated with lipopolysaccharide (LPS) alone, various plasma-derived and recombinant FVIII products alone, or with a combination of LPS plus FVIII product. Subsequently, the maturation and activation status of the treated DC was investigated by studying the expression of selected surface molecules using flow cytometry.

Results and Conclusions:
By testing 29 different donors we could show that co-stimulation of DC with FVIII products in combination with LPS leads to a more pronounced upregulation of the co-stimulatory molecules CD83 and CD86 when compared to FVIII or LPS stimulation alone. This supports the hypothesis that certain risk factors such as subclinical infections promote the immunogenicity of FVIII products. Moreover, by comparing various FVIII products we could demonstrate differences in the immunogenicity among plasma-derived products, whereas tested recombinant FVIII products were not able to activate DC in a danger signal-dependent manner.

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Role of dendritic cells in self tolerance

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Introduction:
Dendritic cells (DCs) link the innate and adaptive arm of the immune system. As professional antigen-presenting cells they prime T cells to exert their effector functions. Beside this DCs play a central role in mediating negative selection of thymocytes and the induction of peripheral tolerance. Insufficient tolerance leads to autoimmune phenotypes.

Objectives:
We sought to investigate whether DCs mediate protection against autoimmunity. Furthermore, we asked whether the number and functionality of Tregs is impaired in DC-deficient mice.

Methods:
Constitutionally DC-deficient mice were generated by crossing CD11c-Cre mice with mice expressing the diphtheria toxin subunit A under the control of a loxP-flanked stop cassette in the ROSA26 locus (R-DTA mice). Flow cytometrical and histological analyses were used to assess the phenotype of these mice.

Results:
CD11c-Cre/R-DTA mice (DDC mice) show a constitutive loss of conventional DCs, plasmacytoid DCs, and Langerhans cells. Frequencies of CD4-single positive thymocytes are increased and CD4 T cell infiltrate the peripheral tissues. DDC mice develop spontaneous autoimmunity with reduced body weight, splenomegaly, autoantibody formation, neutrophilia, expansion of Th1 and Th17 cells, and inflammatory bowel disease. Tregs are only slightly reduced in DDC mice. Current experiments address the question whether Tregs are functional and whether autoreactive T cells or autoantibodies are responsible for the autoimmune phenotype of DDC mice. Using adoptive transfer experiments and depletion studies we further determine whether lack of central or peripheral tolerance can explain the pathological changes.

Conclusion:
DCs are indispensable for protection against fatal autoimmunity under steady state conditions and are essential to maintain a self-tolerant immune system.
Dissecting the role of different dendritic cell subsets in Aldara-induced psoriatic plaque formation in mice


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Introduction:
Psoriasis is a chronic auto-inflammatory skin disease of unknown etiology. The dysregulated immune response is thought to be triggered by environmental and genetic factors but can also be induced by treating patients for unrelated conditions with Aldara cream containing the TLR7 agonist Imiquimod (IMQ). Similarly, in mice topical application of Aldara/IMQ elicits skin inflammation and pathology closely resembling psoriatic plaque formation. Recent reports using this model have challenged currently prevailing concepts concerning the pathophysiology of psoriasis demonstrating that the development of IMQ-induced psoriasis depends on IL-23-mediated production of IL-17/22 by innate lymphocytes.

Objectives:
The role of different dendritic cell (DC) subsets, in particular, plasmacytoid DC (pDC), conventional DC as well as inflammatory monocyte-derived DC and their respective key cytokines including type-I interferons (IFN-I) and IL-23 in the initiation of psoriasis has been suggested, but still remains elusive.

Materials & methods:
To investigate which DC subset(s) drive psoriasiform skin disease, we generated mice deficient for the TLR adaptor molecule MyD88, in which TLR signaling is conditionally switched on in all CD11c+ DC or only in Langerin+ DC. Moreover, we took advantage of pDC-deficient and IFNAR knockout (IFNAR−/−) mouse strains.

Results:
MyD88-deficient mice were resistant to IMQ-induced skin inflammation establishing the strict dependence of this psoriasis model on MyD88 signaling. In contrast, selective activation of CD11c+ DC by IMQ was sufficient to mediate full-blown psoriatic plaque formation, while exclusive TLR7 triggering of Langerin+ DC resulted in attenuated skin disease. Unexpectedly, the course and severity of psoriasiform skin inflammation were not altered in pDC-deficient mice. Moreover, unaffected IMQ-psoriasis in IFNAR−/− revealed that the IFN-I pathway was dispensable for the development of local skin inflammation. Analysis of in vivo cytokine production after IMQ painting onto the skin of wild type mice demonstrated that IL-23 was exclusively produced by a Langerin+ dermal DC subset. In ongoing experiments we are further characterizing the phenotype and function of Langerin+ DC during Aldara-driven psoriatic plaque formation.

Conclusion:
TLR7-activated Langerin+ DC trigger IMQ-induced psoriasis via IL-23 and independently of pDC or IFN-I signaling. These findings may have important implications for treatment of the human disease.
The differentiation and function of a novel subset of IFNβ producing plasmacytoid dendritic cells is independent of IFNAR-mediated signalling

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Introduction:
During the initial response to viral infections type I interferons (IFN) are crucial initiators of subsequent protective immune mechanisms. While it is well established that plasmacytoid dendritic cells (pDCs) are potent producers of type I IFN we have recently shown, using an IFNβ/YFP reporter knockin mouse model (IFNβmob/mob), that after in vivo stimulation with CpG IFNβ production is restricted to a minor fraction of these pDCs. In order to investigate whether IFNβ producing pDCs represent a distinct population, or are simply classic pDCs strongly induced to produce IFNβ through auto- or paracrine activation of type I IFN receptor (IFNA) signalling here we investigate IFNβ producing pDCs on both wildtype (WT) and IFNAR−/− backgrounds. We make use of the increasing variety of described pDC surface-markers to perform a comprehensive study of the phenotype and localization of these up to now ill-defined IFNβ producing pDCs. We suggest that IFNβ producing pDCs define a unique sub-population within classical pDCs. These cells may be of particular importance for protection from viruses.

Objectives:
The objective of our study is to define the specific characteristics and functional relevance of IFNβ producing pDCs and the respective impact of the IFNAR-mediated feedback loop.

Materials and Methods:
C57BL/6, IFNβmob/mob or IFNAR−/−xIFNβmob/mob mice were injected i.v. with TLR9 agonist CpG 1668 complexed to DOTAP or left untreated. For FACS analyses cells were stained with antibodies against CD3ε, CD19, CD11c, mPDCA-1, B220, SiglecH, CCR9 and CD9. For histology spleen sections were fixed with acetone or paraformaldehyde followed by staining for B220, mPDCA-1, SiglecH or YFP and visualized by fluorescence microscopy.

Results:
Our FACS data indicate that only a minor fraction of pDCs that are CD11cintB220’mPDCA-1’SiglecH’CCR9’CD9− produce IFNβ early after TLR9 stimulation in both WT and IFNAR−/− backgrounds. Extending our previous histological findings, IFNβ/YFP+ pDCs can be found at the interface of the T and B cell areas of the spleen whereas staining with different pDC markers like mPDCA-1 or SiglecH shows the vast majority of classic pDCs in the vicinity of the marginal zone. IFNβ/YFP production by pDCs is functional in the absence of the IFNAR indicating that this cellular phenotype and the localization of these IFNβ producing pDCs is independent of the type I IFN mediated positive feedback loop.

Conclusion:
Thus IFNβ producing cells constitute a unique subpopulation within the classical pDC population exhibiting a distinct in vivo localization within the spleen. In addition, we have found that the positive feedback loop via the IFNAR is not required for the production of IFNβ or the differentiation of this distinct pDC subset. We anticipate that this population has a specialized immunoregulatory function that will be investigated in future studies. These studies will include a recently completed microarray based transcriptome profiling where we have found more than 1500 genes with significant differential expression between IFNβ producing and non-producing pDCs following TLR9 stimulation.
Antigen targeting of Fc-receptors induces strong T cell responses in vivo

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Dendritic cells are very important antigen presenting cells and responsible for the initial induction of effective immune responses but also for the maintenance of peripheral T cell tolerance. We recently found that different DC subpopulations are able to induce different T cell responses after targeting antigens to the endocytosis receptors DEC205 and DCIR2 (33D1) in vivo. Beside C-type lectin receptors, Fc receptors are highly efficient endocytic receptors expressed on a variety of antigen presenting cells. We found that Fc gamma receptors were differentially expressed on the DC subpopulations. To explore if Fc gamma receptors are suitable for in vivo antigen targeting, we have cloned the variable regions of a variety of anti Fc gamma receptor antibodies (Ly17.2-FcγRIIB, 9E9-FcγRIV) in frame to a non Fc receptor binding murine IgG1 constant region. For the immunological readout we genetically engineered the ovalbumin model antigen into the C-terminal region of the cloned antibodies. Here, we show that targeting antigens via recombinant ovalbumin carrying Fc receptor antibodies in vivo induces different T cell responses. Although Fc-gamma receptors are also expressed on other lymphoid cell populations (monocytes, B cells, granulocytes) we provide evidence that only the expression of Fc gamma receptors on the DC subpopulations is needed for the induction of T cell responses. Therefore, we suggest antigen targeting to Fc gamma receptors as useful tool for future therapeutic applications.

This project was partly funded by the German Research Foundation (DU548/2-1, SFB643-TPA7), BayGENE, ELAN-Erlangen and the Ria-FreiFrau von Frifisch Stiftung. DD was a fellow of the ‘Förderkolleg’ of the Bavarian Academy of Sciences.
Analysis of Dendritic Cells in human lymphoid organs


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Introduction:
Dendritic Cells (DCs) are important regulators of immune responses. In our previous studies we found differential antigen presentation capacities of murine DC subpopulations using an in vivo antigen targeting system [1]. In contrast to murine DCs, the functional role of human tissue DCs is largely unknown.

Aim:
We are focussing on the characterization of DC subpopulations directly isolated from human lymphoid tissues to understand their functional role in the human immune response.

Patients and methods:
Human tissues (thymus, spleen, bone marrow, tonsils, cord blood, peripheral blood, together around 300 samples) were received from otherwise healthy individuals. For our studies we performed 6 color confocal immunofluorescence analyses, and up to 15 color FACS and cell sort analyses for the study of 284 cell surface molecules (Lyoplate assay). We further investigated the DC’s antigen uptake properties and analyzed the RNA expression by microarrays.

Results:
The percentage of the three main DC subpopulations of mDC1, mDC2, and pDCs was varying depending on the tissue analyzed, indicating different functional roles of the DC subpopulations. Only very view cell surface molecules were uniquely expressed on the different DC subpopulations. Further, future potential antigen targeting receptors of the C-type lectin and Fc receptor family were investigated. Depending on the targeting antibody CD4 or CD8 T cell responses could be initiated. Our microarray data together suggest differential antigen presentation capacities of pDCs, mDC1, and mDC2 cells.

Conclusion. With cutting edge technologies we have characterized directly isolated human tissue DC subpopulations. This study (O.D.) was partly supported by DC-Thera, the German Research Foundation (SFB643-TPA7 and DUS48/2-1, GRK1660), DAAD, and BayGene. D.D. was a fellow of the ‘Förderkolleg’ of the Bavarian Academy of Sciences.

References:
In vivo targeting of human DC-SIGN drastically enhances CD8+ T cell-mediated protective immunity

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Vaccination is one of the oldest and most effective methods to prevent infectious diseases. Yet, eradication of intracellular pathogens and treatment of certain diseases like cancer that require efficient adaptive immune responses remains a medical challenge. In mice, a successful approach to induce strong CTL reactions is to target antigens to DCs using specific antibodies against surface receptors in combination with adjuvants. However, the major drawback for translating this strategy into the clinic is the lack of analogous targets in human DCs. DC-SIGN (DC-specific-ICAM3-grabbing-non-integrin/CD209) is a C-type lectin receptor with potent endocytic capacity and a highly restricted expression on human immature DCs. Therefore, DC-SIGN represents an ideal candidate for DC targeting. Using transgenic mice that express human DC-SIGN under the control of the murine CD11c promoter (hSIGN mice), we explored the efficacy of anti-DC-SIGN antibodies to target antigens to DCs and induce protective immune responses in vivo. We could show that anti-DC-SIGN antibodies conjugated to OVA induced strong and persistent antigen-specific CD4+ and CD8+ T cell responses, which efficiently protected from infection with OVA-expressing Listeria monocytogenes. Our results demonstrate that DC-SIGN is a promising candidate for novel vaccine strategies against intracellular pathogens based on DC targeting.
In Vivo Targeting of HIV Gag to Dendritic Cells Given with Poly ICLC is Safe and Induces Durable CD4+ T Cell And B Cell Responses in Healthy Volunteers

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Introduction:
In vivo delivery of HIV antigens within α-DEC 205 antibodies to maturing dendritic cells in combination with maturation stimuli is a potential new vaccine platform.

Objectives:
This phase-I study evaluates the safety and immunogenicity of DEC-targeting of HIV gag p24 in combination with poly ICLC in healthy volunteers.

Patients & Methods:
45 volunteers aged 18-60 were enrolled. 9 volunteers per dosage group (low: 0.3mg; mid: 1.0mg; high: 3.0mg) received α-DEC205-HIVp24 mAb plus 1.6 mg of poly ICLC s.c., 3 received poly ICLC only, and 3 received saline. Volunteers were vaccinated at weeks 0, 4, 12 and followed for 12 months.

Results:
Transient local and systemic reactogenicity occurred, without vaccine-related serious adverse events to date. Gag p24-specific IgG was induced in 9/15 (60%, 9 received vaccine plus adjuvant) volunteers in all groups. Responses persisted at least for 12 months after last dose in most responders. Four weeks after last immunization, response rates for the vaccine group by IFN-γ ELISPOT were 44%, 56% and 33% in the low, mid and high dose groups, respectively. Responses were detected in 10-25% of vaccinees 12 months after last dose. Intracellular cytokine staining data showed that IL-2 and TNF-α were the predominant cytokines. For CD4+ cells producing IL-2 or TNF-α, response rates for the vaccine group ranged from 37 to 78%, 22 to 55% and 44-100% in the low, mid and high dose groups, respectively. Responses were detectable in 22-100% of vaccinees 12 months after last dose. Among positive responders, the median magnitude across dose groups were similar, with median magnitudes ranging from 0.05 to 0.23% for IFN-γ+ cells, 0.05 to 0.33% for IL2+ cells, and 0.05 to 0.25% for TNF-α+ cells. Minimal response to CD8+ T cells was observed in any cytokine at any visit during the vaccination phase or at week 24.

Conclusion:
This novel DC-targeted protein HIV vaccine in combination with poly ICLC is safe and immunogenic in humans. Antibody and CD4+ T cell responses are induced and remain detectable at 12 months after last immunization in most responders.
Studying the role of DC in human immunity through the manipulation by nanocarriers

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Introduction:
Professional antigen-presenting cells (APC) play a key role in human immunity. By processing and presenting antigens in the context of co-stimulatory molecules, they act as critical regulators in autoimmune diseases and acute Graft-versus-Host Disease (aGvHD) as well as in the generation of responses against infectious agents and cancer. Thus, manipulation of APC appears to be a promising approach for therapeutic application. Nanoparticles (NP) and nanocapsules (NC) carrying incorporated reporter molecules and drugs have become an encouraging tool for specific targeting of human cell populations in research as well as in clinical application. We investigated functionalized polystyrene based NP and hydroxyethyl starch NC (HES NC) for loading APC like dendritic cells (DC) in vitro and their biodistribution in vivo.

Objectives:
It is our aim to identify nontoxic NP/NC that can be functionalized for a specific uptake in different subsets of DC. In a systematic approach, we tested NP/NC with different surface modification on DC in vitro as well as their biodistribution in mice in vivo.

Materials & Methods:
For polystyrene nanoparticle synthesis either ionic sodium dodecyl sulfate (SDS) or nonionic Lutensol AT50 were used as a surfactant. SDS NP did not contain any further functionalization, while Lutensol NP were also tested with covalently linked amino or carboxyl groups. HES NC were used unmodified and with covalently linked polyethylene glycol (PEG) chains, trimannose and galactose. NP/NC were loaded with an IRdye for in vivo analysis and BODIPY or sulforhodamine for in vitro analysis. NP/NC were incubated with DC for several time points and at various concentrations. Cell uptake, DC surface marker expression and toxicity of NP/NC were analyzed by flow cytometry (FACS), while NP/NC localization was investigated using confocal laser scanning microscopy (cLSM). Biodistribution of NP/NC was explored by applying them into mice intravenously (i.v.) following biofluorescence imaging (BFI).

Results:
FACS and cLSM analysis of DC incubated with amino and carboxyl functionalized polystyrene NP revealed strongest cell uptake. Lowest uptake was observed for SDS NP. Uptake of NP was nontoxic but led to slightly increased DC marker expression of CD86 and HLA-DR, while CD83 and CCR-7 were not changed. Following intravenous application in mice, all NP were found in liver, lung, spleen, blood and skin after 4 days. For unmodified Lutensol NP increased presence in lung was observed, while the presence of carboxyl residues led to accumulation of NP in blood. Interestingly, SDS NP showed signal in the skin.

HES NC were taken up by DC nonspecifically. This could be reduced by coupling of polyethylene glycol (PEG) chains. The covalent linkage of trimannose to NC markedly increased cellular uptake. In vivo, functionalization of HES NC led to accumulation in liver, while nonfunctionalized HES NC were predominantly found in spleen and skin.

Conclusion:
In summary, polystyrene NP were taken up nonspecifically by DC, which led to slight differences in DC marker expression. In vivo, their biodistribution depended strongly on their functionalization. Against our expectations, unmodified HES NC were taken up nonspecifically by DC. This could be partially blocked by PEGylation allowing for a more specific targeting by sugar residues. Nonspecific uptake of NP/NC remains a major issue that needs to be solved to allow subsequent targeting e.g. by antibodies.
Dendritic Cells

β-catenin signaling in dendritic cells diminishes immunity during allergic asthma

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Introduction:
Asthma is an allergic inflammatory disease, driven predominantly by T helper (Th) type-2 lymphocytes. The reaction results from a loss of tolerance towards innocuous foreign antigens and is characterized by infiltration of the airways with a plethora of inflammatory cells, including eosinophils (a hallmark of the disease). Dendritic cells (DC) are pivotal mediators in balancing immunity and tolerance and have been shown to be both necessary and sufficient for the induction of allergic asthma. Although immunity is favored after DC activation by microbial or inflammatory stimuli, DC also have the unique capacity to initiate tolerance. However, the underlying mechanisms how DC promote peripheral tolerance are not well understood.

β-catenin forms the central component of the canonical WNT signaling pathway and in vitro experiments demonstrated that activation of β-catenin by cluster disruption of bone marrow-derived DC, results in a regulatory phenotype. Moreover, recent experiments revealed that a DC-specific deficiency of β-catenin aggravates disease in a mouse model of colitis, further suggesting that β-catenin is a key regulator of DC function in vivo.

Objectives:
β-catenin is a promising target to modulate DC function and their regulatory phenotype. While DC-specific deficiency leads to an exacerbation of a Th1/Th17 driven immune response, it remains elusive whether and how β-catenin signaling governs DC function during Th2-mediated allergic asthma.

Materials & Methods:
To this aim, we generated DC-specific mouse mutants expressing either a stabilized form of β-catenin (DC-βcatEX3) or a DC-specific deletion of β-catenin (DC-βcatDEL). These mice were analyzed in the steady state as well as in an OVA-based model of allergic asthma.

Results:
In the steady state, DC-βcatKO mice exhibited similar DC maturation, whereas DC of DC-βcatEX3 mice expressed a more mature phenotype when compared to control mice. During allergic asthma DC-βcatKO mice developed enhanced airway hyper-responsiveness (AHR), associated with increased cellular infiltrates and enhanced T cell activation. In contrast, DC-βcatEX3 mice displayed strongly attenuated AHR accompanied by reduced effector T cell activation, decreased IgE production and diminished eosinophilia. Further analysis of lung tissue and broncho-alveolar lavage fluid revealed decreased levels of Th2 cytokines and chemokines, as well as a striking increase in FoxP3+ regulatory T cells.

Conclusion:
Our data demonstrate that, in addition to regulating Th1/17 responses in the intestine, DC-specific β-catenin signals modulate mucosal tolerance in the lung and contribute to the attenuation of Th2 cell activation. Consequently, augmenting β-catenin signaling enhances the tolerogenic capacities of DC and may improve DC-based strategies to treat allergic or inflammatory diseases.
Dendritic cells are targets of IL-10 to maintain immune homeostasis in the small intestine

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Introduction:
IL-10 is a pleiotropic regulatory cytokine that plays a major role attenuating excessive inflammatory reactions and limiting the magnitude of T-cell responses. Hence, IL-10-deficient (IL-10⁻/⁻) and IL-10 receptor (IL-10R)−deficient mice mount enhanced T helper (Th) type-1/Th17 responses to intestinal bacterial antigens, which induce severe colitis. Likewise, patients with genetic mutations in the IL-10 signaling pathway develop small and large intestinal disease that has resemblance to Crohn’s disease and is driven by Th1/Th17 cells. Similarly, in gluten intolerant celiac disease patients defective IL-10 regulation may be associated with enhanced Th1/Th17 responses and increased numbers of intraepithelial lymphocytes (iELs) in concert with crypt hyperplasia in the small intestine.

Objectives:
Dendritic cells (DC) are professional antigen-presenting cells specialized to control the balance between immunity and tolerance. Although IL-10 mediates a regulatory DC phenotype in vitro, it remains elusive to what extent DC are targets of IL-10 to maintain intestinal tolerance in vivo.

Materials & methods:
To this aim, we analyzed mice with a DC-specific deletion of the IL-10 receptor (DC-IL10R⁻/⁻).

Results:
DC-IL10R⁻/⁻ animals developed spontaneous pathology and inflammation in the small intestine, but not in the colon. Crypt hyperplasia was associated with elevated numbers of iELs and lamina propria IgA⁺ plasmablasts. Moreover, increased numbers of Th1 and Th17 cells in the lamina propria correlated with augmented expression of IFNγ, IL-17 and IL-21 in situ. In particular, the percentage of small intestinal lamina propria effector/memory T cells and iELs secreting IL-17 was significantly higher. Intriguingly, naive T-cell priming in the mesenteric lymph node was not altered. Instead, enhanced T-cell activation was the result of elevated TNFα, IL-6 and IL-23 secretion by IL-10R-deficient lamina propria DC. Furthermore, augmented T-cell function could be attributed mostly to increased local lamina propria T-cell proliferation but also enhanced survival and recruitment into the lamina propria.

Conclusion:
These findings demonstrate that IL-10 signaling in DC is critical to govern effector/memory T-cell reactivation in the small intestine. Therefore, DC-IL10R⁻/⁻ mice represent a novel and unique spontaneous animal model of small intestinal pathology. In addition, our data suggest that harnessing the regulatory function of DC could be used to reestablish tolerance in inflammatory bowel disease or celiac disease patients.
The phosphodiesterase 4 inhibitor roflumilast augments the Th17-promoting capability of dendritic cells, and impairs their T cell stimulatory activity

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Phosphodiesterase 4 (PDE4) inhibitors serve to prevent degradation of the intracellular second messenger cAMP, and have been shown to exert broad anti-inflammatory effects. Accordingly, PDE4 inhibitors like roflumilast (ROF, trade name: Daxas) have been developed for treatment of respiratory diseases like asthma and COPD. Since agents that elevate cAMP levels via activation of adenylate cyclase have been shown to imprint a Th17-promoting capacity in dendritic cells (DCs), we asked for the potential of therapeutic ally relevant PDE inhibitors to induce a pronounced Th17-promoting capacity in DCs, as an unwanted side effect due to the pro-inflammatory role of Th17 cells.

Here we show that of a panel of PDE inhibitors tested, only the specific PDE4 inhibitor ROF exerted a Th17-skewing effect on murine bone marrow derived DCs, treated with that agent for the last 24 h of DC culture during stimulation with LPS (ROF-DCs). ROF enhanced expression of the Th17-promoting factor IL-23 by DCs via activation of protein kinase A, while EPAC activity was dispensable. Neutralizing IL-23 antibodies partially inhibited the Th17-skewing effect of ROF-DCs.

ROF-DCs also displayed a markedly diminished allogeneic T cell stimulatory capacity, although the surface expression of MHCII and of costimulators was not affected. Pharmacological inhibition of protein kinase A, ERK, and MAPK p38 partially restored the T cell stimulatory capacity of ROF-DCs.

Our findings suggest that PDE4 inhibitors have the potential to alter DC functions, namely to impair their T cell stimulatory capacity, but at the same time to imprint a profound Th17-polarizing capacity. Ongoing work is aimed to assess synergistic effects of agents like prostaglandin, which enhance cAMP generation, and ROF as an inhibitor of cAMP degradation.
Casein kinase II regulates the intracellular trafficking of the dendritic cell antigen receptor DEC205 to antigen presenting compartments

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Introduction:
The dendritic cell (DC) antigen receptor DEC205 takes antigens into MHC-class II+ compartments in DC and mediates effective antigen presentation. Its intracellular routing is guided by a short 31 amino acids containing intracellular domain (DEC tail). This domain harbours a putative casein kinase II (CKII) phosphorylation side.

Goal:
We asked whether phosphorylation of the DEC tail by CKII is important for the intracellular targeting of DEC205-antigen complexes to MHC-class II+ endosomal compartments.

Materials:
We generated fusion receptors containing the extracellular HuIgG-binding domain of human CD16 and the intracellular DEC205 domain (CD16:DEC) and transfected the antigen presenting cell line DCEK. In pulse - chase experiments we followed the intracellular routing of HuIgG and tested effects of CKII inhibitors as well as mutated DEC tails on antigen presenting capacities of these cell lines.

Results:
We show, that the CD16:DEC transfected cells bind and endocytose aggregated HuIgG efficiently and within 30 min - 60 min many HuIgG+ vesicles fuse with RAB7+ late endosomal compartments. Moreover, colocalisation of CKII with antigen-loaded vesicles was apparent 10 min after chase. When we applied CKII specific inhibitors to the cells, no changes in CD16:DEC mediated endocytosis was apparent, in contrast the recycling of the CD16:DEC receptors back to the cell surface was abolished. After deletion of the CKII phosphorylation side in the DEC tail by side directed mutagenesis, surface expression of CD16:DEC was impaired and CD16:DEC accumulated in TGN38+ endosomal/trans-golgi compartments. As a functional consequence antigen presentation was drastically reduced in comparison to wild type CD16:DEC transfected cell lines.

Conclusion:
This is the first evidence that phosphorylation of the intracellular domain of a prototypic antigen receptor (DEC205) by CKII is crucial for guiding intracellular targeting and antigen presentation and provides a novel mechanism involved in regulating T-cell immunity by antigen presentation by DC in vivo.
Targeting of myelin oligodendrocyte glycoprotein to DEC-205+ dendritic cells in vivo suppresses allergic experimental encephalomyelitis (EAE) in mice

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Introduction:
The antigen receptor DEC-205 is expressed by dendritic cells (DC) and greatly increases antigen presentation over pinocytosis or phagocytosis. After injection of antigens coupled to antibodies specific for DEC-205 into mice, effective presentation of the antigens to T cell follows. When antigen presentation occurs by non-activated “steady state” DC in vivo, we observed induction of regulatory T cells.

Goal:
We created single chain fragment variables (scFv) specific for DEC-205 fused to the self antigen MOG, to target non-activated DC in vivo and to induce tolerance to EAE.

Materials:
DEC-scFv:MOG fusions proteins, as well as isotype controls were expressed and purified from E.coli and immunohistochemical staining of CD11c+ dendritic cells displayed a positive staining for scFv, colocalizing with MHC class II.

Results:
For functional testing DEC-scFv:MOG was injected into mice and DC were analyzed 3 days later. We found that DC isolated from DEC-scFv:MOG injected mice stimulated vigorous proliferation of MOG-specific 2D2 T cells, indicating presentation of the relevant MOG peptide in vitro. Furthermore, DC isolated from those animals produced significantly more TGF-β as well as IL-10, as compared to isotype-treated or untreated mice, respectively. And when analyzing the T cell compartment we recorded elevated numbers of activated CD4+CD25+FoxP3+ Treg (16% of CD4) in the spleen after injection of DEC-scFv:MOG as compared to controls (12% of CD4). These Treg produced significantly more IL-10 as compared to controls. Most importantly, when EAE was induced in DEC-scFv:MOG-injected mice and in isotype-scFv:MOG treated controls, none of the DEC-scFv:MOG injected mice displayed any EAE symptoms in contrast to controls, which developed severe EAE. In a therapeutical setting, EAE was induced first and after mice had developed mild symptoms, they were treated with respective scFv conjugates. Here injection of DEC-scFv:MOG lead to abrogation of the disease in >90% of the animals tested. In contrast, all animals in the control groups developed severe EAE.

Conclusion:
These data indicate that targeting of MOG to steady state DC in vivo prevents EAE by a DC/Treg-driven mechanism.
Regulation of Flt3-L availability for dendritic cell development

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Flt3-L is a non-redundant cytokine instructing development of conventional DCs (cDCs) and whose availability is controlled by the size of the DC compartment. The aim of this study was to characterise the source of Flt3-L required for DC differentiation and to investigate the mechanisms by which DCs regulate the availability of Flt3-L. All cells and organs produced Flt3-L, likely to ensure optimal cDC generation across different organs. Restricting Flt3-L expression to the hematopoietic or non-hematopoietic compartments using BM chimeras, did not result in changes in the frequency of DC precursors. Both sources equally contributed to the splenic Flt3-L content, although Flt3-L production by immune cells was sufficient and mandatory for normal splenic cDC generation, indicating that the source rather than the total amount of Flt3-L in spleen is dominant for splenic cDC development. The availability of soluble and membrane-associated Flt3-L increased upon DC depletion. Our results indicate that this was not due to transcriptional regulation of Flt3-L expression in Flt3-L-producing cells, but rather due to consumption of Flt3-L by DCs. Thus, DCs appear to serve as a Flt3-L “sink”, thereby regulating the amount of Flt3-L available to instruct DC precursor differentiation.
Annexin 1 on the Surface of Early Apoptotic Cells Suppresses CD8+ T Cell Immunity

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Prevention of an immune response against self-antigens derived from apoptotic cells is essential to preclude autoimmune and chronic inflammatory diseases. Here, we describe apoptosis induced externalization of endogenous cytosolic annexin 1 initiating an anti-inflammatory effector mechanism that suppresses the immune response against antigens of apoptotic cells. Cytosolic annexin 1 rapidly translocated to the apoptotic cell surface and inhibited dendritic cell (DC) activation induced by Toll like receptors (TLR). Annexin 1-inhibited DC showed strongly reduced secretion of pro-inflammatory cytokines (e.g. TNF and IL-12) and costimulatory surface molecules (e.g. CD40 and CD86), while anti-inflammatory mediators like PD-L1 remained unchanged. T cells stimulated by such DC lacked secretion of interferon-γ (IFN-γ) and TNF but retained IL-10 secretion. In mice, annexin 1 prevented the development of inflammatory DC and suppressed the cellular immune response against the model antigen ovalbumin (OVA) expressed in apoptotic cells. Furthermore, annexin 1 on apoptotic cells compromised OVA-specific tumor vaccination and impaired rejection of an OVA-expressing tumor. Thus, our results provide a molecular mechanism for the suppressive activity of apoptotic cells on the immune response towards apoptotic cell-derived self-antigens. This process may play an important role in prevention of autoimmune diseases and of the immune response against cancer.
Changing IL-27 expression pattern during childhood

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Introduction:
Dendritic cells (DCs) have been demonstrated to be a major source for interleukin-12 (IL-12), a pivotal Th1 cytokine. During the first months of life the substantial lack of IL-12 expression results in a Th2-biased T-cellular immune response. Only at the age of 12 years IL-12 levels are as high as in adults. IL-27 has been recognized as an inhibitory cytokine for most T helper cell subsets. At the same time IL-27 has a capacity to induce Th1 polarization.

Objectives:
Recently, we reported on the exceptionally high IL-27 expression levels by neonatal DCs, which let us hypothesize a Th1 polarizing role of IL-27 in the absence of IL-12 during the first years of life. In an effort to substantiate this view we characterized the IL-27 expression levels during childhood.

Material & Methods:
Heparinized whole blood from n=55 healthy subjects aged 0 - 16 years were enrolled after informed consent for an observational study at the University Medical Center Mainz and the Dr. Horst-Schmitt-Kliniken, Wiesbaden. Additionally, 5 blood samples from adult volunteers were collected. After dilution with culture medium capacity to express IL-27 was detected in DCs after a 6 h whole blood cell culture with or without stimulators (IFNg or LPS, IFNg+LPS, PolyIC, ssRNA or SEB). Frequencies of myoid DCs (mDCs; CD11c+), plasmacytoid DCs (pDCs; CD123+) were determined. Percentage of IL-27 positive mDCs and pDCs were detected after intracellular cytokine staining by flow cytometry (LSR II, BD).

Results:
Whole blood collection resulted in eligible sample volumes from 41 subjects. Frequencies of mDCs and pDCs slightly decreased with age in a linear pattern. In contrast, IL-27 expression by mDCs followed a biphasic pattern during childhood, with high levels in the neonatal phase and a second peak in the age range of 10 to 12 years. IL-27 expression levels were lowest in adolescents and adults. This developmental kinetic was present in unstimulated mDCs, only slightly modulated by stimulators, with IFNg+LPS being the most potent positive modulator. pDCs presented with very low levels of IL-27 expression, specifically with no biphasic characteristics and no substantial difference regarding neonatal versus adult IL-27 levels.

Conclusion:
Earlier data showing high neonatal expression levels in contrast to low adult levels could be corroborated. High levels of IL-27 in neonates coincided with the time period of maximum susceptibility for infectious diseases in infants. The second peak expression of IL-27 was unanticipated and had not been shown so far. It prompts further investigations with regard to other developmental factors in this age range. Thus, IL-27 during childhood development doesn't represent a simple substitute for IL-12, but follows a more complex, yet unknown regulative context.
Dendritic Cells

IL-27 induces primary and secondary autocrine effects on neonatal dendritic cells

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Introduction:
IL-27, an IL-12 family cytokine, is a heterodimer composed of the IL-27p28 subunit and Epstein-Barr virus induced gene 3 (EBI-3), and binds to a receptor consisting of WSX-1 and the gp130 subunit to induce signaling. IL-27, a cytokine able to elicit pleiotropic effects, is considered to be critical for the early regulation of T helper (Th) 1 differentiation until it is overruled by IL-12, which also induces IFNγ production in naïve CD4+ T cells. IL-27 is known to be increased considerably in neonatal dendritic cells (DCs) after TLR ligation.

Objectives:
Here, we intended to highlight the particular role of IL-27 in the neonatal immune system compared to the adult by investigating cord blood (CB) and adult blood (AB) monocyte derived dendritic cells (moDCs).

Methods:
PBMCs were isolated from CB of healthy full-term infants born by cesarean sections and from AB of healthy volunteer donors. To generate moDCs, CD14+ cells were magnetically separated (MACS; Miltenyi Biotec) with a purity of >90% and then cultured for 72 h in culture medium containing 100 ng/ml GM-CSF (Miltenyi Biotec) and 20 ng/ml IL-4 (Peprotech). After replacing the medium at day 3, cells were stimulated with IL-27 (R&D Systems) (50 ng/ml or 100 ng/ml) for 24 hours. Total RNA was isolated (High Pure RNA-Isolation Kit™, Roche) and transcribed into cDNA (Omniscript™ Reverse Transcriptase Kit, Qiagen). Relative quantification of IL-27p28, WSX-1, IRF8 and CXCL10 was performed by Real Time PCR (n=7). Furthermore, RT² Profiler PCR Arrays (Qiagen) were performed (n=3). CD86, CD40 and IL-27 FITC (R&D Systems) were analyzed by flow cytometry (n=7-14). Supernatants were collected on day 4 of cultures for detection of secreted cytokines by Human Inflammation CBA Kit (BD)(n=20).

Results:
Exogenous IL-27 transcription in neonatal and adult moDCs, while inhibiting the expression of the IL-27 receptor subunit WSX-1. CB moDCs exhibited a significantly higher basal expression of IL-27 protein than AB moDCs. Autocrine effects of IL-27 on neonatal moDCs emerge primarily as a significant augmentation of IL-27 protein, secondarily as an increased transcription of CXCL10 among other chemokines, chemokine receptor CCR1, IFIT3 among other antiviral genes and Th1 involved transcription factor IRF8. In neonatal cells, HLA-A as well as HLA-DOA and -DMA were upregulated stronger than in adult moDCs by IL-27. Furthermore, neonatal moDCs respond to IL-27 with augmented IL-6 and IL-8. IL-27 also induced the expression of costimulatory molecules CD86 and CD40 in neonatal and in adult moDCs.

Conclusions:
The results suggest that IL-27 has the potential to improve migrational and antiviral capacities of neonatal dendritic cells by its autocrine effects, which it excerts especially in neonates. Moreover, its effect on costimulatory molecules and inflammatory cytokines exhibits its capacity to influence the outcome of immune responses. Induction of IRF8 might play a role in the induction of Th1 responses by DCs. This might be of importance mainly in neonatal DCs, which are able to produce more IL-27 than adult ones.
Does synergistic activation of human dendritic and airway epithelial cells lead to enhanced allergy-protection by different cowshed bacteria?

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Background:
An increasing number of epidemiological investigations show that an early-life contact with cowsheds and farm animals can protect from the development of allergic diseases later in life. Several bacterial species have been isolated from cowsheds - namely Acinetobacter lwoffii F78, Lactococcus lactis G121 and Staphylococcus sciuri W620 - and tested successfully for allergy-protective effects in various mouse models. These bacteria can potently activate human dendritic and airway epithelial cells through different innate recognition systems. Recently, Ege et al. could show that the protective effect of the farm environment correlates with its microbial diversity.

Question:
Aim of this study is to investigate if the enhanced allergy-protection by microbial diversity can be explained by synergistic activation mechanisms.

Methods:
Different combinations and concentrations of A. lwoffii F78, L. lactis G121 and S. sciuri W620 were investigated regarding their activation capacity on human monocyte-derived dendritic cells and airway epithelial cell lines. Real-time PCR and ELISA were used for analyzing the upregulation of cytokines and surface molecules, especially with respect to their T helper cell polarization capacity.

Results:
Different combinations of cowshed bacteria induced an increase in the release of different cytokines in human DCs. Synergistic effects could be detected for all tested cytokines, but the extent of those effects was dependent on the cytokine and the concentration of bacteria used for stimulation. For example, maximal synergistic IL-10-release could be detected when combinations of high concentrations of two bacteria were used for stimulation, whereas maximal TNF-release could be seen by a combination of high and low concentration. The activation of different human airway epithelial cell lines could be shown by expression of different activation markers/molecules. We are currently investigating if a similar synergistic activation pattern can be identified.

Conclusion:
These preliminary data suggest that a combination of bacteria leads to synergistic activation of human dendritic cells, which could be one reason for the correlation of enhanced allergy-protection and microbial diversity in the farming environment.

Characterization of mediators involved in the function of tolerogenic APCs

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Immature dendritic cells (iDCs) can be generated out of CD14+ human monocytes via IL4 and GMCSF. We have shown previously that Toll-like receptor (TLR) agonists interfere with this differentiation process and generate a different cellular phenotype. These APCs (TLR-APCs) fail to express the differentiation marker CD1a, but retain CD14 expression and upregulate PDL-1. Importantly, TLR-APCs are able to block T cell proliferation and induce T regulatory cells. These inhibitory properties are in part dependent on PDL-1. We further observed in TLR-APCs high levels of IL-6 and a prolonged (up to 5 days) STAT-3 activation. Blocking of STAT3 led to a significant decrease in PDL-1, CD14 expression and inhibitory function. Further, we could detect direct binding of STAT3 at the PDL-1 and CD14 promoter. Thus, PDL-1 seems to be involved in the induction of the TLR-APC phenotype.

We were interested in further mechanisms responsible for the divergent phenotype and characteristics of immature DCs (iDCs) and tolerogenic APCs. To this we analysed gene expression pattern in iDCs compared to TLR-APCs using gene arrays. We could identify induction of indoleamine-2,3-dioxygenase (IDO) and adenosinedeaminase (ADA) as a clear hallmark of tolerogenic APC. These gene array results could be validated at the protein level. Moreover first in vitro experiments using selective inhibitors showed that both pathways may contribute to the inhibitory characteristics of TLR-APC. Thus multiple mechanisms account for the tolerogenic phenotype of TLR-APC.
Aryl hydrocarbon receptor as a co-factor in IDO gene expression induction

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The aryl hydrocarbon receptor (AhR) is a ligand activated transcription factor and a sensor of small chemicals. High expression of AhR in dendritic cells is related to its function in immunomodulation, and may link innate/adaptive immune responses to environmental cues. Thus, AhR activation is needed for maturation of dendritic cells (DC), and influences their tolerogenic activity. We and others have recently shown that the constitutive and inducible expression of the immunosuppressive enzyme indoleamine-2,3-dioxygenase (IDO) is absent in AhR-deficient Langerhans cells and BM-derived DC. IDO1 and IDO2 degrade the essential amino acid L-tryptophan, and generate kynurenines as breakdown products. Interestingly, kynurenines are ligands for AhR, and promote Treg differentiation. In general, IDO induction, e.g. by LPS or IFNg, is mediated via NFkB or Stat1 signalling. The failure to induce IDO in AhR-deficient DC was not due to a defect in the respective signalling cascades, as toll-like receptors are present, and LPS or IFNg could induce TNFa, IL6, CXCL10 and IRF1 or the IDO/IDO2 downstream enzyme kynureninase. However, AhR can also dimerize functionally with NFkB subunits RelA and RelB. We hypothesized that AhR is an obligatory co-factor for NFkB-induced IDO induction, and searched the promoters of the IDO genes for putative AhR-binding sites (called “dioxin-responsive elements”, DRES) and RelB/DRE sites. Both promoters of the ido1 and ido2 genes have bona fide DRES (4 in IDO1, 5 in IDO2) and RelB/DRE sites, with the core sequence 5’-GCGTG-3’ and 5’-GGGTGCAT-3’, respectively. Using a luciferase reporter system we assessed functionality of these DRES. Surprisingly, none of the DRE sites of ido1, and only one in ido2 could drive the luciferase gene. Mutation of the core sequence abrogated luciferase inducibility. However, the RelBAhRE core sequence in the IDO1 gene was functional. Our data indicate an important role for AhR in the constitutive expression of IDO, and a role for AhR in IDO induction by inflammatory signals in dendritic cells. The plasticity of the response, including role of persistent versus transient AhR ligands is under investigation.
Mesenchymal stromal cells differentially modulate phenotype and function of plasmacytoid, myeloid and monocyte derived dendritic cells

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Question:
Mesenchymal stromal cells (MSC) show supportive effects in several models of regeneration and are therefore considered promising candidates for novel cell-based therapies. This seems to be mediated mostly by their proangiogenic and immunomodulatory properties rather than by differentiation. However, so far only limited, and in part contradictory, information is available on their effects and the mechanisms behind them. In our study, we extensively compared the impact of human bone marrow derived (BM-MSC) MSC on distinct human dendritic cell (DC) subsets such as in vitro generated monocyte derived (Mo-DC), plasmacytoid (pDC) and myeloid (mDC) DC in terms of their migratory behavior, cytokine and chemokine expression and ability to activate Natural Killer (NK) and T-cells.

Method:
Bone marrow derived MSC were co-cultured with monocytes in the presence of IL-4 and GM-CSF to obtain immature Mo-DC or with in vitro generated immature Mo-DC, blood derived mDC or pDC in the presence of different Toll like receptor (TLR) ligands. Expression of maturation marker and chemokine receptors on DC subtypes was analyzed by flow cytometry. Cytokine and chemokine production by DC was measured by Luminex analysis. The ability of MSC-conditioned DC to activate autologous NK-cells or allogeneic T cells in terms of cytokine production and proliferation has been analyzed after co-culture of DC with the respective cell type.

Result:
We could show that BM-MSC strongly inhibited the in vitro differentiation of monocytes into DC resulting in a lower maturation status, a decreased production of pro-inflammatory cytokines and a reduced ability to activate allo-antigen specific naive T cells. In contrast, maturation of in vitro generated immature Mo-DC was not affected by MSC. Dependent on the type and the differentiation status of DC, MSC showed contrary effects on the expression of CCR7, resulting in either decreased or increased migration towards CCL-21, a chemokine leading DC towards secondary lymphoid organs. A broad analysis of cytokines and chemokines produced by TLR activated Mo-DC, mDC or pDC revealed modulation of many anti- as well as pro-inflammatory factors by MSC. Moreover, mDC activated in the presence of MSC displayed diminished ability to activate NK cells, while pDC induced higher IFN-gamma production in NK cells.

Conclusion:
Our data give an extensive picture on the immunomodulatory effects of MSC on human DC subtypes, pointing out that MSC can inhibit or enhance effector functions of DC dependent on its derived subtype and the context of DC-MSC interaction. The implication of these findings for cell therapies and the underlying mechanisms are currently under investigation.
Understanding migratory capacities of human Dendritic Cell subsets in lymphoid tissues


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Introduction:
Dendritic cells (DCs) are very important antigen presenting cells of the immune system. In humans and mice several DC subsets have been identified. These subsets are specialized to use different antigen presentation pathways to induce different T cell responses. For the interaction of DCs and T cells the expression of chemokine receptors (CCRs) and the secretion of chemokines are essential. However, in humans it is still not known whether the DC subsets differ in the expression of CCRs and Toll-like receptors (TLRs) and whether this potential difference might be responsible for a DC subset-specific recognition of distinct classes of pathogens (e.g. viruses, bacteria, etc.).

Aim:
Therefore, we analyze the CCR and TLR expression profile on different immature and activated DC subsets in a variety of human lymphoid tissues (blood, spleen, thymus, tonsils, bone marrow).

Patients and methods:
Leukocytes were enriched from patient material according to specialized protocols and cells were stored at -80°C or analyzed after overnight incubation at 4°C. Expression of CCRs and TLRs was examined with Multicolor-FACS- and high resolution confocal immunofluorescence-analyses. For comparison of immature and activated DCs cells were stimulated with a maturation cocktail (TNFα, IL-1β, IL-6, Prostaglandin E2) and/or TLR-stimuli.

Results:
Myeloid Dendritic Cells type 1 (mDC1) and plasmacytoid Dendritic Cells (pDC) show a broad expression of CCRs, whereas Myeloid Dendritic Cells type 2 (mDC2) are negative for most of the CCRs. Interestingly, the subsets-specific expression of certain CCRs differs between different tissues. The mDC1 express most of the extracellular TLRs, whereas mDC2 and pDCs mainly express intracellular TLRs.

Conclusion:
The specific expression of CCRs and TLRs could refer to a specialization in pathogen recognition or immune cell interactions. In further experiments we want to determine how the expression profile changes after activation of DCs and which CCRs are important for the interaction with other immune cells and for the homing to different tissues.

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Dendritic Cells

Virus-derived RNA-DNA hybrids are novel ligands for TLR9

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Introduction:
The innate immune system senses viral and bacterial nucleic acids in the cytoplasm and in endosomal compartments via different pattern recognition receptors (PRR) like RIG-like receptors (RLR) or Toll-like receptors (TLR), respectively. Single-stranded (ss) RNA, double-stranded (ds) RNA and CpG-DNA are well known TLR ligands. However, if RNA-DNA hybrids are recognized by the immune system is unknown.

Objectives:
Since RNA-DNA hybrids accumulate during autoimmune disease and may play an important role in viral replication, we investigated the immunostimulatory capacity of RNA-DNA hybrids.

Material and methods:
Human peripheral blood mononuclear cells (PBMC) and murine Fms-related tyrosine kinase 3 ligand-(Flt3L) induced cultures of dendritic cells (DC) derived from wt and TLR9−/− mice were used to analyze cytokine induction. These cells were incubated with RNA-DNA-hybrids as well as their corresponding single strands. All nucleic acids were complexed with DOTAP. Supernatants were harvested after 20h and cytokine induction was analyzed by ELISA.

Uptake and intracellular presence of RNA-DNA hybrids was demonstrated by intracellular staining with an anti-RNA-DNA hybrid-specific antibody.

Results:
RNA-DNA hybrids activate the innate immune system in a TLR9-dependent manner.

Upon stimulation of human PBMCs and Flt3L-induced DCs from wt cultures with synthetic RNA-DNA hybrids derived from HCMV and HIV interferon-α (IFNα) as well as proinflammatory cytokines were induced in a concentration dependent manner. In contrast, cytokine production was totally abrogated in TLR9−/− cells.

Uptake and intracellular presence of intact RNA-DNA-hybrids could clearly be demonstrated by intracellular staining of RNA-DNA-hybrids with an anti-RNA-DNA hybrid-specific antibody.

Conclusions:
In this study we show that TLR9 plays a crucial role in the immune recognition of RNA-DNA hybrids and is therefore highly important in antiviral immune responses.
Human monocytes undergo apoptosis following treatment with temozolomide activating the ATM/ATR pathway while dendritic cells and macrophages are resistant

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One of the most important dose-limiting side effects in cancer chemotherapy is immunosuppression. Aimed at elucidating the sensitivity of immune cells to genotoxicants, we reported that primary monocytes from human healthy donors are highly sensitive towards the chemotherapeutic drug temozolomide (TMZ) while dendritic cells (DCs) and macrophages derived from them are resistant. Because the cells are not proliferating and therefore O6-methylguanine is not a target, we focused on N7-methylguanine and N3-methyladenine as potentially toxic DNA lesions following TMZ treatment. These adducts are repaired by base excision repair (BER) requiring XRCC1, ligase IIIα and PARP-1. We observed that the hypersensitivity towards TMZ in monocytes results from the lack of the expression of these BER proteins and an accumulation of DNA double-strand breaks. These repair factors are upregulated in DCs and macrophages during maturation and, concomitantly, cells become competent for BER again. Immunohistochemical staining of poly(ADP-ribose), which is synthesized by activated PARP-1, shows weak foci in monocytes after genotoxic stress and strong foci in DCs and macrophages. The PARP-1 inhibitor olaparib diminished the PAR signal and significantly sensitized DCs towards TMZ while it was without effect on monocytes. Macrophages showed a tendency to sensitization with olaparib towards TMZ demonstrating that PARP-1 is not the only factor in this cell system conferring drug sensitivity. γH2AX foci are cleared in DCs and macrophages 24 h after TMZ treatment, revealing DNA repair, whereby DNA double-strand breaks (DSB) are continuously present in monocytes. We conclude that TMZ-induced DNA damage is converted into SSBs and later on into DSBs. We further infer that the hypersensitivity of monocytes results from a defect in both BER and B-NHEJ. Further, we elucidated the mechanism of cell kill after TMZ treatment and show that the ATM/ATR-Chk1/Chk2-p53 pathway was activated in monocytes resulting in Fas receptor upregulation and caspase-8 activation, decline of Bcl-2 and activation of caspase-9, indicating participation of the exogenous and endogenous apoptotic pathway in monocyte cell kill. Because monocytes are also hypersensitive to ionizing radiation, which is applied concomitantly in glioma therapy, the sensitivity of monocytes might have impact on the immune response. The findings are important for understanding the killing response of monocytes after TMZ treatment and the immunosuppressive effects of chemotherapy.

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Regulation of endosomal Toll-like receptor signaling by the protein tyrosine phosphatase PTP1B

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Toll-like receptors (TLRs) are key players of the innate immune system. Localised at the cell surface or intracellularly, they trigger immune responses upon recognition of pathogenic patterns. After their synthesis in the ER, intracellular TLRs 3, 7 and 9 need to move to the endosomal compartment in order to meet their ligands. On their way they are escorted by the polytopic membrane protein UNC93B. UNC93B directly interacts with TLRs 3, 7 and 9 and delivers them from the ER to the endolysosome where they initiate signaling cascades leading to the expression of proinflammatory cytokines or type I IFNs upon encounter of nucleic acids. How function and trafficking of UNC93B/TLR complexes is regulated is not entirely understood. In a proteomics approach the protein tyrosine phosphatase PTP1B has been identified as a novel binding partner of UNC93B in macrophages. PTP1B is known to be crucial for cellular trafficking of receptor tyrosine kinases, but has not yet been linked to regulation of TLRs. Interaction of PTP1B with UNC93B and TLRs 7 and 9 was confirmed by coimmunoprecipitation experiments. The IFNα response in plasmacytoid dendritic cells (pDCs) of PTP1B knockout mice was enhanced upon stimulation of TLR9 and TLR7, suggesting that PTP1B may act as a negative regulator of TLR7 or TLR9 signaling in pDCs.
Tolerance induction by a forced expression of MOG-Peptide in the context of EAE

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Introduction:
Mature antigen-loaded dendritic cells (DCs) are well known antigen-presenting cells with a high potential of activating T cells and inducing inflammation, whereas steady-state DCs are known to maintain T cell homeostasis and peripheral tolerance. T cells recognition of self-antigen presented by peripheral immature DC, may lead to Apoptosis, tolerance or the conversion into peripheral-induced Treg (iTreg). The main goals of this study is to define the underlying mechanisms by which DCs control peripheral tolerance of activated T cells.

Methods:
Experimental autoimmune encephalomyelitis (EAE) is a T cell mediated autoimmune disease of the CNS widely used as a model for studying multiple sclerosis. EAE is induction by immunization using Myelin Oligodendrocyte Glycoprotein peptide (MOG33-55) and Complete Freund’s Adjuvant (CFA). To study Ag-induce peripheral tolerance, we used a mouse where the invariant chain CLIP sequence is replaced by MOG33-55 peptide (IiMOG). By crossing the IiMOG to Tamoxifen (TAM)-induced CD11c-creERT2, we generated mice where we are able to induce MOG33-55 presentation on MHC class II by CD11c positive cells after TAM injection without farther maturation/activation signals (DC-IiMOG).

Results:
Upon TAM injection, about 5% of all CD11c+ cells present the MOG peptide all over the body. DC-IiMOG mice exhibit complete resistant to EAE induction by administration of TAM 2 days before active immunization. This is in line with previous studies showing tolerance induction by resting DCs. Tolerance induction in this system is also possible after the active immunization. When we injected the mice with TAM 2 days after active immunization, the DC-IiMOG mice display complete resistance to EAE. The same was true for TAM administration at day 7 post active immunization, a time where the first T cell priming and differentiation is thought to already take place. In contrast, injection of TAM into WT animals 7 days post active immunization did not prevent EAE development. When analysing splenocytes 21 days after immunization (14 days after TAM), we observed a reduced percentage of interleukin-17A (IL-17A)-producing cells as well as IL-17A and interferon-γ (IFNγ) coproducing cells in the DC-IiMOG mice as compared to control littermates. TCR transgenic T cells (2D2) are able to proliferate when adoptively transferred into TAM treated DC-IiMOG mice. However, the 2D2 cells show a strong upregulation of PD-1 and a moderate elevation of BTLA when transferred into DC-IiMOG mice compared to the controls. Both PD-1 and BTLA are known co-inhibitory molecules associated with tolerance.

Conclusion:
Our results show that the MOG-presenting steady state DCs can block the induction of EAE even after the initial priming phase of the disease, in a process that possibly involve PD-1 and BTLA.
Human dendritic cell subtypes interact specifically with the pathogenic mold *Aspergillus fumigatus*

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**Introduction:**
Activities of dendritic cells (DCs) including phagocytosis, expression of co-stimulatory molecules and cytokine release are required for successful clearance of various pathogens. After direct antigen contact, DCs sense other immune cells to interact with pathogens; this reflects one of the most important immune effector functions.

**Objectives:**
We investigated the interaction between different *A. fumigatus* morphotypes (conidia and germ tubes) and subpopulations of human DCs (myeloid DCs (mDCs), plasmacytoid DCs (pDCs) and monocyte derived DCs (moDCs)). Our interest was to explore how the different DC subtypes interact with and affect *A. fumigatus* as well as to determine specific differences among the DC subtypes and their interaction with a fungal pathogen.

**Materials & Methods:**
mDCs, pDCs and monocytes were isolated from the peripheral blood of healthy volunteer donors using antibody bound magnetic MicroBeads. Monocytes were differentiated into moDCs over 6 days with IL-4 and GM-CSF. DCs were co-incubated with resting conidia or germ tubes and afterwards analyzed by time-lapse video microscopy, scanning electron microscopy, plating assays, flow cytometry, transwell assays and multiplex ELISA assays.

**Results:**
Our data show that the different DC subtypes interact with *A. fumigatus* specifically, although, moDCs and mDCs display similar characteristics. mDCs and moDCs were able to recognize and to respond to fungal morphologies. Although, mDCs did not kill conidia or phagocytose fungal morphotypes as efficiently as moDCs, they matured and secreted comparable amounts of cytokines making them both potential T cell stimulators and recruiters of neutrophil granulocytes. In contrast, pDCs did not phagocytose any fungal cells, they did not mature and secreted only limited cytokines upon contact to *A. fumigatus*. Due to rare and random contacts of pDCs with fungal cells during live-imaging it seems that pDCs do not actively recognize *A. fumigatus*. Live imaging (Figure 1) and scanning electron microscopic pictures (Figure 2) of a representative donor after 0 h and 3 h of DC-fungal co-incubation are enclosed.

**Conclusion:**
Our in vitro data elucidate the distinct interaction between DC subpopulations and *A. fumigatus*, which indicate their different roles in the pathogenesis of invasive aspergillosis. moDC’s and mDC’s specific properties are worth to be considered in future studies, analysing the feasibility of DCs as tools for anti-fungal immunotherapy.
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RelB in dendritic cells is important to control proper establishment of an immune reaction

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To target RelB deficiency to professional antigen-presenting dendritic cells (DCs) we crossed RelB<sup>lox</sup> with CD11c-Cre mice. The resulting RelB<sup>Cre<sup>-</sup></sup> mice allow us to dissect the role of RelB specifically in DCs. As suggested by earlier studies of conventional RelB knockout mice we also observed a defective myeloid DC subpopulation (myDCs; CD11c<sup>hi</sup> Sirp1a<sup>+</sup>) in the spleen. In the steady state the defect was characterized by a reduction of CD103<sup>-</sup> and CXCR4<sup>-</sup> myDCs with a decreased expression of the costimulatory marker CD80. Analysis of proliferation (Ki67<sup>+</sup>) did not reveal any change in DC turnover due to the deletion of RelB in DCs. Analysis of cell death (cleaved caspase 3<sup>+</sup>) showed a slight increase in the myDC compartment. Analysis of progenitor cells in RelB<sup>Cre<sup>-</sup></sup> mice indicate a reduction in CD11c<sup>+</sup> Sca-1<sup>-</sup> c-Kit<sup>hi</sup> FLT3<sup>+</sup> precursor cells. The remaining DCs in RelB<sup>Cre<sup>-</sup></sup> mice support the expansion of natural regulatory T cells (nTregs). In contrast to the autoimmune RelB<sup>-null</sup> phenotype these Tregs did not show impaired suppressor activity, neither in vitro nor after transfer into Foxp3<sup>-null</sup> scurfy mice. Experiments utilizing an iso-osmotic pump system to constantly deliver antigens in a non-inflammatory condition in vivo did not show an increased conversion of antigen-specific non-Tregs into iTregs. In addition, we observed an increased expansion of antigen-specific T cells irrespective of FoxP3 expression.

In an immature state, RelB-deficient DCs show normal activation of T cells as observed in polyclonal studies in vitro and in antigen-specific, osmotic pump-assisted studies in vivo. In an adjuvant-driven, antigen-specific activation of OVA-specific transgenic T cells in vivo these DCs were impaired. We further show that RelB<sup>Cre<sup>-</sup></sup> mice are hyporesponsive to delayed-type hypersensitivity (DTH) and to experimental autoimmune encephalomyelitis (EAE). Analyses of antigen-specific T cells indicate a defect in the generation of Th1/Th17 cells resulting in reduced levels of IFN-γ and IL-17A. Initial functional analyses of RelB-deficient DCs revealed impaired upregulation of the CD80 costimulatory molecule and reduced production of IL-6 and TNF in presence of LPS or CD154<sup>+</sup> T cells (after PMA/Iono restimulation).

Collectively, these results suggest that DCs lacking RelB are associated with the generation of a tolerogenic environment accompanied by an enhanced antigen-specific expansion of steady state T cells. In an immunogenic situation RelB-deficient DCs show an intrinsic defect to properly activate T cells in an immunogenic response.
Introduction:
The inflammatory chemokine CCL17 plays an important role in the induction of various inflammatory diseases like atherosclerosis, inflammatory bowel disease and others. Major functions of CCL17 are the initiation of T cell-dendritic cell (DC) interactions and of Langerhans cell (LC) emigration from the skin. Especially in atopic dermatitis (AD), CCL17 deficiency results in a significant reduction of inflammation and decrease of allergen-specific immune responses. In mice, the expression of CCL17 is mainly restricted to DC of barrier organs like skin and intestinal mucosa, including their draining lymph nodes (LN). Interestingly, CCL17 production cannot be detected in the spleen under steady state conditions. Systemic activation of NKT cells via galactosylceramide (GalCer) was shown, however, to increase splenic CCL17, thereby facilitating the local cross-presentation of antigens. Furthermore, recent findings indicate expression of CCL17 by additional, non hematopoietic cell types.

Objectives:
Our aim was to investigate the complex regulation of CCL17 expression by cytokines and environmental stimuli in myeloid cells as well as non-hematopoietic cell types. In addition we study the effects of inducible ablation of CCL17 in mice.

Materials & Methods:
Mice expressing an enhanced green fluorescent protein (eGFP) or a diphtheria toxin receptor (DTR) transgene under the control of the CCL17 promoter were generated to study CCL17 expression as well as possible effects of an inducible ablation of CCL17-expressing cells, respectively. A genome-wide comparative gene expression analysis of sorted CCL17-expressing LN vs. CCL17-negative splenic DC was performed. The influence of IFNg, GM-CSF and IL-4 on CCL17 expression was studied using relevant knockout mice as well as neutralizing antibodies. Bone marrow chimeras were generated introducing CCL17/DTR bone marrow into wild-type mice and vice versa to study consequences of a selective loss of (non-) hematopoietic CCL17-expressing cells.

Results:
Transcriptome analysis revealed a large number of interferon-inducible genes that are differentially expressed between LN and splenic CD8aCD11b+ DC. By analysis of IFNgR-deficient mice, we show that under normal circumstances, splenic CCL17 production is specifically suppressed by IFNg. In contrast, the NKT cell-derived cytokines IL-4 and GM-CSF are the main inducers of the chemokine after GalCer stimulation as shown by treatment with IL-4- and GM-CSF-specific antibodies. CCL17 ablation in non-hematopoietic but not hematopoietic cells resulted in severe weight loss and behavioral abnormalities. Following systemic treatment with CpG oligonucleotides CCL17 expression was detected in the hippocampus, presumably in pyramidal neurons.

Conclusions:
Our findings demonstrate that the local cytokine milieu, in particular IFNg, GM-CSF and IL-4 regulate lymphoid organ-specific immune responses by differential CCL17 expression. Beyond that, our data indicate that CCL17 may also be expressed in non-hematopoietic cells, which appear to be involved in the regulation of behavior and weight maintenance.
Hypoxia and HIF-1α signaling modulate the response of human dendritic cells stimulated with the pathogenic mould *Aspergillus fumigatus*

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Introduction:
Immunocompromised patients are highly susceptible for the development of fungal infections such as invasive aspergillosis (IA), a life-threatening disease most commonly caused by *Aspergillus (A.) fumigatus*. In a murine model of IA, hypoxia has been shown to occur at sites of *A. fumigatus* invasive growth in the lung. Hypoxic microenvironments and signalling via hypoxia-inducible factor (HIF) 1α are known to influence functions of immune cells, including dendritic cells (DCs). DCs are of particular importance in initiating both innate and adaptive immunity against *A. fumigatus* conidia and hyphae during IA.

Objectives:
Here, we evaluate the role of hypoxia and HIF-1α signaling in the immune response of human DCs stimulated with *A. fumigatus* in vitro.

Methods:
Monocytes were isolated from peripheral blood mononuclear cells of healthy donors by magnetic activated cell sorting and differentiated into DCs by five days incubation with IL-4 and GM-CSF. Immature DCs were confronted with *A. fumigatus* germ tubes at normoxia or hypoxia (0.1 and 1% oxygen), followed by characterization of DC functions, including gene expression profiles and analysis of surface marker and cytokine expression. To silence HIF-1α, DCs were electroporated with siRNA directed against HIF-1α mRNA 24 h prior to the experiments.

Results:
Gene expression profiles of DCs stimulated with *A. fumigatus* for 6 h at normoxia or hypoxia (0.1%) revealed a minor influence of hypoxia on DC gene expression compared to stimulation with *A. fumigatus* at either normoxia or hypoxia. Comparing DCs stimulated with *A. fumigatus* at normoxia vs hypoxia, approximately 20% of all regulated genes were exclusively in the hypoxia plus *A. fumigatus* condition. This included genes involved in metabolic and inflammatory pathways, suggesting altered DC function at hypoxia. DCs consumed more glucose and released more lactate in the cell culture media when challenged with *A. fumigatus* at normoxia or hypoxia or when cultivated at hypoxia without stimulus, and this switch in energy metabolism was at least in part dependent on HIF-1α at normoxia as well as hypoxia. Up-regulation of relevant maturation and co-stimulatory molecules (CD40, CD80, CD83, CD86 and HLA-DR) as well as the homing-receptor CCR7 was reduced on DCs stimulated with *A. fumigatus* at hypoxia (both 0.1 and 1%) compared to DCs stimulated at normoxia. This effect seemed to be independent of HIF-1α, as HIF-1α silenced DCs as well as control DCs showed the same results.

Conclusions:
DC maturation was impaired after stimulation with *A. fumigatus* at hypoxia compared to normoxia. Thus, our results suggest that hypoxia has substantial impacts on DC function, which might also influence the course and outcome of IA in immunocompromised patients. The role of HIF-1α in the response of DCs against *A. fumigatus* is not clear yet. Therefore, further studies including gene expression profiling of HIF-1α silenced DCs at normoxia and hypoxia will help to understand the underlying pathways and the role of this transcription factor in the response of human DCs against *A. fumigatus*. 
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Cellular unresponsiveness induced by DNA vaccines encoding DEC205-targeted antigen

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Question:
The increasing incidence of allergy and autoimmune diseases in industrialized countries underlines the need for therapeutic vaccines, which could induce an antigen-specific tolerance by the immune system. Without additional maturation stimuli, proteins can induce antigen-specific tolerance if directly delivered to immature dendritic cells (DCs). A promising approach for a prolonged and continuous antigen delivery to DCs is the use of DNA vaccines encoding antigens targeted to the endocytic uptake receptor DEC-205 via single-chain antibodies. Here, we assessed the immunogenicity of such targeted DNA vaccines after in vivo electroporation. Furthermore we wanted to address the induction of antigen-specific regulatory T cells (Treg) and their influence on the cellular immune response.

Methods:
Plasmids encoding a DEC205-targeted hemagglutinin or a respective control were constructed and used for immunogenicity studies in Balb/c (wt), TCR-transgenic (TCR-HA) and DEREG mice, allowing the selective depletion of Tregs. After DNA electroporation, cellular immune responses were characterized by intracellular cytokine staining and an in vitro proliferation assay. Additionally, Tregs were monitored by phenotypic analysis of CD25 and FoxP3 expression. To address the induction of antigen-specific Tregs, 10⁶ TCR-transgenic CD4⁺ T cells were adoptively transferred to Balb/c mice prior to immunization. Finally, the suppressive capacity of the induced Tregs was analyzed in a murine diabetes model.

Results:
Immunization of Balb/c wt mice with the DEC-targeted antigen resulted in suppressed cellular immune response compared to the non-targeted control, but without obvious alterations in the overall amount of Tregs. Contrary, a rise of antigen-specific Tregs was observed in the blood and in the spleen of vaccinated TCR-HA mice. Similarly, an accumulation of antigen-specific Tregs, dependent on the distance to the site of immunization, was observed in Balb/c mice having received TCR-transgenic CD4⁺ T cells prior to immunization. Nevertheless, transfer of CD4⁺ T cells from draining lymph nodes of immunized TCR-HA mice into INS/HA mice did not reduce or delay the onset of diabetes compared to control mice. Furthermore, depletion of Tregs (DEREG) during the induction phase of the immunization did not restore the cellular immune responses suggesting an alternative mechanism underlying the reduced T cell responses, like anergy or peripheral depletion.

Conclusion:
DNA vaccines encoding DC-targeted antigens induce restricted cellular immune responses, which might be a result of peripheral tolerance. Although antigen-specific Tregs were induced after immunizations in transgenic mouse models, we could not demonstrate any suppressive effect in a diabetes model suggesting regulatory T cells not to be responsible for the reduced T cell response. This is supported by the fact that depletion of regulatory T cells in the early phase does not restore T cell functions after DC-targeted DNA vaccination. Nevertheless, vaccination induced a state of unresponsiveness in antigen-specific T cells after re-stimulation and therefore might be a promising therapeutic approach for allergic diseases.
Distinct modulation of circulating CD1c+ conventional dendritic cells by spingosine 1-phosphate receptor agonism in multiple sclerosis patients

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Objective:
We hypothesize that agonists of spingosine 1-phosphate (S1P) receptor affect accessory cells and potentially influence the adaptive immune response. The objective of our study was to investigate the potential in vivo effects of S1P receptor agonism on circulating CD1c+ conventional dendritic cells (DC) in patients with multiple sclerosis (MS).

Background:
Fingolimod, an S1P receptor agonist leading to segregation of lymphocytes in secondary lymphoid tissues, is a newly approved oral drug for the treatment MS. Data of phase III clinical trials in RRMS patients showed beneficial effects of fingolimod on disease activity as evidenced by slowing the progression of disability and by reducing relapse rates. All five S1P receptors are expressed on DC in mice, yet, to date, the in vivo effect of fingolimod on circulating DC in MS patients has not been characterized.

Methods:
We collected blood from 10 fingolimod-treated MS patients, 13 untreated MS patients, and 15 healthy subjects. Frequencies of conventional CD1c+ DC subsets were determined by flow cytometry. CD1c+ DC were magnetically sorted from PBMC and their expression of surface markers was assessed. For analysis of cytokine and chemokine secretion cells were plated in culture plates and stimulated with LPS and cell culture supernatants were collected 24 h later. For mRNA analysis, DCs were snap frozen after 4 h.

Results:
Fingolimod treatment in MS patients reduced the number of CD1c+ conventional DC in the peripheral blood and was associated with an increased CD86 surface expression by these cells, indicating a modification of DC maturation. Quantitative PCR analyses revealed that the obtained CD1c+ conventional DC upon LPS stimulation expressed lower levels of MIP-1alpha and MIP-1beta, both chemokines being essential for leukocyte recruitment to inflammatory tissue. Analysis of DC culture supernatants further showed a reduced secretion of the pro-inflammatory cytokine IL-1beta that is required for the differentiation of Th17 cells in humans.

Conclusion:
Our data indicate that the immunomodulatory effects mediated by S1P receptor agonism are not limited to lymphocytes, but are also effective on antigen-presenting cells in MS by down-regulating immunogenicity of circulating CD1c+ conventional DC in vivo. A comparable modulation of human dendritic cells as a potential mechanism of action in MS has recently been reported for fumarate by Ghoreschi et al., J Exp Med. 2011.

Support:
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Induced and thymus-derived Foxp3+ regulatory T cells share a common niche within the CD4+ T cell compartment

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Foxp3+ regulatory T cells (Tregs), which play a central role for the maintenance of immune homeostasis and self-tolerance, are known to be both generated in the thymus (thymus-derived Tregs, tTregs) and in the periphery, where they are converted from conventional CD4+ T cells (induced Tregs, iTregs). Recent data suggest a division of labor between these two Treg subsets since combined action of them was shown to be essential for full protection in inflammatory disease models. Here, using the transfer colitis model we examined whether tTregs and iTregs fill different niches within the CD4+ T cell compartment. When naive T cells were co-transferred with either pure tTregs or with a mixture of tTregs and iTregs comparable de novo induction of Foxp3+ Tregs from naive T cells was observed. Moreover, using neuropilin-1 as a surface marker to separate tTregs and iTregs we could demonstrate that iTregs harbour full capacity to fill the complete Treg niche, whereas the size of the Treg niche seemed only to depend on the inflammatory milieu. Together, our data suggest that tTregs and iTregs do not require unique survival factors, but rather share a common peripheral niche.
Polarized Secreted IL-17C Mediates Respiratory Epithelial Innate Immune Response

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Introduction:
The IL-17 family of cytokines consists of at least six members (IL-17A to -F). IL-17 directly activates epithelial cells leading to the expression of inflammatory mediators and antimicrobial factors. Recent studies showed that IL-17C is expressed by epithelial cells. In this study, our aim was to examine the expression and function of IL-17C in respiratory epithelial cells during bacterial infection.

Methods:
Bronchial epithelial cells were cultured in transwells, treated with 2-APB, BTP-2, or with different concentrations of EGTA and incubated with IL-17C, bacteria or bacterial factors. IL-17C was silenced with siRNA. Expression and release of IL-17C and IL-6 was measured by ELISA and qRT-PCR.

Results:
In vitro studies showed that common bacterial pathogens, such as Pseudomonas aeruginosa and Haemophilus influenzae, and ligands of Toll-like receptors 3 and 5 (flagellin, polyI:C) induced the expression and release of IL-17C in cultured human bronchial epithelial cells (HBECS). IL-17C enhanced inflammatory responses of respiratory epithelial cells infected with P. aeruginosa. Differentiated respiratory epithelial cells secreted IL-17C into the basolateral compartment and the secretion of IL-17C was increased after basolateral stimulation than apical stimulation. Pharmacological inhibition of intracellular calcium release and calcium chelators inhibited the secretion of IL-17C whereas IL-6 expression was not affected.

Conclusion:
These data show that IL-17C mediates innate immune responses of respiratory epithelial cells and that calcium-dependent signaling cascades mediate the polarized secretion of IL-17C.
Hypoxia and the hypoxia-regulated transcription factor HIF-1alpha regulate innate immune responses of respiratory epithelial cells

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Introduction:
Acute and chronic diseases of the respiratory tract, such as pneumonia, cystic fibrosis, and COPD, are associated with mucosal and systemic hypoxia. Bacterial infections contribute to exacerbations in chronic respiratory diseases. Innate immune functions of respiratory epithelial cells are required to control and prevent colonization of the lung with bacterial pathogens. Our aim was to examine whether hypoxia and HIF-1alpha regulate innate immune mechanisms of respiratory epithelial cells.

Methods:
Bronchial epithelial cells were cultured under normoxic and under hypoxic conditions (2% oxygen) and incubated with bacteria or bacterial factors. HIF-1alpha was silenced with siRNA. Expression and release of IP-10 and IL-6 was measured by ELISA and qRT-PCR.

Results:
Hypoxia resulted in a significantly reduced expression of inflammatory mediators (IL-6, IP-10) in respiratory epithelial cells in response to bacterial infection with Pseudomonas aeruginosa and ligands for Toll-like receptors (e.g. flagellin, polyI:C). Small interfering RNA-mediated knockdown of HIF-1alpha in bronchial epithelial cells resulted in increased expression of these factors under normoxic and hypoxic conditions.

Conclusion:
These data indicate that hypoxia impacts innate immune mechanism of respiratory epithelial cells via HIF-1alpha.
The intestinal immune system is not only shaped by microbiota but also by food proteins, however, it still remains enigmatic to which degree dietary antigens influence mucosal immunity. We here show that maturation of lymphocytes in Peyer’s patches (PP) and small intestinal lamina propria (LP) depends on stimulation by food protein antigens. Studies on food antigen-free (FAF) mice raised on chow containing free amino acids for several generations demonstrated that food proteins have a critical impact on the intestinal immune system. FAF mice had smaller PP and mesenteric lymph nodes (mLN), fewer CD4+ T and B cells in these organs and lower concentrations of intestinal IgA as compared to conventionally raised mice. The dramatic reduction in T and B cell numbers in FAF mice was observed in small intestine-associated lymphoid tissue but not in spleen and peripheral lymphoid organs. Food protein uptake was associated with a unique CD44+Helios+Foxp3− T cell population accumulating in PP of both SPF and GF mice, indicating that expansion of this population is independent of antigens derived from the commensal microbiota. Notably, these T cells, expressing a CD44+Helios− signature characteristic for activated lymphocytes, represent a dominant percentage (30%) in this organ but they are massively reduced in FAF mice. The food protein-dependent CD44+Helios+Foxp3− T cell population was functionally and phenotypically different from effector T cells, Tregs and follicular T cells. High expression of apoptotic markers in these cells suggests a delicate balance of continuous, food protein driven proliferation and death warranting normal cellular composition and structure of PP.

In conclusion, similar to microbial colonization of the colon, absorption of food protein antigens in small intestine is critical for full expansion of T and B cell numbers and for maintenance of normal intestinal immune system. Thus, not only commensal microbiota but also food protein antigen supply is required for maturation and function of intestinal immune system.
Citrobacter rodentium: from pathogen to commensal - the influence of microflora on lifelong persistence

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Introduction/Objectives:
Citrobacter rodentium (C.rodentium), a natural pathogen of mice is used to study human infections with EPEC and EHEC. Infection of mice with the enteric C. rodentium leads to colonisation via intimate attachment and effacement (A/E) of the colonic epithelium. In normofloric C57BL/6 mice, infection with C. rodentium results in self-limiting colonisation mediated by Citrobacter-specific antibodies. The impact of the normal intestinal microflora on resistance and clearance of C. rodentium is not understood yet. Aim of this project was to study the course of C. rodentium infection in germfree animals with respect to bacterial persistence, expression of virulence genes and tissue specificity.

Results:
Infection of germfree mice with the enteric pathogen C. rodentium leads to lifelong persistence of the pathogen in colon and cecum despite normal levels of Citrobacter-specific antibodies and without obvious signs of inflammation. Persistence of C. rodentium in germfree animals was associated with down-regulation of bacterial virulence and effector genes, demonstrating conversion from pathogenic to commensal life style. On the host side, mono-association of germfree animals resulted in down-regulation of fat metabolism associated genes, including adipsin, adiponectin, and resistin. Transplantation of live but not heat-killed normal microflora to mono-colonised mice resulted in clearance of C. rodentium from the gut accompanied by colonic hyperplasia and increased levels of citrobacter-specific IgA in the gut lumen. The protective effect of normal flora was MyD88 but not Trif dependent and was associated with upregulation of fat metabolism associated genes.

Conclusions:
Infection of germfree animals with C. rodentium show that i) pathogen-specific antibodies are not sufficient to clear C. rodentium ii) the pathogen is able to persist by adopting a commensal lifestyle and iii) normal flora is required for bacterial clearance by an MyD88-dependent mechanism including genes of the fat metabolism.
Induction of Wnt-1 attenuates development of an allergic airway disease

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The role of Wnt signaling during early organ development and lung morphogenesis is already well described. Recent reports reveal that molecules of the Wnt family are involved in the development of different diseases, most likely due to their ability to influence inflammatory responses. In the lung Wnt molecules are involved in repair and dysregulatory processes leading to diseases like COPD or fibrosis. In addition, both in vitro and in the gut Wnt signaling was also shown to be relevant for the development of immunological tolerance.

In the present study we investigated the role of Wnt-1 in allergic airway disease. For this mice with a doxycycline (DOX) inducible lung specific over-expression of Wnt-1 (CCSpTA x tet0-Wnt-1) were systemically sensitized to OVA. In a primary challenge protocol Wnt-1 expression was induced by application of DOX before exposure to OVA by inhalation (prophylactic model). In another protocol sensitized mice were exposed to OVA by inhalation and DOX was given prior to a secondary allergen exposure (therapeutic model).

Sensitized and challenged animals developed airway inflammation and hyperresponsiveness in the prophylactic as well as in the therapeutic model. Numbers of eosinophils in BAL, inflammatory infiltrates in the lung tissue as well as number of mucus producing goblet cells were increased following sensitization and challenge. In both models induction of Wnt-1 expression by DOX application during challenge lead to a reduction of airway inflammation and hyperreactivity. Interestingly, increased expression of Wnt resulted neither in differences in number of regulatory T cell nor concentrations of IL-10.

In summary, increased Wnt expression in the lung demonstrates regulatory properties in prophylactic and therapeutic models of allergic airway disease. This effect seems to be mediated by a mechanism independent of Tregs. Further studies are needed to identify the underlying mechanisms.
The intestinal microbiota triggers epithelial toll-like receptor expression and impairs neuropilin-1 processing and hedgehog signaling in the small intestinal mucosa

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Introduction:
A single layer of epithelial cells in the small intestine forms the barrier between the gut microbiota and the lamina propria. Ligation of microbial-associated molecular patterns (MAMPs) to Toll-like receptors (TLRs) expressed on the surface of epithelial cells promotes epithelial proliferation. Neuropilin-1 (Nrp-1), a co-receptor of VEGFR-2, is expressed on epithelial cells and was recently described as a novel positive regulator of Hedgehog signaling (HH-signaling), a pathway that regulates intestinal homeostasis and gut development. Recently, variation of GLI1, a component of the HH pathway has been associated with inflammatory bowel diseases (IBD).

Objectives:
In this study we use germfree mouse technologies to identify the role and function of Nrp-1 expression in the small intestinal mucosa and its impact on HH-signaling under physiological and pathological conditions.

Material & Methods:
In this study we use germfree mice (GF) that lack bacterial colonization and are reared in sterile plastic isolators. Conventional-raised (CONV-R) mice are colonized at birth and conventionally-derived (CONV-D) mice are ex-germfree mice that are colonized for 14 days with a gut microbiota harvested from the cecum of a CONV-R donor mouse. To decimate the intestinal microbiota CONV-R mice were treated for one week with a cocktail of antibiotics. To investigate the role of Nrp-1 and HH-signaling in experimental colitis, we performed a DSS-induced colitis model. Therefore, mice were treated with 3.5% DSS in the drinking water for 7 days ad libitum. After this time period mice were sacrificed and expression analyses were performed. Additionally, we employed a sterile infection cell culture model with the murine small intestinal cell line MODE-K. These cells were subjected to different MAMPs, like LPS or the synthetic TLR2 agonist Pam3CSK4.

Results:
Since TLRs in the small intestine sense microbial colonization we compared expression levels of TLR receptors in the small intestine of CONV-R mice with GF controls. We found that small intestinal TLR2 mRNA levels and transcript levels of its co-receptor TLR1 increased upon colonization. This effect could be reversed by antibiotic microbiota decimation. When we stimulated the epithelial cell line MODE-K with TLR2 agonists, this resulted in increased TLR2 and TLR1 transcript levels and was associated with increased cellular TNFα levels and increased proliferation. In contrast, knock-down of TLR2 and Nrp-1 in MODE-K cells reduced proliferation. Treatment of MODE-K cells with TNFα resulted in decreased Nrp-1 levels. In line with decreased TNFα mRNA levels detected in the small intestine of GF mice we found decreased Nrp-1 levels in colonized mice. Accordingly, stimulation of MODE-K cells with TLR2 agonists decreased Nrp-1 protein levels. Co-immunoprecipitation analyses suggest that Nrp-1 interacts with TLR2. Localization of Nrp-1 to the cell membrane was reduced upon TLR2 stimulation. Similar to decreased Nrp-1 levels colonization of GF mice led to a drop in HH ligand expression. Short time colonization of GF mice led to decreased expression of Sonic Hedgehog (Shh) and Indian Hedgehog (Ihh). DSS-induced colitis led to reduced expression of Shh in the small intestine pointing to an activation of wound healing processes.

Conclusion:
Collectively, we found that the epithelial TLR2/Nrp1 signaling loop is regulated by MAMPs of the intestinal microbiota. This is associated with altered HH signaling that could be involved in IBD.
Molecular mechanisms underlying the IL17-dependent and -independent formation of bronchus-associated lymphoid tissue

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The bronchus-associated lymphoid tissue (BALT) represents a major component of the larger network of mucosa-associated lymphoid tissues. It is characterized as follicular aggregations of lymphocytes in the lung, containing a follicle-like structure composed of B cells surrounded by parafollicular T cell areas. The successful generation of antigen-specific immune responses against Influenza virus in splenectomised lymphotoxin-a-deficient mice lacking all conventional organized secondary lymphoid organs (SLOs) has indicated that naïve lymphocytes can be primed outside of SLOs. We could recently show that a single intranasal application of the replication-deficient modified vaccinia virus Ankara (MVA) is sufficient to induce BALT in wild-type mice, which represents an organized and functional lymphoid structure, contributing to the initiation of adaptive immune responses in the lung. Despite the parallels between conventional lymphoid organs and BALT regarding the function, the developmental pathways that control the formation and maintenance of BALT remain unclear. Whereas others (Rangel-Moreno et al., 2012) state the formation of LPS-inducible BALT depends on the production of IL-17 by T cells, we could, however, not detect a role for IL-17 in the MVA-induced formation of BALT. The function of IL-17 in the induction of BALT might depend on the BALT-inducing stimuli. In this study, we now present new insights in how IL-17 contributes to the development of ectopic lymphoid tissue in the lung.

We now established new models for the induction of ectopic lymphoid tissue in the lung that allowed us to dissect the molecular mechanisms underlying the IL-17-dependent and IL-17-independent development of BALT.

A PROBIOTIC MODULATES INNATE LYMPHOIDE CELLS IN MICE.

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Introduction:
Innate lymphoid cells (ILCs) are effectors of innate immunity. A subset of ILCs express natural cytotoxicity triggering receptor (NCR) NKp46, NCR+ILC3s 2, and are defined by their capacity to produce interleukin-22 (IL-22). These ILCs depend on the transcription factor RORgt and IL-7Ra for their development. Additionally, evidence suggests that their development is promoted by commensal microbiota. However, what specific microbial species regulate function of these cells is not yet known.

Objectives:
We have therefore sought to study the modulation of these cells in mice fed with a strain of probiotic bacteria, L. rhamnosus JB-1, which we have demonstrated possesses immunomodulatory effects in mice 1.

Methods:
BALB/c mice were fed with L. rhamnosus JB-1 for 5 consecutive days. Peyer’s patches (PP) were harvested and monitored for phenotype and cytokine production of NCR+ILC3s, using flow cytometry technology and ELISA.

Results:
We found that both percentages and total numbers of identified gut NKp46+ cell populations increased in the PP of mice, following oral administration of 10^9/day L. rhamnosus JB-1 for 5 days. The total number of NKp46+CD3 cells in PP of vehicle fed animals was 2.9 x 10^4 (SD=5.7x10^3), which increased to 5.7x10^4 (SD=2.6x10^3) in JB-1 fed mice. Moreover, higher IL-22 generation was detected in these cells in mice fed with L. rhamnosus JB-1, increasing from 2.2 x 10^3 (SD=2.2x10^2) to 2.9 x 10^4 (SD=5.7x10^3) and was associated with a marked up-regulation in their expression of transcription factor RORgt, from 1.4 x 10^4 (SD=4.2x10^3) in the vehicle fed mice to 9.5 x 10^4 (SD=4.4x10^3) in L. rhamnosus JB-1 fed mice. Additionally, an increase in the percentage of NKp46+CD3+RORgt+ cells that express IL-7Ra (CD127) was demonstrated following L. rhamnosus JB-1 feeding.

Conclusions:
We demonstrated that feeding L. rhamnosus JB-1 to mice for 5 days led to a significant increase in IL-22-producing RORgt-expressing NKp46+ cells in the PP. More studies employing an infection mouse model are needed to further clarify the significance of NCR+ILC3s modulation by L. rhamnosus JB-1. Increased IL-22 production following L. rhamnosus JB-1 may be related to the role that these cells play in innate defence against bacterial infection.

References:
Functional properties and de novo induction of Foxp3\(^+\) regulatory T cells in *Yersinia pseudotuberculosis* infection

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**Introduction:**
Foxp3\(^+\) regulatory T cells (Tregs) not only prevent the development of autoimmunity and the immune systems’ overreaction, they also can modulate immune responses against several infectious agents.

**Objectives:**
The current study sheds light on understanding how Tregs are generated and modulated during acute and chronic *Yersinia pseudotuberculosis* (*Yptb*) infections.

**Materials & Methods:**
With the help of a modified strain of *Yptb*, which features a β-lactamase-based reporter, cells targeted in vivo by *Yptb* could be identified. Using the DEREG mouse model to selectively deplete Foxp3\(^+\) Tregs, we further analyzed the functional importance of Tregs for the regulation of immune responses directed against *Yptb*.

**Results:**
Using the β-lactamase-based reporter system we could demonstrate that *Yptb* can target Tregs more efficiently than conventional T cells, and that this modulation can impair the Tregs’ suppressive capacity. Depletion of Tregs 24 hours prior to infection resulted in an increased influx of monocytes and neutrophils in mLN and spleen, however the bacterial burden in these lymphoid organs remained unaltered, suggesting that the immune response against acute *Yptb* infection is not under the control of Tregs. Strikingly, *de novo* Treg induction within gut-draining LNs was drastically reduced during acute *Yptb* infection, and furthermore was still impaired 28 days post infection when a non-lethal *Yptb* mutant strain was used.

**Conclusion:**
Together, these results suggest that *Yptb* infection can have long-lasting effects on the tolerogenic properties of the intestinal immune system.
Epithelial cells control intestinal Treg accumulation through interactions with E-cadherin

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Question:
Intestinal immunity is controlled through different mechanisms to systemic responses. There is increasing evidence that non-hematopoietic cells, including epithelial cells, are active players in the balance between inflammation and tolerance, but the mechanisms mediating their immune activity are still poorly understood. We wanted to know if intestinal epithelial cells can act on gut CD4+ Foxp3+ T cells (Treg), a crucial population controlling intestinal immune homeostasis, through the recognition of epithelial molecules by Treg.

Methods:
We have focussed on E-Cadherin, an epithelial molecule that can be recognised by the integrin CD103 and the lectin KLRG1 on hematopoietic cells. To check the effects of E-cadherin on the mucosal immune system, we used mice in which E-Cadherin had been replaced by the closely related molecule N-Cadherin on intestinal epithelial cells. We compared the intestinal Treg populations of mice maintained under specific pathogen-free and germ-free conditions, and complemented the data from E-cadherin-deficient mice with the analysis of mice deficient for its receptor KLRG1.

Results:
Microscopy analysis show that gut Foxp3+ cells can be found in close contact to intestinal epithelial cells. We find that the absence of E-cadherin on gut epithelial cells leads to lethal intestinal inflammation, which was absent on germ-free E-cadherin deficient mice. Changes in the immune compartment of E-cadherin-deficient mice were mostly attributable to the ongoing intestinal inflammation. In contrast, the absence of epithelial E-cadherin resulted in an accumulation of KLRG1+ Foxp3+ T cells in the gut that was independent of the inflammation or the presence of microbiota. KLRG1+ Foxp3+ CD4+ T cells showed a phenotype indicative of active Treg, and the complementary analysis of KLRG1-deficient mice showed an increase in Foxp3+ cells in the gut.

Conclusions:
The absence of either E-cadherin on intestinal epithelial cells or its ligand KLRG1 on Treg leads to the local accumulation of CD4+ Foxp3+ T cells in the gut, indicating that mucosal regulatory T cells can be controlled by direct interactions with epithelial cells.
C5a but not C3a drives maladaptive Th2 immunity in experimental allergic asthma

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Introduction:
Allergic asthma is a major health concern in western countries. In asthmatic patients, complement is activated locally in the airways resulting in the generation of several proteolytic cleavage products of C3 and C5 including the anaphylatoxins (AT) C3a and C5a. Both ATs exert the biologic functions through distinct receptors, i.e., the C3a receptor (C3aR) and the C5a receptor (C5aR). Previous studies have highlighted opposing roles of C3aR and C5aR during allergen sensitization, in particular with regard to Th17 development. Conventional dendritic cells (cDCs) are necessary and sufficient for the development of allergic asthma in various mouse models. They control allergen uptake and Th cell differentiation towards Th2, Th17 and Th1 effector cells. The precise role of the ATs in cDC-mediated induction of maladaptive immune responses in allergic asthma is unclear.

Objectives:
The aim of this study was to delineate the roles of C3aR and C5aR signaling in DCs for the development of maladaptive Th2, Th1 and Th17 immunity in allergic asthma.

Methods:
GM-CSF-differentiated bone marrow (BM)-derived DCs from wildtype (wt), C3aR⁻/⁻, C5aR⁻/⁻ and C3aR⁻/⁻/C5aR⁻/⁻ mice were generated in vitro. Supernatants from BMDCs stimulated 24h with PBS or crude extract of house dust mite (HDM) were used for cytokine measurement by ELISA. Further, costimulatory molecule expression was determined by flow cytometry. To directly assess the potency of BMDCs to drive allergic asthma in vivo, we adoptively transferred unpulsed or HDM-pulsed BMDCs from wt and AT receptor-deficient mice into wt mice. After 7 days, mice were challenged with HDM extract and 72h later, airway hyperresponsiveness (AHR), broncho-alveolar lavages cell counts, mucus and cytokine production from restimulated lung cells was determined.

Results:
BMDCs from wt and C3aR-deficient mice induced a strong asthmatic response, characterized by a marked increase in AHR, strong Th2 but minor Th17 and Th1 cytokine production and a mixed eosinophilic and neutrophilic infiltration of the lung. In contrast, AHR, Th2 cytokine production as well as eosinophilic and neutrophilic inflammation were significantly decreased following adoptive transfer of C5aR⁻/⁻ BMDCs. The absence of both, C3aR and C5aR in BMDCs recapitulated some effects observed after transfer of C5aR⁻/⁻ BMDCs, in particular the reduced eosinophil, IL-4 and IL-5 levels. In vitro stimulations of BMDCs with HDM induced increased expression of IL-12p40 and lowered the production of TGF-β in absence of C5aR, but had no significant impact on the secretion of other Th17-promoting cytokines or the expression of costimulatory molecules in BMDCs from either wt or AT receptor-deficient mouse strains.

Conclusion:
Our data suggest that C5aR signaling in BMDCs is critical for the development of Th2 maladaptive immune response including the development of AHR, mixed eosinophilic and neutrophilic airway inflammation and mucus production. Surprisingly, the role of C3aR signaling in BMDC-driven Th cell differentiation and pulmonary inflammation appears to be minor. Our findings are in apparent contrast to our previous findings showing an enhanced allergic phenotype in C5aR-targeted or deficient mice. Most likely, BMDCs inappropriately reflect the sensitization process towards aeroallergens such as HDM by lung resident DCs, at least in the context of complement activation. This could be due to the nature of BMDCs, which resemble inflammatory DCs but not resident pulmonary DCs.
Protective role of Nod1 and Nod2 in *Salmonella*-induced Colitis

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Nod1 and Nod2 are cytosolic pattern recognition receptors (PRR) recognizing bacterial peptidoglycan. Mutations in Nod2 are known as genetic risk factors of chronic inflammatory bowel diseases (IBD). The intracellular pathogen *Salmonella enterica* serovar *Typhimurium* (*S. Typhimurium*) causes a systemic infection in mice. When mice are pretreated with streptomycin before infection with *S. Typhimurium* they develop transmural inflammation characterized by edema, neutrophils influx and increased Th1 cytokine expression.

Here, we investigated the role of Nod1 and Nod2 in the development of cecal inflammation *Salmonella*-induced colitis. Streptomycin-pretreated C57BL/6 and Nod2⁻/⁻ mice were orally infected with *S. Typhimurium* wild type or *S. Typhimurium DmsbB* (altered LPS). For further analysis tissue samples were collected 2 or 7 days post infection (p.i.). Subsequently, Nod1⁻/⁻ and Nod1⁻/⁻/Nod2⁻/⁻ mice were orally infected with *S. Typhimurium DmsbB* to investigate the role of Nod1.

To investigate whether different *S. Typhimurium* strains resulted in individual PRR-activation patterns in vitro, Nod2⁻, TLR2⁻ or TLR4⁻ transfected HEK293 cells were stimulated with heat-killed *S. Typhimurium* strains.

Oral infection with *S. Typhimurium* wild type resulted in comparable transmural inflammation in C57BL/6 and Nod2⁻/⁻ mice. However, when mice were infected with *S. Typhimurium DmsbB*, inflammation was significantly exacerbated in Nod2⁻/⁻ mice compared to C57BL/6 mice 2 days p.i. In particular, the edema was more pronounced and we found more neutrophils by immunohistology. Interestingly, *Salmonella* colonization remained similarly high in Nod2⁻/⁻ mice from day 2 to day 7 p.i., whereas it significantly decreased in C57BL/6 mice. Further investigations of Nod1⁻/⁻ and Nod1⁻/⁻/Nod2⁻/⁻ mice revealed, that Nod1 also plays a protective role in *Salmonella*-induced colitis.

To elucidate why *S. Typhimurium DmsbB* but not *S. Typhimurium* wild type induced an exacerbated inflammatory response in Nod2⁻/⁻ mice, HEK293 cells were transiently transfected with different PRRs and stimulated with heat-killed *S. Typhimurium* strains. Stimulation of TLR2-transfected cells with *S. Typhimurium DmsbB* resulted in increased IL-8-production compared to *S. Typhimurium* wild type. Interestingly, IL-8-production was even more increased in Nod2-TLR2-cotransfected cells compared to single transfected cells, indicating a synergistic effect of TLR2 and Nod2.

Our results indicate that Nod1 and Nod2 play an important role in the onset of *Salmonella*-induced colitis controlling *Salmonella* colonization and cecal inflammation. As *S. Typhimurium DmsbB* showed an increased TLR2 reactivity in vitro this is likely dependent on TLR2 signaling.
Identification of microenvironmental factors and molecular signals modulating the tolerogenic properties of stromal cells in gut-draining lymph nodes

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Foxp3⁺ regulatory T cells (Tregs) are critical to avoid deleterious immune responses directed against food antigens and commensal microbiota. Thus, de novo induction of these cells is particularly efficient in gut-draining mesenteric and celiac lymph nodes (mLN and celLN) compared to other sites.

We have used LN transplantation to dissect the contribution of LN stromal cells and environmental factors to the high Treg-inducing capacity of gut-draining LN. Combining LN transplantations with adoptive transfers and selective CD11c⁺ dendritic cell (DC) depletion, we identified cellular players driving efficient Foxp3⁺ Treg induction in the gut immune system.

mLN and celLN retained their high Treg-inducing capacity upon transplantation into the popliteal fossa. In contrast, transplantation of skin-draining LN into the gut mesenteries did not enable efficient Treg induction, suggesting a dominant imprinted effect of stromal cells on the generation of Foxp3⁺ Tregs. This effect seems to be mediated via DC, which were indispensable for Treg induction. Stromal cells themselves are strongly influenced by environmental signals since transplanted celLN from vitamin A-deficient and mLN taken from germ-free donors did not show any superior Treg-inducing capacity. Transplantation of mLN from neonatal, two, four and eight week old mice indicated that the mLN is already imprinted within two weeks after birth. Currently, the impact of Yersinia pseudotuberculosis driven gastrointestinal infection on the tolerogenic properties of mLN is being investigated.

Overall, we could show that stromal cells are shaped early during ontogeny by unique micro-environmental factors and significantly contribute to the superior tolerogenic properties of gut-draining LN by modulating incoming DC and exerting a dominant effect on de novo induction of Foxp3⁺ Tregs.
IL-25 mediated regulation of RORγt+ ILCs at the mucosal barrier

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RORγt+ innate lymphoid cells (ILCs), such as lymphoid tissue-inducer cells and IL-22-producing NKp46+ cells are required for the development of lymphoid tissues, homeostasis with symbiotic microbiota and defense against pathogens. An equilibrated crosstalk between RORγt+ ILCs, microbiota, pathogens and adaptive immunity is critical for intestinal homeostasis, a loss of which leads to inflammatory immunopathology. We have previously shown that commensal microbiota repress the activity of RORγt+ ILCs through induction of IL-25 expressed by intestinal epithelial cells. The mechanisms by which IL-25 acts on RORγt+ ILCs are unclear, as these cells do not express the IL-25-Receptor (IL-25R). In vitro cocultures of purified RORγt+ ILCs in association with stromal and dendritic cells demonstrated that IL-25R+ DCs, found in cryptopatches and isolated lymphoid follicles (ILFs) of the intestinal lamina propria, are sufficient to mediate the repressive activity of IL-25. However, IL-25-mediated repression of RORγt+ ILCs via the DC/ILC axis is only partial. Notably, B cells also express the functional IL-25R+ increasing with ILF maturity along the colonization density. We propose that the spatial accumulation of these cells in organized tertiary structures of the intestine provides a complex network for reciprocal and cooperative regulation.
The C-type lectin receptor SIGNR3 binds to commensal fungi and regulates intestinal immunity

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Inflammatory bowel disease (IBD) is a condition of acute and chronic inflammation of the gastrointestinal tract and can be divided into ulcerative colitis and Crohn’s disease. Epidemiological and genetic studies in patients and IBD-related animal models suggest that a combination of genetic susceptibility, environmental factors, and an altered immune response contribute to IBD initiation and pathology. One important environmental factor that plays a major role in colitis pathogenesis is the interaction of commensal bacteria and fungi with intestinal immune cells, such as dendritic cells. Host pattern recognition receptors sense commensals in the gut, thus are involved in maintaining the balance between controlled responses to pathogens and overwhelming innate immune activation. C-type lectin receptors (CLRs) represent a superfamily of pattern recognition receptors in innate immunity. They are predominantly expressed by antigen-presenting cells and recognize glycan structures on pathogens and self-antigens.

Recent reports demonstrate a role for CLRs such as Dectin-1 or MGL-1 in gut inflammation. In this study, we investigated the contribution of the CLR SIGNR3 to commensal recognition and intestinal immunity. SIGNR3 is the closest murine homologue of the human DC-SIGN receptor and binds to similar carbohydrate ligands such as terminal fucose or high-mannose glycans.

We show here that SIGNR3 recognizes fungi present in the commensal microbiota. To investigate whether the interaction between SIGNR3 and commensal fungi impacts intestinal immunity, we employed the dextran sulfate sodium (DSS) colitis model. Indeed, SIGNR3-deficient mice exhibited an increased weight loss accompanied by more severe colitis symptoms compared to wild-type control mice. The increased inflammation in SIGNR3-deficient mice was mediated by a higher local TNF-α level in the colon.

In conclusion, we demonstrate for the first time that SIGNR3 recognizes intestinal fungi and has an immune regulatory role in colitis.
Functional TLR5 variants affect colorectal cancer progression


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While the role of Toll-like receptors (TLR) has been studied thoroughly in infectious diseases, little is known about their role in cancer, e.g. colorectal cancer (CRC), the third most frequent human malignancy world-wide. We identify here frequent single-nucleotide polymorphisms (SNPs) in the flagellin-receptor TLR5 and in the TLR downstream adaptor genes Myeloid differentiation 88 (MYD88) and TIR-domain-containing adaptor protein (TIRAP) as significantly associated with altered CRC progression and survival. The MYD88 SNP which maps to a promoter region shared by MYD88 and acetyl-Coenzyme A acyl transferase 1 (ACAA1) showed decreased CRC survival and altered transcriptional activity. Both coding TLR5 SNP1 and SNP2 disparately affected CRC survival and modulated TLR5 downstream signaling in cellular model systems in response to flagellin and different commensal and pathogenic intestinal bacteria. Whereas SNP2 showed increased survival and decreased cytokine responses, SNP1 decreased survival. Importantly, in primary immune cells from TLR5 SNP2 minor allele homozygous carriers, flagellin-induced p38 phosphorylation, CD62L shedding and induction of the pro-inflammatory cytokines IL-6 and IL-1β were reduced compared to TLR5 homozygous major allele carriers. This suggests that the well-documented effect of cytokines on CRC progression may be mediated by TLR5 genotype-dependent flagellin sensing. Our study therefore establishes an important link between TLRs and CRC in humans which could have broad implications for TLR-based biomarker and therapy development in CRC.
Impact of intestinal epithelial cell responsiveness to immune cell derived IL-10 on intestinal homeostasis and inflammation

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Introduction:
Inflammatory bowel diseases (IBD) including Crohn’s disease and ulcerative colitis are defined as chronic disorders of the gastrointestinal tract. IBD affects more than 4 million people worldwide. The loss of tolerance and hyper-responsiveness to mucosal antigens leads to an activation of the immune system and consecutive dysregulation of mucosal homeostasis. IL-10 represents an anti-inflammatory cytokine that down-regulates the production of Th1-derived cytokines. Mutations in genes encoding the IL-10R subunit proteins were found in patients with early-onset enterocolitis, involving hyperinflammatory immune responses in the intestine pointing to the fact that defective IL-10 signaling plays a critical role in human IBD [1]. However, the relative contribution of intestinal epithelial cells (IEC) in this IL-10-mediated regulation of intestinal disorders remains largely elusive.

Aim:
To elucidate the impact of IEC responsiveness to IL-10 released by immune cells in the context of intestinal inflammation.

Material and methods:
To explore the impact of intestinal epithelial cell responsiveness to immune cell-derived IL-10 on intestinal homeostasis and inflammation the Cre/lox system for targeted deletion of the IL-10R1 specifically in the gut epithelium was used. For this purpose, a transgenic mouse line expressing exon 1 of the IL-10R1 flanked by two loxP sites was crossed with VillinCre mice expressing the Cre recombinase gene under the control of the villin promoter specifically in IEC. To induce colitis IL-10R1xVillinCre and control mice were treated with dextran sodium sulfate (DSS) for seven days resulting in mechanical disruption in the gut epithelial cell layer. Mice were sacrificed in the acute phase of DSS colitis and after recovery (day 7 and 14) to assess the impact of IEC responsiveness to IL-10 on the course of colitis.

Results:
Cre-recombinase expression under the control of the villin promoter induces efficient deletion of the IL-10R in IEC throughout the entire length of the intestine in IL-10R1xVillinCre mice. As a result of IL-10R deficiency in IEC, IL-10R1xVillinCre showed a higher morbidity following DSS induced colitis as indicated by significant higher body weight loss during DSS treatment and, accordingly, a delayed recovery from acute colitis. In line with this, changes in cytokine levels were observed in intestinal tissue cultures with elevated levels of IFN-γ and IL-10 in supernatants derived from IL-10R1xVillinCre tissue. Currently we are investigating cytokine composition in tissue cultures (small intestine, colon) from IL-10R1xVillinCre and control mice under steady state conditions and following stimulation. Moreover, we are screening for potential differences in cytokines in intestinal lavages and will analyze for differences in intestinal length, intestinal permeability and immune cell composition in the lamina propria.

Conclusion:
Our findings indicate that functional deletion of the IL-10R1 on IEC and therefore their inability to respond to immune cell derived IL-10 affects the course of intestinal inflammation. Therefore, the IL-10RxVillinCre mice represent a suitable model to further improve our understanding regarding the relevance of IL-10 and in particular the responsiveness of IEC to this immune-modulating cytokine in the pathogenesis of IBD.

References:
Activation of resident lamina propria cells in response to epithelial layer damage

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Epithelial layer damage represents an early hallmark of acute and chronic intestinal inflammation. It is associated with the induction of an inflammatory response in resident lamina propria cells including myeloid cells (LPMO) as demonstrated in a human organ culture model. In particular, an up-regulation of pattern recognition receptors, co-stimulatory receptors as well as soluble inflammatory mediators (e.g. IL-1β, IL-8, MIP-1β) can be observed in LPMO emigrated from mucosal specimens in response to loss of epithelial cells (LEL). In order to further characterize the inflammatory response of these LPMO, global gene expression profiles were obtained from these cells. In comparison to peripheral blood monocytes, LPMO expressed genes characteristic of dendritic cells such as FLT3, LAMP3, CD80, CD86, CCR7, CD83. Furthermore, high expression of CCL19, CCL22, IL-23p19, EBI3 as well as matrix metalloproteinases such as MMP12 and MMP9 suggest an important role of these cells in the regulation of an intestinal immune response. Preliminary signaling pathway analysis suggests a potential role of the OX40/OX40L pathway in the activation of LPMO in response to LEL. (Supported by DFG/SFB 938.)
Altered responses of CD8αα TCRαβ intestinal intraepithelial lymphocytes during chronic intestinal inflammation

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Question:
Intestinal intraepithelial lymphocytes (IEL) contain a large proportion of T cells, which differ from circulating T cells. One predominant, unconventional T cell subset are CD8αα CD8β+ TCRαβ IEL. These CD8αα TCRαβ IEL, which are believed to be strictly resident in the intestinal epithelium under homeostatic conditions, contain cells with self-specific T cell receptors and were described to have a hyporesponsive phenotype. As the functions of CD8αα TCRαβ IEL are still largely enigmatic, we aimed to study their responses during chronic intestinal inflammation.

Methods:
We used transgenic mice that contain a monoclonal population of self-specific CD8αα TCRαβ IEL, but no conventional CD4 and CD8 T cells (318xH8xRAG−/− mice) and induced colitis by transfer of naïve CD4 CD45RBhi T cells.

Results:
Compared to RAG−/− mice, 318xH8xRAG−/− mice exhibited a slightly attenuated colitis but still developed intestinal inflammation. Intriguingly, we observed a substantial increase in CD8αα TCRαβ T cells in peripheral blood, lymph nodes, spleen and colon of colitic 318xH8xRAG−/− mice. The appearance of CD8αα TCRαβ T cells at extraintestinal sites in colitic mice was accompanied by vigorous proliferation and changes in homing marker expression. Moreover, CD8αα TCRαβ T cells showed reduced expression of the regulatory genes TGFβ3, Igf2 and LAG3, and gained the ability to produce IFNγ and TNFα after restimulation in vitro. These changes were most prominent in cells obtained from extraintestinal sites.

Conclusions:
These results indicate a so far unanticipated plasticity of the CD8αα TCRαβ T cell population and an unexpected increased ability of CD8αα TCRαβ T cells to respond to stimulation under inflammatory conditions.
Ileus after intestinal surgery depends on Batf3

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Purpose/Objective: Dendritic cells (DCs) are very potent in inducing adaptive immune responses against pathogens, but also cause intestinal autoimmune diseases such as colitis. It has been shown previously that the most severe complication after abdominal operation, the post operative ileus (POI), depends on macrophages (MPs) and memory T helper type 1 (mTh1) T cells, which are initially activated by IL-12 producing DCs. However, the specific POI-inducing DC-subset remains unclear.

Methods: After opening the peritoneal cavity POI was induced in CCR2⁻/⁻ Batf3⁻/⁻ and C57Bl6 mice by manipulating the small bowel with moist cotton applicators once from the oral to aboral direction. After 24h jejunal POI was assessed by oral FITC-Dextran application and measuring its bowel progression after 1.5h. Colonic POI was examined by inoculation of a bead into the colon and measurement of excretion time. Cell numbers were determined using flow cytometry. IL-12 was measured in homogenated muscularis tissue by ELISA.

Results: We found that the presence of CX3CR1-expressing DCs after abdominal operation was dependent on CCR2, but lack of such DCs did not improve POI. However, operated Batf3⁻/⁻ mice lack CD103⁺CD11b⁻ DCs within the muscularis and showed enhanced bowel motility. Nevertheless expression of IL-12 was not altered in manipulated Batf3⁻/⁻ mice.

Conclusions: These data indicate that CCR2-dependent DCs do not play a role in the induction or progression of jejunal POI. However, expression of Batf3 is essential for POI. Furthermore IL-12 expression alone is not sufficient for development of POI. Altogether these findings indicate that other Batf3-dependent cells than IL-12-producing DCs contribute to the induction of POI.
Milieu-adjusted (physiological) oxygen concentration reduces the production of IL-8 and CCL-20 upon stimulation with Flagellin in CaCo-2 cells.

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**Question:**
Intestinal epithelial cells (IECs) line the front to microbial components in the gut lumen. Their close contact to various immune cells as well as their barrier function gives them a central role in maintaining immunological homeostasis in the gut. While ambient air comprises about 20% oxygen, the partial pressure of oxygen in tissues is far below this. As many cellular and immune functions are influenced by oxygen level, it may also be of importance in IECs.

**Methods:**
The colon epithelial cell line CaCo-2 was investigated under a physiological oxygen concentration of 1%. Proliferation was measured by the incorporation of ³H-Thymidin and viability by MTT-assay. Cytokine and chemokine production was measured by ELISA, gene expression by RT-PCR and proteins were detected by Western Blot.

**Results:**
Proliferation and viability of the cells were not significantly altered compared to conventionally cultured cells. While transepithelial electrical resistance (TEER) was initially elevated under low oxygen, it reached comparable levels over time. In contrast, Alcaline Phosphatase-1 as a marker of differentiation was invariably higher under physiological oxygen concentration. When stimulated with the TLR 5-ligand Flagellin, IL-8 and CCL-20 production was diminished at 1% O₂. Interestingly, mRNA levels of IL-8 and CCL-20 were not significantly reduced under physiological oxygen concentration. No changes in TLR 5 expression could be observed. Analysis of MAPK by Western Blot showed a generally higher phosphorylation of p38, JNK and ERK1/2 under low oxygen, which was further enhanced by stimulation with Flagellin.

**Conclusions:**
Understanding the influence of the local oxygen pressure on the physiology of the cells could contribute to a better understanding of the complex immunological network in the intestine.
Regulation of Toll-like receptor signaling by the unfolded protein response

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Question:
Airway epithelial cells are able to recognize pathogen-associated molecular patterns (PAMPs) by Toll-like receptors (TLRs) and respond to potential danger. Subsequent signal transduction pathways result in the increased transcription of host defense genes, such as IL-6 or IL-8. However, respiratory epithelial cells only mount a moderate immune response. We were asking whether this phenotype could change towards a more reactive one during endoplasmic reticulum (ER) stress conditions. ER stress can occur due to accumulations of misfolded proteins in the ER but has also been reported to occur during inflammation, thereby activating unfolded protein response (UPR). UPR is required to restore the ER-homeostasis. It is conducted via three ER transmembrane receptors - PERK, IRE1α and ATF6. Interestingly, UPR has also been shown to act proinflammatory.

Methods:
Human bronchial epithelial (Beas2B) cells are pretreated with DMSO or Thapsigargin (Thaps) for 1 h to induce UPR and subsequently stimulated with a TLR4 agonist lipopolysaccharide (LPS) for 5 h. Secreted IL-6 and IL-8 protein levels in the supernatants are determined by ELISA. Relative mRNA expression of the respective cytokines is measured by qRT-PCR. By silencing the UPR branches using small interfering RNAs (siRNAs), their importance in IL-6 and IL-8 regulation is assessed. MAPKs activation during combined UPR and TLR signaling is investigated by detecting their respective phosphorylated form by Western blot. Detection of UPR marker protein is detected in murine cystic fibrosis (CF) model lung sections by immunohistochemistry.

Results:
We confirm UPR indeed acts proinflammatory. Notably, Induction of UPR by Thapsigargin results in an increased response of airway epithelial cells to LPS with respect to IL-6 and IL-8 induction, indicating a hyperinflammatory status. Furthermore, all three UPR branches seem to be interconnected and involved in modulating IL-6 and IL-8 expression via a dynamic cross-talk. However, IL-6 and IL-8 mRNA and protein levels are influenced mainly by PERK and ATF6. UPR induction results in an increased p38 and ERK activity by LPS, whereas NFκB activation is not affected. Furthermore, both IL-6 and IL-8 mRNA are stabilized during simultaneous UPR and TLR stimulation. A human pathophysiological condition where UPR is observed is cystic fibrotic lungs. By immunologically detecting Chop, an UPR marker, UPR was confirmed to take place in the murine CF model.

Conclusions:
Combined UPR and TLR signaling leads to hyperreactivity of bronchial epithelial cells. Regulation of IL-6 and IL-8 is mostly orchestrated by PERK and ATF6 through ERK and p38 MAPK mediated mRNA stabilization. UPR-induced hyperreactivity might be important in cystic fibrosis.
The role of *Moraxella catarrhalis* in asthma development

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**Introduction:**
Asthma, a disease of unknown etiology is characterized by hyper-responsiveness of the airways with eosinophilic and neutrophilic inflammation. Often, exacerbation of asthma and COPD is associated with bacterial infections, such as the gram-negative bacterium *Moraxella catarrhalis*. The aim of this study is to investigate the role of *M. Catarrhalis* during acute and chronic asthma development in the murine OVA asthma model.

**Methods:**
The effects of infection with *M. catarrhalis* were investigated in acute and chronic mouse models of experimental, OVA-induced asthma. Shortly, *M. catarrhalis* infected or naive Balb/c mice were sensitized and challenged with OVA/Alum, both in acute or chronic experimental asthma. Lung function (airway resistance), cytokines of the BAL, serum immunoglobulins, T lymphocytes and the histology of the lungs were analyzed.

**Results:**
In the acute model, infection with *M. Catarrhalis* resulted in increased BAL neutrophils but reduced influx of eosinophils and significantly decreased IL-5 and IL-13 in BAL and reduced OVA-IgE levels in comparison to non-infected animals. In chronic experimental asthma, infection with *M. catarrhalis* lead to reduced metacholine induced airway resistance in comparison to the non-infected asthma group. However, the number of goblet cells and the T-cell profile in the lungs of Moraxella infected mice was not altered as compared to non-infected asthma controls.

**Conclusion:**
In this study we show that i) intranasal infection with *M. catarrhalis* decreases acute asthma by reducing eosinophils, Th2 cytokines IL-5 and IL-13, IgE and IgG1 antibodies. ii) In the chronic form, we show that *M. catarrhalis* lowers the airway resistance in the lung.
Prevention of an asthmatic phenotype by gene-based vaccines encoding for DEC205-targeted antigen


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Question:
Asthma is a respiratory illness with an ever increasing prevalence, affecting about 300 million people worldwide. Glucocorticoid therapy is only symptomatic and cost intensive, while antigen specific immunotherapy (SIT) is time consuming and poses a high risk of side effects in asthmatics. The mechanisms of SIT are not known yet, but probably include the induction of tolerance towards the antigen. Targeting of an antigen to dendritic cells by coupling to an antibody against the scavenger receptor DEC205, leads to increased uptake, processing and presentation in complex with MHC class II molecules. By protein-based immunization in the absence of maturation- and danger signals, this was shown to induce tolerance towards the antigen. The aim of this study was to investigate the tolerogenicity of DEC-targeting in a gene based approach.

Methods:
Here we employed a murine ovalbumin (OVA)-specific asthma model to compare the efficacy of different genetic immunization regimens: DNA subcutaneously, DNA intramuscularly followed by in vivo electroporation, and replication-deficient adenoviral vectors given either intramuscularly or intranasally. All vaccines encode for DEC-targeted OVA, and mice were immunized either before the sensitization (prophylactic) or before the challenge phase (semi-therapeutic) of the asthma model.

To measure the efficacy of the immunizations, we assessed the airway reactivity to methacholine by unrestricted whole body plethysmography, as well as different immunological parameters. Among these were the cytokine production of OVA-stimulated splenocytes, cell composition in bronchoalveolar lavage fluid (BALF), and OVA-specific antibody titers and isotypes in serum and lung.

Results:
In the prophylactic setting, all immunizations led to a reduction of the airway hyperresponsiveness (AHR). Along with improved airway conditions, mice receiving the prophylactic immunization had less eosinophilia in the lung and produced lower amounts of Th2 cytokines and OVA-specific IgE. In contrast, the semi-therapeutic immunization had nearly no influence on the production of Th2 cytokines and IgE, and only the immunizations based on adenoviral vectors, had an ameliorating effect on the eosinophilia. In accordance, these mice showed an AHR comparable to the control mice.

Conclusions:
A single dose of an unadjuvanted gene-based vaccine, which encodes for DEC205-targeted OVA, can prevent the onset of asthmatic symptoms if given prior to the sensitization. While all prophylactic regimens inhibited the development of AHR and eosinophilic inflammation, the exact mechanisms have to be further examined in future studies. The immunologic parameters indicate different mechanisms for the regimens tested, including the induction of tolerance as well as a possible Th1-shift. The diminished efficacy of the semi-therapeutic immunization needs to be addressed e.g. by addition of immune modulating adjuvants and/or repeated immunizations.
Fms-like tyrosine kinase 3 ligand promotes lymphoid tissue inducer cell and secondary lymphoid organ development

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**Question:**
Fms-like tyrosine kinase 3 ligand (Flt3L) is a cytokine promoting the growth of various hematopoietic lineages. The cytokine receptor Flk2 (fetal liver kinase 2) is expressed on hematopoietic progenitor cells. Although the effect of Flt3L for B cells, T cells and myeloid cells has been well characterized, little is known about its role in the development of lymphoid tissue inducer cells (LTi cells) and the newly characterized NKp46+ LTi cells.

**Methods:**
Since Flt3L acts synergistically with other cytokines such as Interleukin 7 (IL-7) we crossed Flk2−/− and Flt3L−/− mice to IL-7−/− mice. Single and double knock out mice were injected with Chicago Blue ink and the number of lymph nodes and Peyer’s patches was counted. To analyse the impact on lamina propria LTi cells and NKp46+ LTi cells, knock out mice were compared with wild type mice and mice overexpressing Flt3L under the control of the human beta-actin promoter.

**Results:**
Lymph node development was normal in both Flk2−/− and Flt3L−/− mice. In contrast, the number of Peyer’s patches was reduced to 60% in Flk2−/− and to 30% in Flt3L−/− mice compared to WT mice. IL-7−/− mice already show a reduction in the number of lymph nodes. IL-7−/− Flk2−/− mice had even less lymph nodes than IL-7−/− mice while in IL-7−/− Flt3L−/− mice lymph nodes were completely absent. IL-7−/− mice show a reduced number of LTi cells and NKp46+ LTi cells in the lamina propria of the small intestine. The additional deletion of Flk2 and Flt3L in these mice led to even lower numbers of these cells in double knock out mice. Contrary, in mice overexpressing Flt3L a 10-fold increase in the number of LTi cells and NKp46+ LTi cells was observed.

**Conclusion:**
Altogether, our data show that Flt3L promotes the generation of LTi and NKp46+ LTi cells and collaborates with IL-7 in the development of secondary lymphoid organs.
Immunomodulation of human intestinal T-cells by the synthetic CD80 antagonist RhuDex

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Deregulated activation and expansion of mucosal lamina propria T-cells play a critical role in the pathogenesis of chronic intestinal inflammation. One of the pathways to modulate T-cell activation and thus, potentially reduce such inflammation is via blocking the CD28/CD80 co-stimulatory pathway. Therefore, the fusion protein CTLA-4-Ig (Abatacept) has been evaluated in clinical trials as a therapy for ulcerative colitis and M. Crohn, yet was ineffective in treating patients with moderate to severe disease.

Here, we investigated the effect of RhuDex®, a low-molecular-weight molecule that binds CD80, as an alternative to Abatacept to modulate CD28 signaling in lamina propria T-cells, generated in a human organ culture model of intestinal inflammation.

To this end, lamina propria lymphocytes (LPL) and peripheral blood lymphocytes (PBL), respectively, were stimulated via the T cell receptor (TCR/CD3) using a monoclonal antibody. RhuDex® caused a profound reduction of LPL and PBL proliferation after stimulation in a dose-dependent manner. Abatacept, however, inhibited LPL proliferation to little degree, but had no effect on PBL proliferation. Further, release of IL-2, IL-17, TNF-alpha, and IFN-gamma after CD3/TCR stimulation was analyzed. Cytokine release from LPL was more inhibited by Abatacept, while PBL were more sensitive to RhuDex® inhibition. Specifically, Abatacept significantly inhibited IL-2, TNF-alpha and IFN-gamma release from LPL in response to TCR/CD3 stimulation, whereas in PBL, Abatacept had no effect on cytokine release. In contrast, in the presence of RhuDex®, secretion of IL-17 and IFN-gamma was inhibited in both LPL and PBL after TCR/CD3 stimulation.

We conclude that in this initial study RhuDex® inhibits both, PBL and LPL activation in vitro. The observed differences between Abatacept and RhuDex® in their respective inhibitory activity, especially the stronger inhibitory effect of RhuDex® on LPL proliferation, makes it a promising drug development candidate also for chronic intestinal inflammation.
SMAD7 overexpression in T cells leads to spontaneous intestinal inflammation in CYLD mutant mice

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CYLD plays an important role in different signaling pathways such as NF-κB, JNK and TGF-β signaling. Hövelmeyer et al. generated CYLDex7/8 mice, which express excessively a naturally occurring splice form of CYLD (sCYLD), while the full length protein (FL-CYLD) is missing. Data illustrated the important role of sCYLD in B cells, T cells and DCs using the CYLDex7/8 mouse model. To further examine the role of sCYLD in TGF-β signaling, we crossed CYLDex7/8 mice with CD2 SMAD7 mice, which overexpress SMAD7 specifically in T, NK and NKT cells.

By analysing T cells of CYLDex7/8 CD2 SMAD7 mice, we found a strong reduction of the CD4 and CD8 T cell compartment in thymus, spleen and lymph nodes, similarly to the data shown for the CYLDex7/8 mice. Interestingly, a majority of the CD4+ T cells in CYLDex7/8 CD2 SMAD7 mice are already activated in the periphery, even without any further stimulation. By analysing regulatory T cells (Tregs) in these mice, we detected an increased population of Foxp3+ CD4+ Tregs in the thymus and periphery. Of note, most of the Tregs were negative for the expression of the IL-2Rα-chain, which is known to be expressed by Tregs. Further, by examined the cell number of thymus derived natural Tregs (nTregs) versus peripheral induced Tregs (iTregs), we observed a drastic decrease of iTregs in the mLN of CYLDex7/8 CD2 SMAD7, indicating an essential role for SMAD7 in iTreg differentiation.

At the age of 8 weeks, the CYLDex7/8 CD2 SMAD7 mice started to develop a severe intestinal inflammation accompanied by a reduction of the body weight and a massive infiltration of inflammatory cells into the lamina propria of the colon. At the age of 12 weeks, these mice reached a very high clinical score of colitis with even a more dramatic infiltration of T cells, macrophages, DCs and neutrophils. In addition, a marked up-regulation of inflammatory cytokines such as IL-12, TNF-α, IL-6 and IFN-γ could be detected in the colonic tissue of those mice. Meanwhile, we did not observe any development of intestinal inflammation in WT, CYLDex7/8 and CD2 SMAD7 mice. All together, these data indicated that overexpression of SMAD7 in T cells accompanied by overexpression of sCYLD plays a crucial role in the pathogenesis of colonic inflammation.
Expression of the NKC-encoded C-type lectin-related glycoprotein Clr-a

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Introduction:
The ‘C-type lectin-related’ (Clr) molecules (Clr-a to Clr-h) are C-type lectin-like receptors encoded by Clec2 genes in the mouse Natural Killer Gene Complex (NKC). Except for Clec2d encoding for Clr-b, little is known about expression and function of members of the Clec2 gene family. Interestingly, Clr-b is a ligand of the inhibitory NK cell receptor Nkrp1d with genes of Nkrp1d and other Nkrp1 receptors interspersed with Clec2 genes in the centromeric region of the mouse NKC.

Objectives:
To unravel the immunological significance of Clr molecules we are aiming at the characterization of expression and function of Clr molecules, including the orphan receptor Clr-a encoded by the Clec2e locus.

Materials and methods:
Clr-a cDNA and mutants thereof were transfected in 293T and CHO transfectants. Clr-a expression was characterized by immunoblotting, immunoprecipitation and flow cytometry. Tissue-specific abundance of Clr-a transcripts was studied by quantitative RT-PCR (qPCR) analysis of various tissues of C57BL/6 and BALB/c mice. By immunizing rats with soluble Clr-a ectodomains, Clr-a specific mAb were raised.

Results:
Tissue-specific expression of Clr-a was delineated from qPCR data and confirmed by immunohistochemistry. Low cell surface expression of Clr-a on transfectants (as compared to other Clr transfectants) was attributed to certain Clr-a domains by domain swapping experiments.

Conclusion:
Cell surface expression of Clr-a is impaired due to sequence-intrinsic characteristics of Clr-a. Clr-a expression confined to specific epithelia suggests involvement of Clr-a in modes of tissue-specific immunosurveillance. Attempts to identify the (yet unknown) receptor for Clr-a are ongoing and will aid in testing this hypothesis.
Cylindromatosis (Cyld) gene mutation in T cells exacerbates the development of an allergic phenotype in experimental asthma

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The cylindromatosis gene (Cyld) is a deubiquitinating enzyme which acts as a regulatory element in NF-kB activation. The solely and excessively expression of the naturally occurring short splice variant of (CYLDex7/8), which can be achieved by deletion of the exons 7 and 8, results in positive regulation of NF-kB signal transduction and homeostasis in lymphocytes.

In the present study we investigated the function of sCYLD in a murine model of allergic airway disease towards the model antigen Ovalbumin (OVA), using mice with a T cell-specific expression of CYLDex7/8 (CYLD-fl x CD4-Cre). In this model of allergic asthma, T cell-specific expression of CYLDex7/8 leads to a marked enhancement of lung eosinophilia, accompanied by increased goblet cell metaplasia and elevated levels of antigen-specific IgE and IgG1. In addition, these mice develop an increased airway hyperreactivity. These particularly pronounced allergic symptoms could be attributed to increased numbers of IL-9, IL-4 and IL-5 producing CD4+ T cells found in lung tissue of genetically modified mice. Thus, T cell-specific overexpression of the short isoform of CYLD predisposes to the development of an allergy-prone phenotype.
The role of IL-1β in the intestinal homeostasis and the effect of IL-1β on the composition of the intestinal microbiome

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The surface of vertebrates is colonized by manifold microorganisms. Especially the gut is populated by a variety of different bacteria, fungi and other microorganisms. Over millions of years many different microbes and higher organisms have coevolved to a close symbiosis. The advantage of this symbiosis is bought at the price of a perpetual threat for the host by bacterial overgrowth or the penetration of microorganisms into the mucosa. For maintaining the homeostasis of host-microbial interaction vertebrates have developed several mechanisms of defense. IL-1β, a key component of the inflammatory response, possesses diverse biologic effects contributing to acute and chronic inflammation. To investigate the impact of IL-1β on the intestinal microbiome, the composition of the microorganisms living in the intestine of IL-1β-deficient mice were compared to that of wild type animals. For the microbiome profiling, combinatorial sequence tags attached to two different PCR primer pairs amplifying the V4 and the V6 region of the bacterial 16rRNA gene were used. PCR products were sequenced using an illumina paired-end protocol to generate millions of reads. A total of 12 samples (6 wild type and 6 IL-1β-deficient mice) were sequenced. It could be shown that the two most prominent bacteriological orders of bacteria found in the faeces, the order of Bacteroidales and Clostridiales differ not significantly between wild type and knockout mice. However, major differences between wild type and knockout animals were observed within the orders of Deltaproteobacteria, Cyanobacteria and Deferribacterales. To get information about the implication of the IL-1β knockout on the intestinal immune system, expression of cytokines and chemokines was compared between wild type and IL-1β knockout mice. IL-1 knockout mice showed a significant decrease in cytokines involved in Th17 immune response. In conclusion, the present data indicates that IL-1β is involved in controlling intestinal immune responses and that IL-1β dysregulation can selectively respond to specific components of the complex luminal microflora.
Evidence for a role of the histamine H₃- and H₄-receptors in DSS-induce colitis

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Introduction:
Inflammatory bowel diseases (IBD) are a growing health problem which still lack causal therapies. Main manifestations of IBD are ulcerative colitis (UC) and Crohn’s disease (DC). A mouse model of IBD is the dextran sulfate sodium (DSS)-induced colitis. Acute DSS-induced colitis reflects mostly the involvement of innate immunity in the mucosal inflammation. Histamine, mainly produced by mast cells, is an inflammatory mediator which is found in high concentrations in intestinal samples of colitis patients as well as of animals of colitis models. Histamine affects the activity of target cells via four different receptor subtypes, histamine H₁-receptor (H₁R), H₂R, H₃R, and H₄R. Antagonists at the H₁R and the H₂R, which are used successfully in the clinic to treat allergic and acid-induced gastrointestinal disorders, respectively, are of only very limited efficacy in the treatment of colitis.

Objective:
In the present study we aimed at elucidating a possible role of H₃R and H₄R in DSS-induced colitis in mice.

Materials & Methods:
Colitis was induced in 10 week old BALB/c mice by feeding water supplemented with 2.5 % [w/v] DSS over a period of 7 days. Control mice received water without supplementation. Body weight of the mice was recorded every day and at day 8, mice were sacrificed and ceaca and colons were prepared for histological examination. Pharmacological intervention was performed using the H₃R-selective antagonist JNJ 5207852 and the H₄R-selective antagonist JNJ 7777120. Due to pharmacokinetic reasons the antagonists were applied by osmotic pumps, which were implanted 12 hours prior to starting DSS-feeding. The pumps delivered the antagonists continuously over a period of 7 days with a rate of 12 µl/day, corresponding to 300 nmol of antagonist per day.

Results:
DSS-feeding induced a dramatic weight loss mainly in the last 3 days of the observation period. This weight loss was reduced by application of either the H₃R antagonist JNJ 5207852 or the H₄R antagonist JNJ 7777120. Co-administration of both antagonists, however, demonstrated neither additive nor synergistic effects. The DDS-induced reduction in ceacum length was not affected by the antagonists, while histological signs of inflammation appearing after DSS feeding were reduced in JNJ 5207852- and in JNJ 7777120-treated mice.

Conclusion:
We conclude that histamine regulates inflammation not only directly via the H₃R expressed on innate immune cells such as eosinophils or mast cells, but also indirectly via the H₄R expressed in the nervous system.
Regulatory T cells promote a protective Th17 cell response to intestinal infection with C. rodentium

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Intestinal infection with the mouse pathogen C. rodentium induces a strong local Th17 response. While this inflammatory immune response helps to clear the pathogen, it also induces inflammation-associated pathology in the gut and thus has to be tightly controlled. In this project, we therefore studied the impact of Foxp3⁺ regulatory T cells (Treg) on the infectious and inflammatory processes elicited by C. rodentium. Surprisingly, we found that depletion of Treg by diphtheria toxin in the Depletion of Regulatory (DEREG) mouse model resulted in impaired bacterial clearance in the colon, enhanced body weight loss and systemic dissemination of bacteria. Consistent with the increased susceptibility, we found that the local colonic Th17 cell response was impaired in Treg-depleted mice, suggesting that the presence of Treg is crucial for the establishment of a functional Th17 response in the gut. As a consequence of the impaired Th17 response, we also observed less inflammation-associated pathology in the colons of Treg depleted mice. Interestingly, anti-Interleukin (IL)-2 treatment of infected Treg-depleted mice fully restored the colonic Th17 response, indicating that Tregs support the induction of a protective Th17 response during intestinal infection by consumption of local IL-2.
NAD(P)H fluorescence lifetime imaging reveals the contribution of NADPH oxidase activation in Multiple Sclerosis pathogenesis


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Question:
Multiple Sclerosis (MS) is a chronic inflammatory disease of the central nervous system (CNS). Despite of the relapsing-remitting course seen in most patients, these patients develop lasting disabilities over time. Even though neurons may look healthy in histopathologic samples, studies show an early impairment of neuronal function in the course of disease. Direct neuronal damage seems to be mediated by reactive oxygen species (ROS). Nikic et al. showed that focal axonal damage could be induced by applying ROS-producing agents and was reversible by neuralising ROS (1). Direct evidence of the cellular source of ROS, however, is still missing and it is unclear what is the cellular source of ROS? Is there an effect on neuronal dysfunction before we see morphological changes? Are MS-patients more susceptible to NOX-activation and can this susceptibility be influenced therapeutically?

Methods:
We employed parallelized time-correlated single-photon counting (TCSPC) to perform intravital marker-free FLIM. We analyzed the activation of NAD(P)H oxidase by intravital NAD(P)H FLIM (2) and the FRET-FLIM signal of a neuronal calciumsensor in the brain stem of EAE affected mice (3). To determine whether NOX-activation is affected in peripheral blood cells during MS, we isolated CD11b+monocytes from EAE splenocytes and MS patient blood and analyzed the activation of NAD(P)H oxidase by intravital NAD(P)H FLIM.

Results:
Thereby found that neurons, astrocytes and microglia, as well as peripheral macrophages can activate NAD(P)H oxidase (NOX). We could show an increased proportion of tissue with NOX activity at onset of clinical symptoms to the peak of disease and compared to healthy controls. We correlated the NOX activated cells with neuronal dysfunction indicated by increased neuronal calcium signal detected by FRET-FLIM in the brain stem of EAE affected animals. In healthy human and mice CD11b+monocytes were not activated after isolation. Untreated EAE affected mice in the chronic phase had elevated normalized NOX activation area. MS patients and EAE mice treated with glatiramer acetate (GA) had less but still increased normalized NOX activation area compared to healthy controls. This could be counteracted by additional intake of green tea extract (epigallocatechin-3-gallate = EGCG). Similar to the enhanced activation of NOX in the CNS parenchyma in mice during the onset of EAE, humans with clinical isolated syndrome showed almost normal NOX activation in peripheral monocytes.

Conclusion:
We demonstrated that by using intravital FLIM we can investigate the role of NAD(P)H activation in EAE. Our results showed that the patholgical activation could be therapeutically targeted by oral intake of EGCG. This mechanism could participate in the observed benefit of EGCG treatment of EAE affected mice(4) and further studies are needed to evaluate the clinical benefit of EGCG treatment in Multiple Sclerosis patients.

(1) Nikic et al., A reversible form of axon damage in experimental autoimmune encephalomyelitis and multiple sclerosis Nat. Med., 2011
(2) Niesner et al., Selective detection of NADPH oxidase in polymorphonuclear cells by means of NAD(P)H-based fluorescence lifetime imaging, J. Biophys., 2008
ADAP-deficient mice are resistant to Experimental Autoimmune Encephalomyelitis (EAE)

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The adhesion and degranulation-promoting adapter protein (ADAP) is expressed in T cells, myeloid cells, and platelets. ADAP is known to regulate receptor-mediated inside-out signaling leading to integrin activation and adhesion of T cells and platelets. EAE is a proinflammatory autoimmune disorder that targets the central nervous system (CNS) and serves as an animal model for the human disease Multiple Sclerosis.

In this study, we investigated the EAE in ADAP-deficient mice. ADAP-deficient mice developed a significantly milder EAE compared with their wild-type counterparts after active immunization with MOG₃₅-₅₅ peptide. This observation was accompanied by markedly reduced leukocyte infiltration in the CNS of ADAP-deficient mice. Moreover, ADAP-deficient recipients failed to induce passive EAE after adoptive transfer of MOG-specific TCR transgenic T cells (2D2 T cells). Monitoring the adoptively transferred 2D2 T cells over time demonstrated that they accumulated in the lymph nodes of ADAP-deficient mice. Application of an anti-CD62L antibody to prevent lymph node entrance of T cells resulted in enhanced severity of EAE in ADAP-deficient mice. Therefore, we concluded that ADAP-deficient mice are resistant to EAE due to accumulation of encephalitogenic T cells in the lymph nodes. Further studies are necessary to elucidate the exact mechanism of the observed lymph node trapping of T cells in ADAP-deficient mice during EAE.
Neuro-immune interaction in stress: where, when and how much matters

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Stress activates adaptive neuro-immune mechanisms that allow the organism to survive under ever changing life-conditions. Mal-activation however leads to dysbalanced neuro-immune activation and contributes to disease. Since the description of the hypothalamus pituitary adrenal axis (HPA) in the 50ies of the past century we have come to understand that this response is not restricted to the central nervous system or primarily immune competent organs such as the spleen. Peripheral organs at the self-environment border such as the skin are hardwired with peptidergic nerves which undergo life-long plasticity guided e.g. by neurotrophins. However, if pro-inflammatory or anti-inflammatory neuro-immune interaction is initiated greatly depends on activation time point, place of encounter and neuro-immune competence of the affected tissue. Here we summarize work done in a mouse model of experimentally induced atopic-dermatitis like allergic inflammation combined with noise stress exposure. In this model we found time-point, exposure-frequency and site dependent changes firstly on local level: neurogenic inflammation and initiation of adaptive and regulatory immune responses; and secondly on systemic level: hypothalamus and spleen. Thereby, singular acute stressors (restraint, noise, allergen provocation) led to depletion of peptidergic nerve fibers, mast cell degranulation and inflammation. By contrast, repeated intermittent stress exposure led to activation of regulatory mechanisms. Moreover, TH1/TH2 balance depended on type and combination of stress exposures. Neuro-immune modulation differed between skin, peripheral blood and spleen and presence of allergic inflammation in skin altered HPA activation. Employing neurokinin 1 receptor blockade and neurotrophin neutralizing antibodies peripherally reversed the majority of the observed changes locally as well as systemically suggesting the presence of an additionally stress axis acting through neuropeptides and neurotrophins, which interacts with the HPA and potently modulates immune function in peripheral organs.
BACL is a novel brain-associated, non-NKC-encoded mammalian C-type lectin-like receptor of the CLEC2 family

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Introduction:
Natural Killer Gene Complex (NKC)-encoded C-type lectin-like receptors (CTLRs) are expressed on various immune cells including T cells, NK cells and myeloid cells and thereby contribute to the orchestration of cellular immune responses. Some NKC-encoded CTLRs are grouped into the C-type lectin family 2 (CLEC2 family) and interact with genetically linked CTLRs of the NKR/P1 family. While many CLEC2 family members are expressed by hematopoietic cells (e.g. CD69 (CLEC2C)), others such as the keratinocyte-associated KACL (CLEC2A) are specifically expressed by other tissues. Here we provide the first characterization of CLEC2L, another orphan gene of the CLEC2 family. Notably, the CLEC2L locus is adjacent to another orphan CTLR gene (KLRG2), raising the question for the functional significance of this genetic linkage.

Objective:
Characterization of the orphan gene CLEC2L by studying its expression and function.

Materials & Methods:
CLEC2L tissue expression was addressed by quantitative real-time PCR and in situ hybridization. CLEC2L mutants were generated by site-directed mutagenesis. Wild-type and mutated CLEC2L proteins were ectopically expressed in 293T cells for characterization by flow cytometry and immunoblotting. Soluble CLEC2L ectodomains were expressed in 293 cells and used for the generation of CLEC2L-specific antisera in chicken. Endogenous protein expression was addressed by immunohistochemistry and immunoprecipitation using CLEC2L antisera. BWZ reporter assays were exploited to investigate the interaction of CLEC2L- and KLRG2-encoded proteins. A syngeneic mouse tumor model served for analysis of an immune-related function of CLEC2L.

Results:
In contrast to other CLEC2 family members, CLEC2L is conserved among mammals and located outside of the NKC. We show that CLEC2L is readily expressed on the cell surface as a non-glycosylated homodimer linked by two disulfide bonds in the stalk region. CLEC2L expression is fairly tissue-restricted with a predominant expression in the brain. Thus CLEC2L-encoded CTLRs were designated BACL (brain-associated C-type lectin). Combining in situ hybridization and immunohistochemistry, we show that BACL is expressed by neurons in the CNS, with a pronounced expression by Purkinje cells. Notably, the CLEC2L locus is adjacent to another orphan CTLR gene (KLRG2), but reporter cell assays did neither indicate interaction of BACL with the KLRG2 ectodomain nor with human NK cell lines or lymphocytes. Along these lines, growth of BACL-expressing tumor cell lines in immunocompetent mice did not provide evidence for an immune-related function of BACL.

Conclusion:
Taken together, here we provide the first characterization of the orphan gene CLEC2L that encodes a homodimeric CTLR which stands out among CLEC2 family members by its conservation among mammals, its biochemical properties and the predominant expression in the brain, and hence was termed BACL. Future studies will have to reveal insights into the functional relevance of BACL in the context of its neuronal expression.
Glutamate receptors in EAE and microglia activation

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Objective:
Treatment of animals with antagonists of the NMDA as well as the AMPA/Kainate family of glutamate receptors ameliorates the clinical signs of EAE, the animal model for MS and results in reduced inflammatory infiltrates and blood brain barrier disruption. Microglia are highly activated during EAE and upregulate MHCII and secrete inflammatory mediators. In EAE they are thought to act as local APCs in the reactivation of the CNS-infiltrating T cells.

Methods:
We analyzed microglia activation and the role of NMDA receptors in this process using the microglia cell line BV2 and cortical slice cultures. The in vivo analysis was performed in two models: first, in oDTR mice that express the diphtheria toxin receptor specifically in oligodendrocytes (ODCs) which allows induced killing of ODCs (Locatelli et al., 2012) and second in mice that were immunized with MOG/CFA to induce EAE.

Results:
The proinflammatory cytokine IL-17A plays a pivotal role in the pathogenesis of MS and EAE. We showed IL-17A activates microglia in vitro. Therefore, we activated the microglia cell line BV2 and cortical slice cultures with IL-17A to and subsequently treated the cultures with the NMDA receptor antagonists MK801 and AP5 to block NMDA receptor signaling. This treatment inhibited IL-17A-mediated activation of microglia and in particular ROS production, proliferation and migration. Additionally, we detected increased secretion of IL-6 and G-CSF. Furthermore, IL-17A enhanced activation of the NMDA receptor through phosphorylation leading to higher influx of calcium upon ligand binding. To further analyze NMDA receptor-dependent microglia activation we used two different mouse models that provoke different mechanisms of microglia activation. Whereas in EAE a strong inflammatory process activates microglia, in oDTR mice, activation occurs due to induced ODC death. Interestingly, in the context of EAE microglia activation was clearly diminished when mice were treated with MK801 whereas inhibition of the NMDA receptor had no effect on microglia activation in the oDTR model. In line with the in vivo data we show that inhibition of microglia activation takes place only when the cells were stimulated with IL-17A and not upon stimulation with LPS.

Conclusions:
Our findings show that NMDA receptors are not involved in general microglia activation but rather play a role in microglia activation in an inflammatory context with specific cytokines such as IL-17A.
Signalling mechanisms shared between B cells and osteoclasts

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Toxigenic Pasteurella multocida strains produce a protein toxin, the Pasteurella multocida Toxin (PMT), which deamidates distinct G protein α subunits and initiates constitutive downstream signalling. In porcine atrophic rhinitis PMT destabilises the equilibrium between bone build-up and bone degradation by stimulating the differentiation of bone marrow cells into osteoclasts and inhibiting osteoblast function, respectively.

Co-culture experiments revealed that B cells enhance the PMT-induced differentiation of macrophages into osteoclasts. This is achieved by enhanced B cell-mediated secretion of cytokines such as IL-1, IL-6, TNF-α and sRANKL that are known to support osteoclastogenesis. Recent findings in the literature point to the possibility that osteoclasts may also differentiate from a B-lymphoid progenitor population when the commitment-determining transcription factor Pax5 is knocked-down. This notion challenges the classical model of osteoclastogenesis describing cells of the myeloid lineage as being progenitors for osteoclasts. We found Pax5 down-regulated in B cells and investigated therefore, whether PMT has the potential to interfere with fate-determining transcription factors in B cells and transdifferentiates them into cells of the myeloid lineage. Supporting this hypothesis, we find that isolated B cells stimulated with the toxin can differentiate into multinucleated cells that are positive for Syndecan-1, a classical plasma cell marker. Additionally, the tartrate-resistant acid phosphatase (TRAP), an enzyme regarded as an important marker for osteoclasts, can be found in vesicles in the cytoplasm of these cells pointing out the possibility that they might be osteoclasts with lymphoid origin. On a molecular level, the processes involved in terminal plasma cell differentiation and osteoclast differentiation show many similarities. We hypothesize that PMT utilizes the same signalling pathways in macrophages and B cells to drive differentiation into osteoclasts. We found that PMT acts on two transcriptional repressors Bcl6 and Blimp1, which have very recently been shown to be involved in the regulation of bone homeostasis by controlling the transcriptional programme of osteoclastic genes. Bcl6 is a transcriptional repressor of this program, while Blimp1 in turn targets Bcl6 to start the osteoclastic transcription program by silencing the suppressive pathway. Blimp1 and Bcl6 are also well known transcriptional regulators of the B cell lineage involved in the terminal differentiation of B cells to plasma cells, therefore supporting the potential link between B cells and osteoclasts. We find that PMT up-regulates Blimp1 in bone marrow derived macrophages on the protein-level as well as on the mRNA level. Consistent with this we observed a down-regulation of the target suppressor Bcl6. In plasma cells the activation of STAT-3 is sufficient to trigger Blimp1 expression and plasma cell differentiation. We could show that PMT phosphorylates STAT3 in macrophages and suggest this as a shared mechanism for Blimp1 induction between B-cells and macrophages.
The IL-22/STAT3 axis as protective principle in necroinflammation

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Introduction:
Interleukin (IL)-22 is a member of the IL-10 cytokine family that gained attention in connection with its tissue protective properties observed in disease models driven by death of epithelial-like cells and necroinflammation. Specifically, STAT3-mediated proliferation, anti-apoptosis, and anti-microbial action are regarded as principal functions of IL-22 at host/environment interfaces and crucial for tissue protection associated with this cytokine.

Objectives:
Here, we set out to characterize the tissue protective function of IL-22 in experimental acetaminophen (paracetamol, APAP)-induced liver injury, a prototypic acute syndrome with a pathology initiated by hepatic toxicity and significantly enhanced by release of damage-associated molecular patterns (DAMPs) and subsequent activation of innate immunity.

Materials and Methods:
Drug-induced liver damage was initiated in C57Bl6/N mice by administration of APAP at 500 mg/kg or 750 mg/kg (i.p.). Mice were prophylactically or therapeutically treated with recombinant murine IL-22 (3.5µg/mouse, i.v.). Liver pathology was evaluated by histology, immunohistochemistry, Western blot analysis, and determination of alanine aminotransferase (ALT) activity. Cytokines and expression of STAT3-dependent genes were assessed by ELISA or PCR. CCL20 and IL-36 receptor (IL-1Rrp2) expression in human Huh7 hepatoma cells were evaluated by PCR. IL-1Rrp2 in human liver tissue was analyzed immunohistochemically.

Results:
Administration of prophylactic IL-22 in mice resulted in hepatic STAT3 activation that associated with reduced serum ALT and liver damage under the influence of APAP. Concomitant gene expression analysis demonstrated hepatic induction of genes prototypically induced by the IL-22/STAT3 axis such as SOCS-3, lipocalin-2, and α1-antichymotrypsin. In a translational protocol of therapeutic treatment 2h post APAP, IL-22 enhanced protection in the context of suboptimal dosing of N-acetylcysteine. Notably, application of N-acetylcysteine is standard therapy of clinical intoxication. Protection by IL-22 from APAP-induced liver damage coincided with increased hepatocyte proliferation. Thorough analysis of hepatic gene expression upon IL-22 treatment demonstrated modulation of IL-36γ/IL-1F9 expression. This member of the IL-1 family may, alike IL-1α, serve DAMP-like functions. Cell culture experiments actually revealed that IL-36γ upregulates CCL20 in human Huh7 cells. This chemokine is crucial for hepatic necroinflammation. Interestingly, CCL20 and IL-36γ were co-regulated in APAP intoxication. Finally, we demonstrate IL-1Rrp2 expression in human liver tissue.

Conclusion:
APAP-induced liver damage is a prototypic DAMP-related syndrome and the leading cause of acute liver injury in the Western world. Herein, we demonstrate protective action of the IL-22/STAT3 axis in this detrimental condition and introduce IL-36γ as novel player in APAP intoxication.
Distinct TREM-1 signaling cascades in human PMN and monocytic cells

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The triggering receptor expressed on myeloid cells 1 (TREM-1) is an important mediator of innate inflammatory responses in microbial infections and sepsis. TREM-1 ligation on neutrophils (PMN) or monocytes results e.g. in the production of proinflammatory cytokines. Towards cellular mechanisms, TREM-1 engagement induces the activation of MAP kinases as well as rapid Ca²⁺ mobilization. However, a detailed understanding of TREM-1 signaling pathways is currently missing.

In our present work, we evaluated the TREM-1 signaling hierarchy in monocytic cells and found that the acute myeloid leukaemia cell line MUTZ-3 expresses TREM-1 in a natural and functional manner. We compared essential signaling molecules of the TREM-1 cascade in MUTZ-3 cells as well as primary monocytes or PMN by Western Blot analysis. These studies confirmed the essential role of phosphatidylinositol 3-kinase (PI3K) and p38MAPK in the TREM-1 cascade of monocytic cells. Importantly, PI3K and p38MAPK signals in monocytic cells both control Ca²⁺ mobilization and are directly connected in the TREM-1 signaling hierarchy, which contrasts previous results obtained in PMN.

Taken together, our results indicate cell type specific differences in the TREM-1 signaling cascade and contribute to an enhanced understanding of the regulation of innate inflammatory responses.
Dusp16 gene trap mice reveal an essential function of MKP-7 in perinatal survival and regulation of TLR-induced cytokine production

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MAPK activity is negatively regulated by members of the Dual specificity phosphatase (Dusp) family, which differ in expression, substrate specificity and subcellular localization. Here, we have investigated the function of Dusp16/MKP-7 in the innate immune system. The Dusp16 isoforms A1 and B1 were inducibly expressed in macrophages and DC following TLR stimulation. A gene trap approach was used to generate Dusp16-deficient mice. Homozygous Dusp16tp/tp mice developed without gross abnormalities but died perinatally. Fetal liver cells of Dusp16tp/tp embryos reconstituted the lymphoid and myeloid compartment by Dusp16-deficient hematopoietic cells efficiently. However, GM-CSF induced proliferation of bone marrow progenitors in vitro was impaired in the absence of Dusp16. In vivo challenge of with E. coli LPS triggered higher production of IL-12p40 in mice with a Dusp16-deficient immune system. In vitro, Dusp16-deficient macrophages but not dendritic cells, selectively over-expressed a subset of TLR-induced genes, including the cytokine IL-12. Production of IL-10 and its inhibitory effect on IL-12 production were unaltered in Dusp16tp/tp macrophages. However, increased expression of the transcription factor IRF-1 in Dusp16tp/tp macrophages suggested a potential mechanism of heightened IL-12p40 production. Together, the Dusp16 gene trap mouse model identifies an essential role in perinatal survival and revealed selective control of differentiation and cytokine production of myeloid cells by the MAPK phosphatase Dusp16.
The class II transactivator CIITA is stabilized by binding to an N-terminal ubiquitin-like domain of the chaperone BAT3/BAG6 which governs HLA class II gene expression

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Question:
Antigen presentation by human lymphocyte antigen (HLA) class II peptide receptors initiates CD4+ T cell responses to combat infections. The class II transactivator (CIITA) exclusively regulates HLA class II, HLA-DM and other genes involved in class II antigen processing. Adjacent to the HLA class II loci, the HLA class III gene region encodes several proteins involved in immune defense and inflammation. Recent studies identified the class III encoded BAT3/BAG6 as a multi regulator of numerous, although diverse cellular processes. We discovered that BAT3 stabilizes CIITA, which controls transcription of class II genes. In addition, treatment of cells with interferon γ simultaneously induces transcription of BAT3 and CIITA and moreover initiates translocation of CIITA and BAT3 from the cytosol to the nucleus. In this present study we analysed interaction of several BAT3 deletion mutants with the class II transactivator.

Methods:
We constructed recombinant BAT3 molecules with deletions at the N- and at the C-terminus and several N- and C-terminal BAT3 fragments. COS-7 cells were transiently co-transfected with CIITA-GFP and the recombinant BAT3 constructs. Subsequently CIITA-GFP was immunoprecipitated and co-isolated BAT3 was identified by western blotting.

Results:
A C-terminal BAT3 deletion mutant showed interaction with CIITA, whereas the N-terminal deleted BAT3 was not able to bind to the transactivator. By employing additional BAT3 constructs, we identified an ubiquitin-like domain of BAT3, which mediates binding to the class II transactivator.

Conclusions:
We identified one of two N-terminal ubiquitin-like domains of BAT3 to be important for binding to CIITA. This interaction of CIITA to BAT3 stabilizes the class II transactivator and permits translocation from the cytosol to the nucleus.

References:
An siRNA screen for NFAT activation identifies septins as coordinators of store-operated Ca\(^{2+}\) entry

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The STIM1-ORAI1 pathway of store-operated Ca\(^{2+}\) entry is an essential component of cellular Ca\(^{2+}\) signaling\(^1\). STIM1 senses depletion of intracellular Ca\(^{2+}\) stores in response to physiological stimuli, and relocates within the endoplasmic reticulum (ER) to plasma membrane (PM)-apposed junctions, where it recruits and gates open plasma membrane ORAI1 Ca\(^{2+}\) channels. Here we used a genome-wide RNAi screen to identify filamentous septin proteins as critical regulators of store-operated Ca\(^{2+}\) entry. Septin filaments and phosphatidylinositol 4,5-bisphosphate (PIP2) rearrange locally at ER-PM junctions prior to and during formation of STIM1-ORAI1 clusters, facilitating STIM1 targeting to these junctions and promoting the stable recruitment of ORAI1. Septin rearrangement at junctions is required for PIP2 reorganisation and efficient STIM1-ORAI1 communication. Septins are known to demarcate specialized membrane regions such as dendritic spines, the yeast bud, and the primary cilium, and to serve as membrane diffusion barriers and/or signaling hubs in cellular processes including vesicle trafficking, cell polarity, and cytokinesis\(^2,4\). Our data show that septins also organise the highly localised plasma membrane domains important in STIM1-ORAI1 signaling, and indicate that septins may organise membrane microdomains relevant to other signaling processes.
Nicotinic Acid Adenine Dinucleotide 2’-Phosphate (NAADP) binding proteins in T-lymphocytes

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Nicotinic acid adenine dinucleotide phosphate (NAADP) is a second messenger involved in T cell calcium signaling (reviewed in 1,2) and T cell activation. NAADP regulates calcium release from intracellular stores. Although several channels, including two-pore channels (TPC), ryanodine receptor (RYR) and mucolipin (TRP-ML1) have been implicated in NAADP regulation of calcium signaling, the NAADP receptor has not been identified. In this study, the photoaffinity probe, [³²P]-5-azido-NAADP ([³²P]-5-N₃-NAADP), was used to study NAADP binding proteins in extracts from NAADP responsive Jurkat T-lymphocytes. [³²P]-5-N₃-NAADP photolabeling of Jurkat S100 cytosolic fractions resulted in the labeling of at least ten distinct proteins. Several of these S100 proteins, including a doublet at 22/23kDa and small protein at 15kDa displayed selectivity for NAADP as the labeling was protected by inclusion of unlabeled NAADP, whereas the structurally similar NADP required much higher concentrations for protection. Interestingly, the labeling of several S100 proteins (60, 45, 33 and 28kDa) was stimulated by low concentrations of unlabeled NAADP, but not by NADP. The effect of NAADP on the labeling of the 60kDa protein was biphasic, peaking at 100nM with a five-fold increase and displaying no change at 1µM NAADP. Several proteins were also photolabeled when the P100 membrane fraction from Jurkat cells was examined. Similar to the results with S100, a 22/23kDa doublet and a 15kDa protein appeared to be selectively labeled. NAADP did not increase the labeling of any P100 proteins as it did in the S100 fraction. The photolabeled S100 and P100 proteins were successfully resolved by two-dimensional gel electrophoresis. [³²P]-5-N₃-NAADP photolabeling and two-dimensional electrophoresis should represent a suitable strategy in which to identify and characterize NAADP binding proteins.

Taken together we confirm expression of the 22/23 kDa doublet NAADP BP with high affinity binding and high specificity over NADP in Jurkat T-lymphocytes, as shown previously for 2 other mammalian cell lines and pancreatic tissue. As the central aspect of the unifying hypothesis (2) NAADP needs to bind to specific binding proteins to modulate different ion channels, e.g. RyRs, TPCs, TRP-ML1 and perhaps others, too.

References:
STIM2 drives Ca\(^{2+}\) oscillations through store-operated Ca\(^{2+}\) entry caused by mild store depletion

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The main source of Ca\(^{2+}\) in T cells is through a mechanism known as store-operated calcium entry (SOCE). STIM1 and STIM2 serve as endoplasmic reticulum Ca\(^{2+}\) sensors that, upon store depletion, activate Ca\(^{2+}\) release-activated Ca\(^{2+}\) (CRAC) channels (Orai1-3, CRACM1-3) in the plasma membrane. In the absence of sustained Ca\(^{2+}\)-influx through CRAC channels, lymphocyte activation, proliferation, and effector functions are severely compromised, as demonstrated in the most extreme case by patients suffering from a form Severe Combined Immune Deficiency (SCID). Agonist-induced calcium oscillations in many cell types are triggered by Ca\(^{2+}\) release from intracellular stores and driven by store-operated Ca\(^{2+}\) entry (SOCE). However, their relative roles in agonist-mediated Ca\(^{2+}\) oscillations remain ambiguous. We here report that while both STIM1 and STIM2 contribute to store-refilling during Ca\(^{2+}\) oscillations in mast cells (RBL), T cells (Jurkat) and human embryonic kidney (HEK293) cells, they do so dependent on the level of store depletion. Molecular silencing of STIM2 by siRNA or specific inhibition by G418 selectively suppresses SOCE and agonist-mediated Ca\(^{2+}\) oscillations at low levels of store depletion, without interfering with STIM1-mediated signals induced by full store depletion. Thus, STIM2 is preferentially activated by low-level physiological agonist concentrations that cause mild reductions in ER calcium levels. We conclude that with increasing agonist concentrations, store-operated Ca\(^{2+}\) entry is mediated initially by endogenous STIM2 and incrementally by STIM1, enabling differential modulation of Ca\(^{2+}\) entry over a range of stimulus intensities and levels of store depletion.
Calcium-dependent signals enable selective induction of TLR-triggered IL-10 production in human B cells

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Question:
Suppressory B cell function prevents premature and exaggerated immune activation and is essential for the termination of immune responses. The regulatory properties exerted by B cells mainly depend on the secretion of IL-10 and Toll-like receptors (TLR) contribute to the development of these cells. In the present study we sought to investigate the molecular mechanisms underlying TLR-triggered IL-10 production.

Methods:
We studied the response of human peripheral blood B cells to TLR9 ligand CpG ODN in the presence and absence of calcineurin inhibitors, rapamycin and other small molecule inhibitors. This includes the assessment of cytokine synthesis, proliferation and calcineurin and NFAT activity.

Results:
The results demonstrated that IL-10 synthesis in human peripheral blood B cells could be triggered via TLR7/9 ligands and BCR stimulation. Interestingly, TLR9-triggered IL-10 production could be selectively blocked by the calcineurin inhibitors. By contrast, BCR-mediated IL-10 release was not altered by these agents. Other immunosuppressants such as rapamycin were unselective, i.e. they also affected IL-6 secretion and proliferation. Further analysis suggested that TLR-induced IL-10 production is stimulated in a Ca²⁺/calmodulin-dependent, but calcineurin/NFAT-independent manner: we observed no change in calcineurin activity after B cell stimulation with CpG ODN and NFAT transcription factors were only found in the phosphorylated, e.g. inactive form. Moreover, competitive antagonization of NFAT with VIVIT revealed a negative regulatory role for NFAT transcription factors in the modulation of TLR-induced cytokine production in human B lymphocytes. Further experiments favored a contributory role of Ca²⁺/calmodulin kinase II (CaMKII) and/or immunophilins in the induction of TLR9-triggered IL-10 in human B lymphocytes.

Conclusions:
Calcium-dependent signaling may, thus, represent a target for selective pharmacological manipulation of B cell-specific IL-10 synthesis and might be therapeutically useful in autoimmunity and infection.
NMDA Receptor Antagonists: Effective Modulators of T Cell Signaling and Effector Function

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Introduction:
Glutamate receptors of the N-methyl-D-aspartate type (NMDARs) are ligand-gated calcium-permeable ion channels and play an important role in neuronal development, plasticity and disease. NMDARs are hetero-tetramers consisting of the obligatory NR1 and homo- or heterodimeric NR2A-D subunits. NMDAR activity is inhibited by the non-competitive NR2B antagonist ifenprodil or the open channel blockers MK801 and memantine. These pharmaceuticals have been shown to be neuroprotective in animal models of stroke, epilepsy and experimental autoimmune encephalomyelitis (EAE) and memantine is used to treat Alzheimer’s disease.

Question:
In order to understand how NMDAR antagonists influence disease severity, their possible effects on non-neuronal cells have to be known. Indeed, the reported expression of NMDARs in immune cells suggests glutamate regulates both, nervous and immune cells. We analyzed the influence of ifenprodil, MK801 and memantine on TCR-induced signaling events and the effector function of CD4⁺ and CD8⁺ T cells.

Results:
The antagonists reduced antigen-specific T-cell proliferation, cytotoxicity, migration, and changed the cytokine secretion profile of TH cells. At the molecular level, NMDAR antagonists reduced the activation of major TCR-induced signaling pathways, including Ca²⁺-mobilization, activation of Erk1/2, Akt and NFATc1. We found mRNA of NMDAR subunits and NMDA-induced Ca²⁺-flux, providing evidence for NMDAR expression in murine T cells. However, we did not detect NR1 protein expression, indicating NMDARs are either expressed at an extremely low level or are very unstable. Assuming additional targets for the employed NMDAR antagonists, we discovered an inhibitory influence on other channels expressed on T cells.

Conclusions:
Since NMDAR antagonists are potent modulators of T cell function in vitro, their in vivo use to divert immune responses could be envisaged. In addition, when they are applied in the treatment of neuronal or immune diseases, their effects on other channels needs to be (re)considered.
The calcium-dependence of killing


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CD8+ cytotoxic T cells (CTL) play a key role in eliminating virus-infected or malignantly transformed cells. The release of lytic granules, containing perforin and granzymes, into the cleft of the immunological synapse is thought to be the main killing mechanism. The second apoptosis-inducing pathway, FASL (CD178)/FAS (CD95), is involved in immune tolerance as well as tumor suppression, at least within the lymphoid compartment. It has been previously reported that store-operated ORAI1 channels are needed for proper CTL function. The exocytosis of lytic granules appears to be calcium-dependent similarly to other cellular systems like mast cells or chromaffin cells where the calcium dependence of exocytosis has been studied. In addition to that however, not much is known about the calcium-dependence of killing. Here, we report a bell-shaped calcium-dependence of killing. We purify PBMC out of the LRS chamber from human thrombocyte apheresis from the local blood bank. CD8+ T cells were stimulated by superantigen Staphylococcus aureus Enterotoxin A (SEA) within the PBMC population and after five days CTL were positively isolated by antibody-coated magnetic beads. To analyze CTL killing function, we use a sensitive, calcein-based assay for quantification of killing kinetics developed in our laboratory. We first analyzed killing efficiency of CTL using different external calcium concentrations with the population killing assay over four hours in Ringer’s medium where calcium can be easily adjusted. We found that the optimal calcium concentration for killing is within the range of 0.5 mM to 1 mM, both very low calcium concentrations (lower than 100 µM) and high calcium concentrations (higher than 2 mM) reduced killing drastically. To mimic more physiological conditions, killing was also analyzed in medium (AIMV, 37°C and CO2 conditions). Different calcium concentrations were adjusted by the addition of different amounts of EGTA. Calcium concentrations were determined by either MagFura-2 measurements or with a Blood Gas system. Similarly to the experiments in Ringer’s solution, we observed a bell-shaped calcium dependence of killing in AIMV under more physiological conditions. Our work sheds new light on understanding the regulation of CTL killing efficiency by changing the calcium concentration in the surrounding microenvironment.
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Analysis of spatiotemporal calcium signaling events in T-lymphocytes

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Introduction:
T-lymphocyte activation and clonal expansion is stimulated via \( \text{Ca}^{2+} \) release and entry events. The associated gene expression may be affected e.g. by the amplitude or duration of the preceding \( \text{Ca}^{2+} \) signals (1). Localized \( \text{Ca}^{2+} \) events, e.g. in T-lymphocytes upon TCR activation, may induce recruitment of further receptors such as ryanodine receptors, thereby triggering global \( \text{Ca}^{2+} \) signals (2). The understanding of subcellular \( \text{Ca}^{2+} \) signals preceding global \( \text{Ca}^{2+} \) signals and the underlying receptor activities are crucial in T-cell activation.

Objectives:
Fura-2 is a frequently used ratiometric \( \text{Ca}^{2+} \) dye with advantages e.g. in sensitivity or stability in comparison to selected single wavelength dyes. However, measurement with two separate excitation wavelengths slows the acquisition speed making Fura-2 unsuitable for the measurement of spatiotemporal \( \text{Ca}^{2+} \) events. Further, ratioing of 2 images acquired at different excitation time points may lead to systematic errors. Aim of the present study was the optimization of high-resolution life cell imaging of spatiotemporal \( \text{Ca}^{2+} \) signals in T-lymphocytes using a photostable \( \text{Ca}^{2+} \) dye.

Materials & methods:
\( \text{Ca}^{2+} \) imaging was performed in Jurkat T-cells using a Leica IRBE2/ Hamamatsu Imaging system. The cells were loaded with the membrane permeant AM esters of the \( \text{Ca}^{2+} \) dyes Fluo-4, Fluo-8, Indo-1, Asante Red (all 10 \( \mu \text{M} \)) or Fura-Red (20 \( \mu \text{M} \)). T-cells were stimulated with a final concentration of 10 \( \mu \text{g/ml} \) OKT3. \( \text{Ca}^{2+} \) signals were recorded with an acquisition rate of approximately 40 frames/sec at 153x153 nm/pixel spatial resolution.

Results:
Two major problems were encountered during analysis of local \( \text{Ca}^{2+} \) signaling events in T-cells: first, morphological changes of the cells during activation and second, extensive photobleaching of the \( \text{Ca}^{2+} \) dyes. The single wavelength dye, Fluo-4, was found to be more stable during high-speed acquisition particularly in comparison to Fluo-8 and characteristic spatiotemporal \( \text{Ca}^{2+} \) signals were observed. However, morphological changes of the cells may interfere with the subsequent ratiometric analysis, particularly in the proximity to the plasma membrane, thereby disturbing subcellular signal analysis. Therefore, the ratiometric dyes Indo-1 and Asante Red were tested, using a DualView imaging system allowing instantaneous acquisition of two different emission wavelengths. However, following 30 sec of high-speed acquisition, Indo-1 bleached below light detection level. Fluorescence intensity of Asante Red was too low for high-resolution acquisition at short exposure times. Due to the good photostability of Fluo-4, a combination of Fluo-4 and Fura-Red was tested using the DualView acquisition mode. Analysis of spatiotemporal \( \text{Ca}^{2+} \) signals and photobleaching indicated that cell shape and subcellular signals e.g. in the proximity to the plasma membrane were reproducibly detected during high-speed acquisition.

Conclusion:
Methods for high-speed imaging of spatiotemporal \( \text{Ca}^{2+} \) signals need to be carefully evaluated since photobleaching and morphological changes may lead to systematic errors. The combination of the \( \text{Ca}^{2+} \) dyes Fura-Red and Fluo-4 described here enables the ratiometric acquisition of subcellular \( \text{Ca}^{2+} \) events despite cellular movement and changes in cell shape.

(2) Bootman et al. (2007) Cell; 3(91); 367-373
Ligand induced opening of TRPM2 channel requires terminal ribose of ADPR and involves R1433, Y1349 and T1347

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Introduction:
TRPM2 is a Ca²⁺-permeable non-selective cation channel of the TRP ion channel family with expression in a variety of immune cells including monocytes/macrophages, neutrophiles, dendritic cells and T lymphocytes. The channel is activated by binding of adenosine diphosphate ribose (ADPR) to it's cytoplasmic NUDT9H domain. Activation due to the increase in cellular ADPR after exposure to oxidative stress has been shown to result in apoptosis. Recently more physiological functions of TRPM2 in cytokine secretion, regulation of production of reactive oxygen species and chemotaxis began to emerge, but investigation of the role of ADPR/TRPM2 in these processes is largely hampered due to the lack of specific inhibitors.

Objectives:
Rational design of ADPR/TRPM2 antagonists might greatly benefit from a better understanding of the structure-activity relationship. We therefore tried to obtain insight into the way in which ADPR binds to the NUDT9H domain by synthesis and evaluation of ADPR analogues, focusing on the terminal ribose.

Material & Methods:
Synthesis and electrophysiological evaluation of ADPR analogues. Homology modeling of the NUDT9H domain based on the crystal structure of human NUDT9. Computational docking of ADPR. Evaluation of point mutants by Ca²⁺ imaging of transfected HEK293 cells stimulated with H₂O₂.

Results:
We tested ADPR analogues with modifications in the terminal ribose at a concentration where ADPR induces a robust current. None of the analogues was able to activate the channel. In contrast when tested for antagonist activity two of the compounds resulted in a significant reduction of current when applied at excess over ADPR, indicating that these compounds, while not able to activate the channel, were competing with ADPR for binding to the NUDT9H domain. From the modelling studies we identified amino acids in proximity to the terminal ribose potentially involved in hydrogen bonding. Analysis of the mutants using Ca²⁺ imaging indicated a role for Arg 1433, Tyr1349 and Thr1347 in the molecular interaction between the NUDT9H domain and ADPR.

Conclusion:
Our findings show that interactions between the terminal ribose of ADPR and amino acids of the NUDT9H-domain of TRPM2 while required for activation are less important for binding. Similar compounds might be turned into inhibitors suitable for elucidation of the role of TRPM2 in the immune context.
Antagonistic regulation of human Th17 and GM-CSF-producing T helper cells

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The pathogenicity of Th17 cells in mouse models of autoimmunity has recently been associated with their production of granulocyte-macrophage-colony-stimulating factor (GM-CSF). Here, we have analyzed the expression of GM-CSF by human T helper cells. The induction of GM-CSF expression by human T helper cells is constrained by the IL-23/STAT3/ROR-gt/Th17 cell axis but promoted by the Th1 axis. While STAT3 blocks GM-CSF induction, STAT5 promotes it. Ex vivo, most GM-CSF⁺ T helper cells co-express IFN-g and T-bet. A distinct subset of GM-CSF⁺ T cells does not express Th1, Th2 and Th17 signature cytokines or master transcription factors, and thus constitutes an independent T helper cell lineage, the Th-GMCSF subset. Our findings suggest a role for GM-CSF⁺ T cells in the inflamed brain of multiple sclerosis patients as well as in the healthy skin.
Overexpression of CREM alpha in CD4+ T cells accelerates acute ConA-induced hepatitis but does not influence fibrosis

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Background and aims:
The cAMP response element modulator CREMα is overexpressed in T cells of patients with autoimmune diseases such as systemic lupus erythematosus (SLE). In SLE T cells produce decreased amounts of IL-2 and increased amounts of IL-17. Similarly, mice overexpressing CREMα specifically in T cells (CD2-Cremtg) also show enhanced IL-17, decreased IL-2 production and T cell proliferation, leading to accelerated contact dermatitis compared to wildtype (wt) mice. Based on the involvement of IL-17 producing T cells (Th17 cells) for autoimmune hepatitis, we investigated in this study the functional relevance of CREMα in immune-mediated hepatitis and liver fibrosis.

Methods:
Wildtype (wt) and CD2-Crem-overexpressing (CD2-Cremtg) mice were subjected to Concanavalin A (ConA)-induced hepatitis or chronic liver fibrosis by repetitive carbon tetrachloride (CCl4) injections. Liver damage, inflammation and fibrosis development were assessed by biochemical methods, histology, flow cytometry and qPCR.

Results:
Upon ConA treatment CD2-Cremtg mice showed elevated transaminase levels and increased mortality compared to wt mice. In contrast, chronic injury and fibrosis development upon CCl4 were comparable between wt and CD2-Cremtg mice. In both models, CD2-Cremtg mice displayed less infiltration of immune cells to the liver, while immune cell numbers in the spleen remained similar to wt mice. Cremα expression in hepatic and splenic CD4 T cells decreased after ConA or CCl4 treatment, alongside increased IL-2 and IL-17 expression. Interestingly, Crem-overexpressing CD4 T cells from the liver showed higher levels of IL-2 and lower levels of IL-17 compared to wt T cells, while in splenic CD4 T cells expression of these cytokines did not differ between both strains. We could not detect differences in CD4 T cell differentiation or activation between CD2-Cremtg and wt mice. Reduced hepatic T cell numbers in CD2-Cremtg mice was not caused by overactivation and cell death through higher levels of IL-2, because pretreatment of wt mice with IL-2 did not accelerate ConA-hepatitis.

Conclusions:
Overexpression of CREMα in CD2+ T-cells enhances acute immune-mediated hepatitis but does not influence fibrosis. CREMα-overexpression does not induce a predominant Th17 response in intrahepatic T-cells, thus suggesting compartmental differences of T cell activation pathways between liver and other organs in autoimmunity.
Epigenetic signature of ex vivo Th17 cells

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Introduction:
Heritable epigenetic modifications play a key role for the terminal differentiation of effector T cell subsets. Interleukin 17 (IL-17)-producing CD4+ effector T cells (Th17 cells) are involved in both immune responses against pathogens and the promotion of autoimmune responses. Several studies analysed the epigenetic modifications in Th17 cells. A study by C.J. Cohen showed that in vitro-expanded human Th1, Th1/17 and Th17 subsets have a distinct methylation pattern at the cytokine loci of IFNG and IL17A, and at the transcription factor loci of TBX21, RORC, and RORA. Another publication revealed that a partial demethylation of the Il17a and Il17f promoter region is lineage specific for in vitro differentiated murine Th17 cells. However, none of the studies so far have identified any Th17-specific demethylated regions in ex vivo-isolated cells.

Objective:
Genome-wide screening for differentially methylated DNA regions within ex vivo-isolated Th17 cells and studying how the identified epigenetic modifications contribute to Th17 lineage commitment.

Materials and Methods:
We isolated ex vivo murine Th17, Th1, Tregs and naive CD4+ T cells with cytokine secretion assays. Differentially methylated regions were identified by in silico methylome comparison using methyl-CpG binding domain protein sequencing (MBD-seq) and validated by methylation-sensitive high-resolution-melting analysis (MS-HRM). Detailed methylation analysis by pyrosequencing was carried out in six candidate regions including Dclk1, Dpp4, Cmklr1, Chn2, Bik and Il17a. Differentially-methylated CpG motifs were identified by comparing different ex vivo-immune cell subsets and in vitro-differentiated Th17, Th1 and Tregs. RNA microarrays with ex vivo-isolated Th17, Th1 and naive T cells were performed to correlate the methylation status of a validated candidate region with the expression pattern of the corresponding locus.

Result:
The CpG motifs in Il17a showed a homogeneous demethylation in ex vivo-isolated Th17 cells whereas they were only partially demethylated in in vitro-differentiated Th17 cells and mostly methylated in all other ex vivo-isolated T helper subsets. As expected, Il17a region was homogeneously demethylated in murine γδ T cells but not in B cells, CD8+ T cells, CD4+ T cells, NK cells, neutrophils, dendritic cells, macrophages or monocytes. The methylation pattern of Bik and Cmkl1r1 in Th17 cells showed similarity to Th1 cells, whereas four motifs in Dpp4, two motifs in Chn2 and one motif in Dclk1 were uniquely demethylated in ex vivo-isolated Th17 cells but not in other immune cell subsets. Preliminary transcriptome analyses showed an upregulation of Il17a, Dpp4 and Bik in Th17 cells and a downregulation for Chn2 and Dclk1.

Conclusion:
We have successfully identified the epigenetic signature of ex vivo Th17 cells. Further molecular characterization (eg. luciferase assay for transcriptional activity) will show the functional role of the identified epigenetic signature for the unique phenotype of Th17 cells.

References:
GARP has regulatory impact on the differentiation process and function of CD4+ T cells *in vitro* and *in vivo*

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GARP (glycoprotein A repetitions predominant) is an activation maker on human regulatory T cells (Treg) which binds and activates latent TGF-β, a well known suppressor of inflammatory T cell activation. To investigate the potential role of GARP as an immunomodulator of T cells we generated soluble GARP protein (sGARP) and analyzed the functional assignment of sGARP in the activation and differentiation of human CD4+ T cells. Here we show that sGARP suppresses proliferation of naive CD4+ T cells, leads to Smad2/3 phosphorylation and induces Foxp3 expression. In addition sGARP inhibits effector cytokine production such as IFN-γ and IL-2. Foxp3 induction and Smad2/3 phosphorylation by sGARP can be inhibited by blockade of TGF-β signalling pathway, suggesting that sGARP function is at least in part dependent on TGF-β. Furthermore repetitive stimulation of naive cordblood-derived CD4+ T cells in presence of sGARP induces their differentiation into adaptive regulatory T cells which suppress the activation of T effector cells in coculture. To reveal the relevance of these findings *in vivo*, we analyzed the effect of sGARP to Treg-mediated suppression with the help of a well established xenogenic graft-versus-host disease (GvHD) model. Herein, we can show that the repetitive application of sGARP efficiently prevent GvHD onset and T cell-mediated destructive inflammation.

Taken together, our results indicate a crucial role for GARP in the modulation of peripheral tolerance and open the possibility to use sGARP as an immune modifier in the treatment of transplant rejection and inflammatory disorders such as T cell-mediated autoimmunity and allergic diseases.
Histone modifications represent a promising new approach in cases where cell functions are to be modulated as in autoimmune diseases or cancer. While several histone deacetylase (HDAC) inhibitors are currently in clinical cancer studies, we demonstrated an additional anti-inflammatory potency in murine colitis models. Here we describe a possible cellular mechanism for this effect.

Murine naïve T helper cells were isolated via magnetic cell sorting and macrophages were derived from bone marrow (BMMΦ). T cells were stimulated using coated anti-CD3/CD4 antibodies, macrophages via LPS. Cells were analysed using flow cytometry, cytometric bead array or western blot. Acute DSS colitis was performed.

In the presence of ITF2357, the generation of FoxP3+ cells from naïve T helper cells could be enhanced, the polarization to the pro-inflammatory Th17 cells suppressed. In parallel, we demonstrated a dose-dependent down-regulation of the IL-6 receptor on naïve CD4 T cells treated with ITF2357. This effect could be observed on the mRNA expression level and on the protein level via flow cytometry. These results were confirmed in murine colitis models, where the IL-6R expression was diminished on naïve T cells within the lymphnodes, paralleled by a significant reduction of Th17 cells in the lamina propria of ITF2357-treated animals. Consequently, HDAC inhibition resulted in a reduced amount of activated/phosphorylated STAT3 in T cells identifying the IL-6/STAT3/IL-17 pathway as an important target of HDAC inhibitors.

In parallel, ITF2357 treatment of BMMΦ leads to a dose-dependent down regulation of TNFα, IL-6 and IL-12p70 secretion by BMMΦ. TLR4-dependent IL-6R up regulation was significantly impaired by ITF2357, while expression of the signaling transducer CD130 was unchanged. ITF2357 reduced the ability of antigen-specific, MHC-II-dependent T-cell activation.

The present study demonstrates that inhibition of HDAC exerts an anti-inflammatory potency by modulation in T cell polarization directly, but also via affecting macrophage differentiation, leading to impaired IL-6 signalling, reduced T-cell activation, thus representing a novel therapeutic approach for inflammatory bowel disease.
T helper Cell Differentiation

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Treg development depends on the combined activity of cRel and IκBNS

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Introduction:
Nuclear factor κ B (NFκB) represents one of the most important regulatory networks, as it controls cytokine expression, cell activation, survival and differentiation. Canonical triggering of NFκB leads to the activation of the inhibitor of NFκB (IKK) complex. It consists of catalytically active IKKβ and IKKα and the regulatory protein IKKγ/NEMO. Once activated, it phosphorylates the inhibitor of NFκB α (IκBα), which normally inactivates NFκB transcription factors via sequestration within the cytoplasm due to masking of the nuclear localization signal. Upon phosphorylation, IκBα undergoes a conformational change, which ultimately leads to its proteasomal degradation. Afterwards, the released NFκB transcription factor translocates into the nucleus and binds to the DNA. The dimeric NFκB transcription factor is assembled of two Rel subunits, a protein family which consists of RelA, RelB, cRel, p52 and p50.

Objectives:
NFκB governs the generation of regulatory T cells via control of the induction of the forkhead box P3 (Foxp3) transcription factor. Recent studies revealed that loss of the upstream signaling components, Carma1 or IKKβ, abolishes nearly the entire Treg compartment. However, loss of the downstream signaling protein cRel causes a reduction of Treg cells by only 50% and, thus, cRel is not the sole downstream signaling target of Carma1 and IKKβ. IκBNS, an atypical nuclear IκB protein, also referred to as Nfkbid, is necessary for Treg development, since Treg cells in these mice are reduced by about 50% as well. The interplay of cRel and IκBNS and NFκB-mediated chromatin rearrangement during Foxp3 induction are barely understood and require further investigation.

Materials/Methods:
On the cellular level we performed flow cytometry, Treg suppression assays and adoptive transfer models. To study the molecular events in cRel- and IκBNS-dependent signal transduction we did qPCR, Western Blots, ChIP and DNA-pulldown experiments. These analyses were performed using IκBNS- and cRel single- as well as double-deficient mice.

Results:
cRel- and IκBNS-deficient mice display a comparable reduction of Treg cells, throughout the lymphoid organs, but do not spontaneously develop signs of autoimmunity. We recently reported that IκBNS is necessary for the transition of thymic Treg precursor cells into mature Tregs, as Foxp3 induction in these cells is impaired in IκBNS-deficient mice [1]. As a result Treg precursors remain longer in their immature state. As we detected binding of IκBNS to the promoter and conserved non-coding sequence (CNS) 3 of the Foxp3 gene IκBNS is a novel factor, which drives Foxp3 induction. Analyses of cRel/IκBNS double-deficient mice provide evidence that cRel and IκBNS act in part independently of each other during Foxp3 induction, as the Treg compartment is nearly completely abolished in these animals. Furthermore, analyses of thymic Treg precursors revealed a major impact of cRel for precursor generation, but of IκBNS for their further maturation.

Discussion:
Precursor generation and maturation are steps, which are critically regulated via NFκB. Our results demonstrate that cRel and IκBNS have at least partially non-overlapping functions for two points in Treg development: precursor generation and maturation.

Plasticity of inflammatory CD4 T cell subsets in human autoimmune disease, rheumatoid arthritis

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Question:
Whereas T helper (Th) cell subsets were previously regarded as irreversibly differentiated endpoints, accumulating evidence suggests that Th cell differentiation is a plastic process potentially dependent on inflammatory conditions. Mechanisms leading to the predominance of Th17 cells in rheumatoid arthritis (RA) are not yet fully understood. We hypothesized that an altered T cell plasticity might contribute to the shift towards the Th17 phenotype observed in RA, and therefore analyzed the re-differentiation flexibility of CD4 T cells in patients with RA and healthy controls (HC).

Methods:
26 HC and a unique cohort of 24 patients with very early, untreated RA (to exclude effects of immunosuppressive drugs on T cells) were included in the study. Viable Th1, Th2 and Th17 cells were FACS-sorted based on cytokine secretion assay for IFNg, IL-4 and IL-17 either after a first round of in vitro differentiation of naive CD4 T cells or directly ex vivo. Pure Th1, Th2 and Th17 cell populations were then re-differentiated under Th1-, Th2- or Th17-inducing conditions. The cytokine secretion profile of the re-differentiated cells was assessed by flow cytometry.

Results:
When in vitro-generated Th1 cells from patients or healthy individuals were differentiated under Th17-inducing conditions, 12 ± 13.6% vs 16.1 ± 8.8% IL-17-producing cells were generated, respectively (n.s.). In contrast, re-differentiation of in vitro-generated Th17 cells under Th1-inducing conditions resulted in populations of 84.6 ± 2.8% vs 76.3 ± 7.9% of IFNg-producing cells in RA and HC, respectively (n.s.). After re-differentiation of Th2 cells under Th17-inducing conditions 5.1 ± 4.8% vs 5.4 ± 2.9% IL-17-producing cells were found, while re-differentiation of Th17 cells under Th2-inducing conditions gave rise to minor numbers of IL-4 producing cells 4.7 ± 2% vs 3 ± 1.1% in RA and HC, respectively (n.s.). Together, the degree of plasticity for all the in vitro-generated Th cells was comparable between RA patients and HC. In marked contrast, in vivo-generated Th17 cells from RA patients re-differentiated under Th1-inducing conditions kept their Th17 phenotype to a significantly higher degree compared to HC. After Th1 re-differentiation of Th17 cells 83.3 ± 5.6% vs 70.4 ± 7% IL-17-producing cells were detected in RA patients compared to HC, respectively (p<0.01). Consistent with this, lower Th1 and Th2 frequencies were observed when Th17 cells were re-differentiated under Th1 and Th2-inducing conditions (8.1 ± 4.1% vs 17.5 ± 5.4%, p<0.01 and 0.9 ± 0.5% vs 2.5 ± 1.1%, p<0.05, respectively), which indicates a decreased plasticity of RA Th17 cells to acquire a Th1 or Th2 phenotype. Also, the re-differentiation of in vivo-generated Th1 cells into Th17 cells yielded higher frequencies of IL-17-producing cells in RA compared to HC reflecting a shift towards Th17 cells (22.4 ± 10.7% vs 9.2 ± 8.8%, p<0.01). Interestingly, even when RA Th1 cells were primed under non-Th17 conditions (e.g. Th2) higher frequencies of IL-17 producers were found as compared to HC (p<0.05).

Conclusions:
Taken together these data show that in RA in vivo-generated Th17 cells are impaired in their ability to re-differentiate towards a Th1 or Th2 phenotype, whereas Th1 and Th2 cells are more prone to re-differentiate into Th17 cells in RA. This suggests a resistance of Th17 cells in vivo to plasticly change and a propensity of other Th subsets to acquire the Th17 phenotype, which together might contribute to the observed Th17 predominance in RA.
miR-17–92 promotes T follicular helper cell differentiation and represses subset-inappropriate gene expression

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Introduction:

T follicular helper (Tfh) cells are the prototypic T helper cell subset that enables B cells to form germinal centers (GCs) and produce high affinity antibodies. Tfh cell differentiation begins very early in the immune response, coinciding with rapid proliferation that expands the pool of responding cells. MicroRNAs (miRNAs) have emerged as important regulators of many aspects of immune cell differentiation and function. The cell fate decisions of activated T helper cells are very sensitive to precise dosing of regulatory factors, and are therefore subject to regulation by the fine-tuning activity of miRNAs. There is some evidence that miRNAs might regulate the Tfh cell gene expression program and the plasticity of Tfh cells. However, the precise contribution of miRNAs to Tfh cell differentiation and function remains unknown.

Objectives:

To analyze the requirements of global and specific miRNA expression in CD4+ T cells for Tfh cell differentiation and function.

Materials & Methods:

We combined genetic ablation of all microRNAs (Dgcr8-deficiency) or genetic ablation or overexpression of a specific miRNA cluster (miR-17–92) specifically in T cells with adoptive CD4+ T cell transfers to study Tfh cell differentiation and function in vivo.

Results:

We found that miRNA expression by CD4+ T cells was essential for Tfh cell differentiation. More specifically, we show that after protein immunization the miRNA cluster miR-17–92 was critical for robust Tfh cell differentiation and function in a cell-intrinsic manner that occurred regardless of changes in proliferation. Conversely, miR-17–92 overexpression in T cells promoted Tfh cell differentiation and GC B cell induction. Early in the response to protein antigen, these effects were partially dependent on altered expression of the miR-17–92 target gene Pten, a known inhibitor of Tfh cell differentiation. In a viral infection model, Tfh cells depended on the miR-17–92 cluster to restrain the expression of Tfh subset-inappropriate genes, including Ccr6, Il1r2, Il1r1, and Il22. In addition, we found that conserved regions in the RAR-related orphan receptor alpha (Rora) 3’ UTR were directly targeted by all 4 miRNA families in the cluster. Genetically removing one Rora allele significantly rescued the inappropriate gene expression signature in miR-17–92-deficient Tfh cells.

Conclusions:

Our results identify the miR-17–92 cluster as a critical regulator of T cell-dependent antibody responses, Tfh cell differentiation, and the fidelity of the Tfh cell gene expression program.
The NF-κB regulator MALT1 determines the encephalitogenic potential of Th17 cells


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Effector functions of inflammatory IL-17-producing Th (Th17) cells have been linked to autoimmune diseases such as experimental autoimmune encephalomyelitis (EAE), a mouse model of multiple sclerosis. However, what determines Th17 cell encephalitogenicity is still unresolved. Here, we show that after EAE induction, mice deficient for the NF-κB regulator MALT1 (Malt1−/− mice) exhibit strong lymphocytic infiltration in the CNS, but do not develop any clinical signs of EAE. Loss of Malt1 interfered with expression of the Th17 effector cytokines IL-17 and GM-CSF both in vitro and in vivo. In line with their impaired GM-CSF secretion, Malt1−/− Th cells failed to recruit myeloid cells to the CNS to sustain neuroinflammation, whereas autoreactive WT Th cells successfully induced EAE in Malt1−/− hosts. In contrast, Malt1 deficiency did not affect Th1 cells. Despite their significantly decreased secretion of Th17 effector cytokines, Malt1−/− Th17 cells showed normal expression of lineage-specific transcription factors. Malt1−/− Th cells failed to cleave RelB, a suppressor of canonical NF-κB, and exhibited altered cellular localization of this protein. Our results indicate that MALT1 is a central, cell-intrinsic factor that determines the encephalitogenic potential of inflammatory Th17 cells in vivo.
A Genomic Regulatory Element That Directs Assembly and Function of Immune-Specific AP-1-IRF Complexes

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IRF4 and IRF8 regulate B, T, macrophage, and dendritic cell differentiation. They are recruited to cis-regulatory Ets-IRF composite elements (EICE) by PU.1 or Spi-B. How these IRFs target genes in most T cells is enigmatic given absence of specific Ets partners. ChIPseq in Th17 cells reveals that IRF4 targets sequences enriched for AP-1-IRF composite elements (AICE) that are co-bound by BATF, an AP-1 factor required for Th17, B, and dendritic cell differentiation. IRF4 and BATF bind cooperatively to structurally divergent AICEs, promote gene activation and Th17 differentiation. The AICE motif directs assembly of IRF4 or 8 with BATF heterodimers and is also utilized in Th2, B, and dendritic cells. This genomic regulatory element and cognate factors appear to have evolved to integrate diverse immuno-modulatory signals.
Regulation of memory T helper type 1 cell survival by microRNA-148a


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Auto-antigen specific chronically activated T helper type 1 (Th1) lymphocytes are critically involved in the development and maintenance of chronic inflammation in autoimmune diseases. These cells function independently of antigenic stimulation and proliferation and are therefore resistant against physiological regulation and conventional immunosuppressive therapies. The acquirement of these properties is probably mediated by reduced expression of the pro-apoptotic protein Bim.

Our recent results suggest that microRNA (miRNA) mediated regulation of Bim may play an important role for the development, function and persistence of chronically activated Th1 cells in autoimmune disease.

Assuming that Th1 cells involved in autoimmune inflammation have a history of repeated restimulation by persistent (auto-)antigens, we use in vitro generated acutely (once) activated and chronically (four times) activated murine Th1 cells. By using high-throughput sequencing of miRNA expression libraries, we have identified miRNAs differentially expressed between once and repeatedly reactivated Th1 cells. We found that among Th subsets chronically activated Th1 cells uniquely express microRNA-148a. MiR-148a regulates expression of the proapoptotic gene Bim leading to a decreased Bim/Bcl2 ratio. When inhibiting miR-148a using antagomirs in Th1 cells Bim expression increases, leading to enhanced apoptosis and reduced expansion of repeatedly reactivated EM Th1 cells. Knockdown of Bim expression by siRNA in miR-148a antagonir treated cells restored viability of the Th1 cells. This clearly proofs that miR-148a controls viability exclusively by regulating Bim expression. T cells isolated from the synovium of arthritic patients exhibit elevated miR-148a expression. Interestingly, Tbet (Th1 master transcription factor) and Twist1 (marker for chronically activated memory Th1 cells) induce expression of miR-148a. Taken together the data imply that Tbet and Twist1, besides controlling pathogenicity of Th1 cells, also regulate the longevity in chronic inflammation via the miR-148a-Bim-axis. MiR-148 plays an important role for the survival of chronically activated memory Th1 cells in autoimmunity and chronic inflammation, thus, represents a highly potent molecular target for therapeutical treatment.
T helper Cell Differentiation

IRF4 but not RORγt is indispensable for Th17 cell development \textit{in vivo}

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\textbf{Introduction:}
The transcription factors IRF4 and RORγt are both known to play a crucial role in T cell development and function as was shown by the analysis of \textit{Irf4} and \textit{Rorc} knockout mice, respectively. Expressed not only in T cells but also in B cells, macrophages and dendritic cells (among others) IRF4 is a pleiotropic regulator of immune responses. In contrast, RORγt expression is restricted to T cells and lymphoid tissue inducer cells. Nevertheless, disruption of the \textit{Rorc} gene has systemic consequences as these mice do not have lymph nodes.

\textbf{Objectives:}
To better understand the roles of IRF4 and RORγt in T cell biology we have used the Cre/loxP system to specifically delete these transcription factors in all conventional T cells.

\textbf{Materials & methods:}
For this purpose we crossed mice carrying floxed alleles of \textit{Irf4} and \textit{Rorc} genes, respectively, to the CD4 Cre transgenic mouse strain. The resulting \textit{Irf4}\textsuperscript{ΔT} and \textit{Rorc}\textsuperscript{ΔT} mice are characterized by a T cell-specific loss of function of the indicated factors.

\textbf{Results:}
Whereas \textit{Irf4}\textsuperscript{ΔT} mice present a normal thymic development of T cells and lymphadenopathy, CD4+ T cells in \textit{Rorc}\textsuperscript{ΔT} mice are underrepresented beginning from the early stages of development. Both mouse models show an increase in Th1 and Tc1 cell populations compared to wild type controls. As a result of \textit{Irf4} deletion, T cells fail to differentiate into Th17 cells under both \textit{in vitro} polarizing conditions as well as after MOG immunization of \textit{Irf4}\textsuperscript{ΔT} mice \textit{in vivo}. As a consequence, \textit{Irf4}\textsuperscript{ΔT} mice are fully resistant to the induction of experimental autoimmune encephalomyelitis (EAE), a mouse model for multiple sclerosis. Interestingly, T cells lacking RORγt expression can be triggered towards Th17 by \textit{in vitro} polarization involving the cytokines TGFβ, IL-6 and IL-23. Moreover, \textit{Rorc}\textsuperscript{ΔT} mice are susceptible to EAE induction, although the disease severity is reduced compared to wild type mice. At the peak of disease CNS-infiltrating CD4+IL17A+ cells can be found in sick \textit{Rorc}\textsuperscript{ΔT} mice, pointing to the pathogenic potential of RORγt-deficient T cells. To further check the necessity for this transcription factor in Th17 generation and/or expansion \textit{in vivo} we used a model of systemic anti-CD3 treatment. Indeed, after polyclonal T cell activation in the periphery we could observe Th17 cells in the small intestine of \textit{Rorc}\textsuperscript{ΔT} mice.

\textbf{Conclusion:}
In this work we show the preferential differentiation of T cells towards Th1 and Tc1 cells in mice lacking transcriptional factors essential for Th17 cell development, thereby pointing to T cell-intrinsic effects of IRF4 and RORγt function. Our data emphasizes the importance of IRF4 for Th17 differentiation during EAE and suggest that Th1 cells developed independently of the “Th17 program” are not pathogenic. In contrast, RORγt seems to be at least partially dispensable for Th17 cell differentiation and pathogenicity \textit{in vivo}. 
The transcription factor E47 suppresses Foxp3 transcription in regulatory T cells

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Regulatory T cells (Treg cells) are crucial for the maintenance of immunological tolerance. In particular, Foxp3+ Treg cells possess potent suppressive activities. The expression of the transcription factor Foxp3 is not only important for the differentiation of CD4+ T cells into Treg cells, but also for the stable maintenance of their suppressive functions. However, the transcriptional control of Foxp3 expression is only partially understood. Here, we show that overexpression of the basic helix-loop-helix transcription factor E47 in Treg cells led to a loss of Foxp3 expression in vitro. Interestingly, using the Id3-GFP transgenic mouse line, expressing GFP under the control of the Id3 gene locus, we found that Foxp3 expressing Treg cells express high levels of the E47 inhibitor Id3. Detailed analysis of regulatory regions in the Foxp3 gene revealed the presence of three conserved non-coding sequences (CNS), which serve as enhancers of Foxp3 transcription. It is well established that T cell receptor (TCR) stimulation of Treg cells lacking the CNS2 region results in loss of Foxp3 expression. Accordingly, we found that TCR stimulation of Id3-deficient Treg cells led to a loss of Foxp3 expression. With the aid of luciferase assays, we demonstrated that E47 represses Foxp3 transcription by acting on the CNS2 region of the Foxp3 gene. However, using a tamoxifen inducible E47 construct and the protein synthesis inhibitor cycloheximide, we showed that E47 does not directly suppress Foxp3 transcription, but regulates the transcription of a still unknown negative regulator of Foxp3 gene expression. Understanding the detailed transcriptional control of the Foxp3 gene is a prerequisite for generating stable Treg cells for therapy.
Stable balance of Th1 and Th2 differentiation programs limits the pathogenic potential of hybrid Th1/2 cells

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T helper (Th) cells differentiate into effector lineages by committing to one of several differentiation programs. Th cell differentiation programs were thought to be mutually exclusive, however, many of them have been found to exhibit plasticity upon secondary polarizing challenges. We showed recently that fully committed Th2 cells could be reprogrammed by virus-induced signals into a hybrid Th1/2 phenotype with combined Th1 and Th2 functions (1).

Here we demonstrate that these combined functions are each manifested in hybrid Th1/2 cells at a quantitatively intermediate level. The Th1 and Th2 differentiation programs remain stably balanced in the hybrid subset in the absence and presence of perturbations. Furthermore, functional characterization of hybrid Th1/2 cells in vivo revealed that the modulated effector functions of hybrid Th1/2 cells translate into attenuated immunopathology. Thus, we propose that the cell-intrinsic balance of two opposing differentiation programs may act as a self-limiting mechanism of Th cells to prevent excessive inflammation.

Atypical IkB proteins in regulatory T cell development

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Introduction:
Regulatory T (Treg) cells are involved in the maintenance of the immune homeostasis and self-tolerance by suppressing excessive immune responses. They are generated in the thymus or extrathymically by conversion of naive CD4⁺ T cells into Treg cells. Expression of the Forkhead box P3 transcription factor (Foxp3) is a prerequisite for Treg cell development and maintenance of suppressive capacity. Deficiencies in Treg cells due to mutation or genetic deletion of Foxp3 cause fatal systemic autoimmunity in mice. Mutations of Foxp3 gene in humans produce a dysfunction of Treg cell development and in consequence polyendocrinopathy enteropathy X-linked syndrome develops. Previous studies have shown that Treg cell development depends on the transcription factor NF-κB and among others the influence of IkBₙₙ has been demonstrated in this process. IkBₙₙ binds to conserved non-coding sequences (CNS) of the Foxp3 locus and drives its expression. IkBₙₙ belongs to a family of proteins known as inhibitors of NF-κB (IkBs), and together with Bcl-3 and IkBz form a group within this family called “atypical IkBs”. These proteins have a nuclear localization and are able to induce and repress NF-κB activity, in contrast to the classical IkBs, which are located into the cytoplasm and inhibit NF-κB.

Objectives:
Previous studies performed in our lab revealed the importance of IkBₙₙ in Treg cell development. The aim of this work is to assess whether other atypical IkB proteins, particularly Bcl-3 and IkBz, are also involved in this process.

Materials and Methods:
Bcl-3-deficient mice and IkBz-deficient mice were analyzed by flow cytometry to determine the frequencies of T cell populations and T cell activation markers in different lymphoid organs as well as Treg cell precursors in the thymus. In vitro Foxp3 induction via TGF-β stimulation was performed in naïve T cells in order to compare the capacity of these cells to develop into induced Treg cells in Bcl-3-deficient mice and IkBz-deficient and the respective wild type littermates.

Results:
Flow cytometry analyses of the different T cell population as well as T cell activation markers did not reveal significant differences among wild type animals, Bcl-3-deficient and IkBz-deficient mice. Likewise, in vitro differentiation of naive CD4⁺ T cells into Foxp3 expressing Treg cells was unaltered. However, we observed a mild reduction of thymic Treg cell precursors in Bcl-3-deficient mice compared with wild type animals. In addition, we detected a significant increase of the T cell population in the spleen of Bcl-3-deficient mice.

Conclusion:
According to our analyses, IkBₙₙ is the sole member of the BCL-3 family, which is necessary for Treg cell development. Analysis of Treg cells in vitro and ex vivo displayed no differences between Bcl-3-deficient, IkBz-deficient and wild type mice, indicating that the development of Treg cells in the organism is similar. Although Bcl-3 may play a role in the development of thymic Treg precursor cells, this defect is compensated by normal Foxp3 induction.
Indexing the T cell crowd: epigenetic features for clinically relevant T cell subsets


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Immune-mediated diseases are one of the emerging types of illnesses of the industrialized world. However, the current therapeutical options are unsatisfying as they utilize largely unspecific immunosuppressive drugs with strong site effects or antibody-based "biologicals" which only a fraction of patients respond to. The need of new therapeutical approaches demands a more detailed understanding of the underlying mechanisms leading to pathological chronic immune activation. We therefore focus our research on CD4+ T helper (Th) lymphocytes, which are known to orchestrate responses of the adaptive immune system and thereby contribute significantly to the chronification of inflammatory reactions, resulting in devastating immune pathology of the affected organs.

Many studies in recent years have firmly established a strong epigenetic regulation of different aspects of Th physiology, such as lineage-commitment, memory development, effector function and migratory behavior. These findings prompted us to characterize the epigenetic profile of clinically relevant Th populations more systematically and on a genome-wide scale. As part of the "German Epigenome Programm (DEEP)”, an associated partner of the International Human Epigenome Consortium (IHEC), we are now analyzing the epigenomes of isolated human Th subsets from chronically inflamed tissues. We expect that these epigenetic signatures will mark crucial developmental steps, regulators and effectors on the way from physiological states to a terminally differentiated stage associated with chronicity. This approach will not only significantly increase our knowledge about pathogenic T cells subsets but should highlight new biomarkers and therapeutical targets for inflammatory disorders.

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Crohn’s Disease (CD) is a chronic inflammatory disorder of the intestine that is connected to severe symptoms, such as weight loss, diarrhea and anemia. A role for the adaptive immune system, especially CD4+ T cells, in disease pathogenicity has long been established. Interestingly, in both CD and a mouse model for CD, the T cell transfer model of colitis, a special type of T cells coproducing IFN-γ and IL-17A, so-called Th17+1 cells, is abundant. We hypothesized that by coexpressing RORγt and T-bet, the master transcription factors of Th17 and Th1 cells, respectively, these cells gain capabilities from both lineages beyond simple IFN-γ and IL-17 coproduction. To address this, we sorted live Th1, Th17 and Th17+1 cells from the inflamed mucosa of colitic mice using the cytokine secretion assay and analyzed their transcriptional profile with Affymetrix arrays. Th17+1 cells coexpressed many genes with Th1 cells, among them effector molecules such as Ifng, Ccl3, Ccl4, Ccl5 and Fasl. Likewise, Th17+1 cells and Th17 cells shared the expression of many genes such as Il17a, Il22, Cxcl3 and Ccl20. However, there was a substantial number of genes that were expressed by either Th1 cells or Th17 cells but not by Th17+1 cells arguing against a simple additive effect of RORγt and T-bet on transcriptional activity. Conversely, Th17+1 cells expressed genes that were not expressed by either Th1 or Th17 cells. Whether their unique transcriptional profile confers a non-redundant role to Th17+1 cells for colitis pathogenicity will be subject to future investigations.
Selective inhibition of the immunoproteasome influences Th17 differentiation in a murine model of systemic Candida albicans infection

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The immunoproteasome has recently been shown to be involved in the pathogenesis of autoimmune diseases and in the modulation of T helper cell differentiation. Selective inhibition of the LMP7 (beta5i) subunit of the immunoproteasome ameliorated clinical signs in mouse models of rheumatoid arthritis, multiple sclerosis, systemic lupus erythematosus, inflammatory bowel disease and diabetes. Moreover, LMP7 inhibition or deficiency was shown to suppress the differentiation of the pro-inflammatory T helper subtypes Th1 and Th17 while it enhances the generation of anti-inflammatory regulatory T cells (Tregs) in vitro. In this study we investigated the role of the immunoproteasome in Th17 differentiation in vivo in a murine model of systemic infection with Candida albicans. IL-17 mediated immunity has been demonstrated to play a central role in host defense against fungal infections most probably by the means of activating the polymorphonuclear neutrophil compartment. Treatment of mice with the LMP7-selective inhibitor ONX 0914 led to reduced differentiation of Th17 and Th1 cells in kidney and spleen. Additionally, the number of neutrophils in the blood was reduced. Inhibition of LMP7 had only a minor influence on the fungal burden in the kidney while it led to enhanced weight loss and negatively affected the mean survival of mice. These results demonstrate a strong influence of LMP7 inhibition on Th17 and Th1 differentiation in vivo.
Interferon gamma ameliorates EAE development but is not essential for TH17 plasticity

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Th17 cells have been shown to play a major role in the pathology of MS and EAE and recently a population of IFNγ expressing Th17 cells has been identified in humans and mice. However, little is known about the function and exact development of this population. We have previously shown that in vivo generated Th17 cells switch towards IFNγ expression when transferred into naive mice. Additionally, it was shown that IFNγ synergizes with IL-12 to induce effective IFNγ expression from in vivo generated Th17 cells, after in vitro reactivation. We therefore used mice with T cell specific knock-out of the Ilner2 gene (CD4/IFNγR2 KO) to analyze the role of IFNγ signaling in CD4⁺ T cells in regard to Th17 plasticity and EAE development. Our adoptive transfer experiments showed, that loss of IFNγ signaling in CD4⁺ T cells leads to severe disease outcome and high accumulation of Th17 cells in the CNS. However, IFNγ signaling seems not to be essential for effective Th17 plasticity in vivo, as proposed by the high number of IL-17A⁺ IFNγ⁺ T cells in the CNS of mice which received Th17 cells from CD4/IFNγR2 KO animals. We therefore suggest IL-27 to be involved in Th17 plasticity and to act independent of IFNγ, as we found IL-27 to be a potent cytokine, synergizing with IL-12 in TH1 differentiation and TH17 plasticity in vitro in control as well as CD4/IFNγR2 KO mice.
Improved method to retain cytosolic reporter protein fluorescence while staining nuclear proteins

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Introduction:
To address the functions of cells and certain genes, reporter mice are available, such as direct knockin mice in a specific gene or conditional loxP-flanked versions in the Rosa26 locus. These mice often express cytosolic soluble fluorescent proteins under the specific promoter or in the case of fate mapping mice a fluorescent protein, which is only expressed in cells in which a specific Cre recombinase is active. The latter are a very useful tool to study cell differentiation and their destiny, due to the irreversible tagging once the respective Cre was produced.

Objective:
To establish a protocol, which fixes soluble cytosolic fluorescent proteins such as eYFP, GFP and RFP in cells with retention of their fluorescence activity while staining for transcription factors, such as Foxp3, Rorgt and T-Bet for flow cytometry.

Methods:
To reach this aim, we had to find an alternative fixation reagent and the crossing point of harsh and soft fixation. The harsh fixation keeps the fluorescence proteins in the cell, while the soft fixation prevents antigen masking allowing staining of nuclear transcription factors.

Results:
The provided kits for staining transcription factors by companies (eBiosience) are insufficient to keep the soluble fluorescent proteins inside the cell. A replacement of the provided fixation/permeabilization buffer is therefore necessary. Paraformaldehyde fixation, also in low concentrations and with short fixation times was too harsh and incompatible with nuclear antigen staining. We defined an alternative fixation method to keep the fluorescence protein functional inside the cells and to stain for different nuclear proteins. This was demonstrated for FoxP3, Rorgt and T-bet in different reporter mice expression eYFP, GFP or RFP using thymocytes and splenocytes.

Conclusion:
In summary, we provide a new protocol, which enables easy and reliable staining of nuclear transcription factors retaining soluble cytosolic reporter proteins with fluorescence activity.
Phosphoproteomic profiling identifies Treg-specific signal control of TCR activation

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CD4⁺Foxp3⁺ regulatory T cells (Treg) and CD4⁺Foxp3⁻ conventional T cells (Tconv) require activation via the T cell receptor (TCR) for full acquisition of their immunosuppressive and effector functions, respectively. To date most of our understanding about the composition and sequence of the TCR signaling pathway has been derived from studies on cell lines or Tconv. Since most of the TCR signaling pathway components investigated so far display comparable expression in Treg and Tconv, alternative regulatory mechanisms have to be responsible for the apparently different phenotypes of these two T cell subsets. We, therefore, aimed to unravel Treg-specific posttranslational modifications, namely phosphorylation patterns, following TCR engagement by quantitative high-throughput LC-MS/MS phosphopeptide sequencing. In total, we obtained unambiguous data from 3888 phosphorylation sites from murine Treg and Tconv under resting and CD3/CD28-activated conditions. Careful inspection identified 144 Treg-specific phosphosites that belong to central TCR signaling modules as well as microtubule-associated proteins. In sum, our data from murine Treg and Tconv suggests that these modules contribute to the Treg-specific formation, composition and function of the immunological synapse.
The switch in T cell survival: Cross-regulation between homeostasis and antigen-induced activation

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The survival of mature T cells in the periphery is a tightly regulated process governed by two major processes: homeostasis of naïve T cells mediated by IL-7 and antigen-induced activation. How the transition between these two states is controlled is not well understood. Here we investigated the events controlling survival of T cells shortly after antigen activation before cells enter the proliferative state. We found that T cell survival is reprogrammed upon TCR-mediated stimulation by actively inhibiting homeostatic survival signals while initiating a new, dominant survival program. Activation through the TCR antagonised IL-7/IL-7R mediated STAT5 phosphorylation and Bcl-2 expression and induced pro-apoptotic molecules Bim and Bax, while simultaneously promoting survival through induction of alternative anti-apoptotic Bcl-2 family members A1 and Bcl-xL. Stimulation strength determined the kinetics of the transition between the survival programs. Calcineurin or MEK pathway inhibitors prevent the initiation of the new survival program while permitting the dominant repression of Bcl-2. Thus, in the presence of these drugs the response to antigen receptor ligation is cell death. Using calcineurin inhibitors Cyclosporin A or FK506 we were able to induce antigen specific loss of activated T cells in-vitro and in-vivo. Our results identify a molecular switch that can serve as an attractive target for inducing antigen-specific tolerance in treating autoimmune disease patients and transplant recipients.
Early Signalling dynamics driving the generation of induced Tregs from naïve T cell pools

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Introduction:
Activation of naïve CD4⁺ T-cells by naive peptide loaded B cells (ToFB) leads to the production of T cells with a CD62-L<sup>high</sup> phenotype and regulatory properties (Treg) in vivo and in vitro. This is in contrast with the functional outcome following activation of naïve T-cells with dendritic cells as APC (ToFDC), which induces an effector type of T-cell activity.

Objective:
Using this phenomenon as a model to study how induced Tregs develop from a naïve T-cell pool we investigated the molecular mechanisms governing this differential regulation of CD62-L and the induction of a regulatory phenotype in freshly activated naïve T-cells.

Materials and Methods:
8-12 weeks old DO11.10 and OT-2 mice were sources of naïve CD4 T-cells specific for a peptide of chicken ovalbumin. Wildtype BALB/c and C57/Bl6 mice were used as sources of DC and naïve B-cells. Splenic T- and B-cells were negatively enriched using the MACS system. DC were obtained from bone marrow precursors differentiated with GM-CSF and IL4 or GM-CSF alone and treated with LPS when further functional maturation was desired.

Results:
Following differential activation using both APC types and dissection of underlying signaling pathways, it was observed that pERK driven TACE activity was responsible for early shedding of CD62-L in both cell systems. However, after 24h CD62-L was re-expressed in ToFB while it maintained at low levels in ToFDC. The long term downregulation of CD62-L ToFDC was mediated by sustained PI3K/mTOR activity. In contrast in ToFB we identified an attenuated phosphorylation of the hydrophobic motif of Akt occurring at the time of divergence in CD62-L kinetics between ToFB and ToFDC but returning to the same activity as seen in ToFDC later. This was accompanied by the transient upregulation of PHLPP1, a phosphatase specific for pAKT Ser473.

To approach a more physiological stimulus for the induction of Tregs we also activated naïve T-cells with immature DC which are known to tolerize T-cells. These TofImmDC also a transiently attenuated Akt profile in addition to a high CD62-L phenotype, which were both indistinguishable from the effects seen in ToFB. It was also possible to induce regulatory behavior in T-cells triggered with matured DCs via fine tuning of the PI3K but not the mTOR pathway. Interestingly, while CD28 triggering turned out to be decisive for inhibiting Treg development, it did not have an impact on CD62-L kinetics.

Conclusions:
Thus, cell lineage decision after initial onset of TCR triggering involves both, PI3K/Akt and mTOR signaling; however only the PI3K/Akt axis and CD28 seem to be responsible for the induction of a regulatory phenotype, while long-term CD62-L downregulation is controlled via PI3K/Akt alone. This shows that rather than completely distinct signaling pathways a differential timing of relatively similar pathways mediate the development of Tregs from naïve T-cells.
Alternative IL-7-mediated NFAT activation is critical for early thymus development

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Interleukin 7 (IL-7) controls the development of early double-negative (DN) thymocytes and homeostasis of mature T cells. Inactivation of genes encoding IL-7, IL-7 receptor (IL-7ra) or JAK3 led to a block in early thymic development at the stage of DN1 thymocytes. While the transcription factor STAT5 is an important component of IL-7 signaling, differences between the phenotypes of STAT5 and IL-7/IL-7ra/JAK3-deficient mice suggest the existence of STAT5-independent IL-7 signaling. Here we show that in DN thymocytes IL-7/JAK3-mediated signals activate the transcription factor NFATc1 which, in part, can substitute for defects of IL-7 ablation. IL-7/JAK3 signals phosphorylate one conserved tyrosine residue within the regulatory region of NFATc1 and, thereby, support its nuclear translocation and transcriptional activity. This alternative IL-7-dependent NFAT activation pathway differs from ‘conventional’ TCR-Ca²⁺-calcineurin-mediated NFAT activation and is critically involved in the survival and development of early DN thymocytes. Inactivation of NFATc1 in Vac-cre mice bearing Nfatc1 fl/fl alleles led to a strong reduction in thymus size. One prominent NFATc1 target in DN thymocytes is the anti-apoptotic Bcl2 gene which is directly regulated by NFATc1. Our results show that in DN thymocytes NFAT factors are independently activated from (pre-) TCR signals. They suggest that in addition to their role in mounting immune responses upon Ca²⁺-calcineurin signaling, NFAT factors control also the survival and homeostasis of lymphocytes by IL-7. Our findings favor NFATc1 as a target for developing therapies to treat viral infections and immunodeficiencies in which IL-7 treatment showed promising effects.

Reference:
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**Differential post-selection proliferation dynamics of αβ T cells, Foxp3⁺ Treg cells, and invariant NKT cells monitored by genetic pulse labeling**

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The thymus generates two divergent types of lymphocytes, innate and adaptive T cells. Innate T cells such as invariant (i)NKT cells provide immediate immune defense whereas adaptive T cells require a phase of expansion and functional differentiation outside the thymus. Naive adaptive T lymphocytes should not proliferate much after positive selection in the thymus to ensure a highly diverse TCR repertoire. In contrast, oligoclonal innate lymphocyte populations are efficiently expanded through intrathymic proliferation. For CD4⁺Foxp3⁺ regulatory T (Treg) cells, which are thought to be generated by agonist recognition, it is not clear whether they proliferate upon thymic selection. Here, we investigated thymic and peripheral T cell proliferation by genetic pulse labeling. To this end, we used a mouse model in which all developing αβ thymocytes were marked by expression of a histone-2B-eGFP fusion-protein (H2BeGFP) located within the Tcrd locus (TcrdH2BeGFP). This reporter gene was excised during TCR α-chain VJ-recombination and the retained H2BeGFP-signal was thus diluted upon cell proliferation. We found that innate T cells such as CD1d-restricted iNKT cells all underwent a phase of intense intrathymic proliferation whereas adaptive CD4⁺ and CD8⁺ single positive thymocytes including thymic Treg cells cycled on average only once after final selection. After thymic exit, retention or loss of very stable H2BeGFP-signal indicated the proliferative history of peripheral αβ T cells. There, Treg cells showed lower levels of H2BeGFP compared to CD4⁺Foxp3⁺ T cells. This further supports the hypothesis that the Treg cell repertoire is shaped by self-antigen recognition in the steady state.
Regulation of CD8 T cell function by the small GTPase Arl4d

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Members of the ADP-ribosylation factor (ARF) family of guanine-nucleotide-binding (G) proteins, including the ARF-like (ARL) proteins, regulate membrane traffic and organelle structure by recruiting cargo-sorting coat proteins, modulating membrane lipid composition and interacting with regulators of other G proteins. Arl4D is mainly localized to the plasma membrane and can modulate actin remodeling via regulating ARF6 activity. It has furthermore, recently, been implicated in the regulation of mitochondrial membrane potential.

The liver is known to induce immune tolerance rather than immunity. Priming of CD8 T cells by liver sinusoidal endothelial cells (LSEC) induces non-responsive CD8 T cells incapable of immediate effector function, but possess memory function in infectious immunity. We found Arl4d mRNA to be highly induced during CD8 T cell stimulation by LSEC. Using CD8 T cells from Arl4d-deficient mice we found the lack of Arl4d to result in the overproduction of IL-2. As the amount of IL-2 produced by naive CD8 T cells during antigen-specific interaction with LSEC is an important factor for the induction of non-responsive CD8 T cells, the action of Arl4d may be central to dampen IL-2 production and for subsequent abrogation of immediate effector function in naive CD8 T cells by LSEC.
Regulation of the early thymic presursor pool by the small GTPase Arl4d

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Members of the ADP-ribosylation factor (ARF) family of guanine-nucleotide-binding (G) proteins, including the ARF-like (ARL) proteins, regulate membrane traffic and organelle structure by recruiting cargo-sorting coat proteins, modulating membrane lipid composition and interacting with regulators of other G proteins. Arl4D is mainly localized to the plasma membrane and can modulate actin remodeling via regulating ARF6 activity. It has furthermore, recently, been implicated in the regulation of mitochondrial membrane potential.

Analyzing Arl4d-deficient mice, we observed that the thymus is greatly enlarged when Arl4d is absent. Although thymic cellularity was up to 3-fold increased, we did not observe major differences in the percentages of the double negative, double positive or single positive subsets within wild type and Arl4d-deficient thymocytes. We did find an increase in CD24<sup>int</sup>,CD117<sup>hig</sup> early thymic T cells (ETP) progenitors within the (Lin)<sup>low</sup>, CD25<sup>neg</sup>,CD44<sup>pos</sup> DN1 subset, which has been suggested to correlate with thymic cellularity. Together, these data implicate the small GTPase Arl4d to be involved in thymic homeostasis via the regulation of the size of the early thymic progenitor pool.
Quantitative analysis of receptor clustering by flow cytometry

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Question:
Transmembrane receptor activation results in many cases in receptor clustering and internalization. A quantitative description of this process can provide important information about basic signaling mechanisms and of disease states.

Methods:
In principle, cells are marked with fluorescently labeled antibodies, for instance Fab fragments, against surface receptors. Cross-linking and clustering are subsequently induced by receptor specific antibodies for the BCR and CD11b, or by bivalent cations (Mn²⁺) and phorbol myristoyl acetate (PMA) in the case of CD11b, respectively. Before clustering, the ratio of the time of flight of the fluorescence (TOF₉₅) - that is, the time of the fluorescent signal when a cell passes the laser beam in the flow cytometer - and the TOF of the cell body (TOF₉₅), is calculated and set as one. When the receptor clusters, the ratio TOF₉₅/TOF₉₅ should decline if the cell size does not change (Fig. 1). These changes can be monitored in real time by standard laboratory flow cytometers.

Results:
We established a facile flow cytometric real time method to quantify clustering of the murine B cell receptor (BCR) on murine B cells, and human CD11b on neutrophils. The anticipated decrease in the ratio between TOF₉₅ and TOF₉₅ could be found for both the BCR and CD11b after antibody induced cross-linking, and for CD11b after Mn²⁺ and PMA induced clustering. We revealed a dependence of this process on energy, as necrotic cells did not follow this relationship. We furthermore corroborated our results by immunofluorescence and by imaging flow cytometry.

Conclusion:
Here, we present a novel technique to quantify receptor clustering by standard flow cytometry. This method will help to gather real time information about basic signaling mechanisms involved in receptor clustering. Furthermore, this method will allow to monitor disease related receptor activation.

figure 1
 Friend virus (FV) infection of mice induces the expansion and activation of regulatory T cells (Treg) that dampen acute immune responses and promote the establishment and maintenance of chronic infection. Current results indicate that the responding Treg are Neuropilin+ natural “nTreg” rather than induced “iTreg” converted from conventional CD4+ T cells. High affinity, virus-specific CD4+ T cells were excluded from the Treg repertoire responding to FV infection. Analysis of Treg TCR VB chain usage revealed a broadly distributed polyclonal response, but with a disproportionate expansion of the VB5+ Treg subset. VB5+ Treg are specific for an endogenous retrovirus-encoded superantigen, and the VB5+ Treg displayed a unique phenotype of highly activated (CD43+, CD11a+), terminally differentiated (KLRG1+) effector T cells. The expansion of VB5+ Treg was more associated with the level of the CD8+ T cell response than the level of FV infection, and most interestingly, the expansion and accumulation of the VB5+ Treg was IL-2 independent, whereas the expansion of VB5+ Treg was. We could show that the production of IL2 by FV-specific conventional CD4 T cells was able to drive the expansion of VB5+ Treg. IL2 neutralization led to the abolishment of this expansion. A marker of interest is TNF receptor 2 (TNFR2), as it was recently described to be important for Treg development and suppressive function. Interestingly, the blockage of its ligand TNFα resulted in a substantial loss of the disproportionate expansion and activation of the VB5+ Treg subset, whereas the VB5+ Treg subset was not influenced. During FV infection CD8+ T cells produce significantly more TNFα, which could provide the vital signal for VB5+ Treg expansion. Thus the stimuli that drive VB5+ Treg expansion and marker expression during FV infection are different than the bulk population of Treg. To test for the biological importance of the VB5+ Treg subset, we depleted them with a monoclonal Antibody during infection. The results show better CD8+ T cell cytotoxic functions as well as increased cytokine production. This shows the biological importance of this VB5+ Treg subset and has important implications on further research in the Treg field, as the existence of different Treg subpopulations with distinct characteristics could be important for human diseases in which Treg play a role.
Setting an intrinsic brake on T cell division number: Roles for TCR affinity and IL-2 concentration

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Kinetic mechanisms mediate the selective expansion of high-affinity T-cells during an immune response. Such processes include changes in time to first division but not times through subsequent divisions, which are typically constant. The proportion of cells that survive through each division also alters with stimulation affinity and is thought to be a dominant form of affinity-based regulation. Here we investigate an additional mechanism where the number of divisions that a T cell undergoes following stimulation is ‘programmed’ and, as a consequence, higher affinity cells progress further and come to dominate the response.

We first quantified the effect of TCR-Ag affinity on T-cell division progression and survival by tracking proliferation of wild-type or Bim-deficient OT-I T-cells stimulated with a range of different affinity OVA peptides. We found high-affinity T-cells underwent greater expansion even when survival was enforced by Bim deficiency. This difference in expansion between affinities was traced to differences in the maximum number of divisions the T-cells were able to undergo before reverting to the quiescent state. We examined other T-cell stimuli and identified IL-2 as a potent modulator of the T-cell division limit, contributing towards selective expansion. By progressively subculturing cells in vitro, and applying mathematical modeling we determined a typical dose response between IL-2 concentration and the average division number that ranged from ~2 to over 12 divisions.

Although we cannot exclude a role for survival in mediating selective expansion, these results show that the programmed division capacity of T-cells plays an important survival-independent role in regulating selective expansion.
IL-35 is induced in human T cells upon co-stimulation via CD43 and PD-1

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Regulatory T cells (Treg) are not only crucial for the maintenance of immunological self-tolerance but are key mediators of infectious diseases. IL-35, a member of IL-12 family is an inhibitory cytokine associated with natural regulatory T cells (nTreg) in mouse models but not in humans. It is a heterodimer of EBV-induced gene 3 (EBI3) and p35 subunit of IL-12. Recently, we have shown that human rhinovirus (HRV) - a common cold associated pathogen, induces IL-35 producing Treg via up-regulation of B7-H1 (CD274) and sialoadhesin (CD169) on rhinovirus treated DCs (R-DC) as one of the immune evasion mechanisms. Thus, a combined set of co-stimulatory signals for T cells seems to be critical to induce IL-35. Therefore, by using a panel of plate-bound monoclonal antibodies (mAbs) against putative accessory T cell surface receptors, for T cell activation followed by qPCR, intracellular staining, sandwich ELISA and Luminex® assay for T cell signature cytokines, it has been so far shown that co-engagement of CD3 and CD43 along with PD-1 agonist mAb most potently induces IL-35 in human peripheral (PBT) as well as naïve cord blood (CBT) T cells. Furthermore, induction of IL-35 is not accompanied by specific T cell polarization but characterised by a low level T cell signature cytokine profile (IL-2, IL-4, IL-10, IL-17, IL-22, IFN-γ) expression compared to the other prominent T cell activation pathways. Unlike mouse counterparts, induction of IL-35 in human is not restricted to CD4+CD25+FOXP3+nTreg as co-stimulation via CD+CD43+PD-1 induces up regulation of EBI3 subunit in CD4+ as well as CD8+ peripheral blood T cells. The induction of IL-35 in T cells via CD3+CD43+PD-1 co-stimulation also seems to be independent of FOXP3 expression.

Though the primary effect of IL-35 is suppression of T cell proliferation, our data suggests that mechanism of suppression by such IL-35+T cells is rather dependent on the presence of antigen presenting cells (APC). Thus, our findings identify a novel pathway to induce immune-inhibitory T cells.

References:
Lymphocyte Activation

The human cationic amino acid transporter 1 (hCAT1) is essential for activation-induced arginine uptake and function of human T lymphocytes.

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Introduction:
The activation and efficient function of human T lymphocytes is strongly dependent on the presence of the semi-essential amino acid L-arginine. Under L-arginine-depleted conditions, as found in tumor microenvironment and sites of inflammation, it comes to a distinct loss of conventional T cell function. T cells arrest in the G0-G1 phase of the cell cycle in the absence of L-arginine, which results in the down-regulation of their proliferation. Furthermore, the secretion of INF-γ by human T cells is strongly decreased.

Objectives:
The dependence of T cells on extracellular L-arginine led us to the assumption that transmembranous transport of L-arginine must be crucial for human T cell function. We thus wondered which transporter may be responsible for the influx of L-arginine into activated human T lymphocytes.

Materials & methods:
Resting human primary T cells were highly purified by negative selection from healthy blood donors. The cells were stimulated with anti-CD3/anti-CD28 coupled beads. Expression of known arginine transporter mRNA was determined by quantitative Real Time PCR and proteins by Western Blot. Plasma membrane localisation of specific proteins was determined by biotinylation and consecutive Western Blot analysis. L-arginine transport was quantified by uptake of [3H]-L-arginine. Finally, siRNA technology was used in primary human T cells to study cellular functions in the absence of specifically downregulated proteins.

Results:
Our analysis revealed in activated T cells a strong induction of the expression of human cationic amino acid transporter 1 (hCAT1), whereas in unstimulated T cells almost no hCAT1 expression was detectable. Of note, hCAT1 expression was even higher in stimulated T cells under L-arginine-depleted conditions compared to physiological arginine availability. The cell membrane biotinylation analysis showed that the proportion of hCAT1 protein detected in the plasma membrane was comparable in cells stimulated in the absence or the presence of L-arginine. Quantification of [3H]-L-arginine uptake revealed a strong induction of L-arginine influx into stimulated T cells compared to unstimulated cells. Experiments with T lymphocytes, which were stimulated for 6, 24 and 48 hours, showed a positive correlation of L-arginine uptake and stimulation time. Transport was completely inhibited by the irreversible CAT-inhibitor N-ethylmaleimide, demonstrating that the L-arginine transport was indeed hCAT-mediated. To confirm our results, we performed L-arginine uptake experiments with T cells, in which hCAT1 expression / induction was efficiently suppressed. In these cells, activation-induced L-arginine uptake was severely reduced and T cell proliferation as well as IFN-γ secretion were partially downregulated.

Conclusion:
The amino acid transporter hCAT1 is primarily responsible for influx of L-arginine into activated human T lymphocytes and thus plays a crucial role in T cell function.
Lymphocyte Activation

Insight into the molecular basis of the unique T cell signaling induced by the CD28 superagonistic antibody TGN1412

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Introduction:
In 2006 the CD28 superagonistic antibody TGN1412 caused a severe and life-threatening cytokine storm during a phase I clinical trial. The TGN1412 incidence was unexpected, since the rodent precursors of TGN1412 had been shown to suppress autoimmune diseases by preferentially inducing an expansion of regulatory T cells (Treg).

Objectives:
Although some insights into the signaling signatures and functional properties of TGN1412 have meanwhile been provided, the molecular basis for its unique properties remains largely elusive.

Materials & Methods:
Primary human CD4+ T cells, CD28-deficient Jurkat T cell line JCH7C17; CD28 superagonist TGN1412, anti-CD3 antibodies OKT3 and UCHT1; Ion channel (BTP2, Margatoxin) and mitochondrial (CCCP, Ru360) inhibitors; 3H-thymidine incorporation-based proliferation assays, Western Blotting, ELISA, FACS analyses, wide-field and total internal reflection fluorescence (TIRF) microscopy.

Results:
We here report that in contrast to the situation in rodents, TGN1412 does not induce a preferential expansion of human Treg. Rather, conventional effector Th cells appear to primarily respond to TGN1412 stimulation. The low reactivity of human Treg towards TGN1412 does not result from selective expression of negative regulatory surface molecules such as Siglecs on this T cell subpopulation.

TGN1412 is known to induce a long-lasting and oscillating Ca2+ flux in human T cells whose molecular basis is until now unclear. By using specific ion channel blockers and inhibitors we show that TGN1412-induced Ca2+ flux involves the same ion channels that are activated upon CD3 stimulation. Furthermore, similar to CD3 stimulation, the Ca2+ flux appears to be regulated by mitochondria. In addition, TIRF microscopy of TGN1412 stimulated human T cells revealed that, similar to CD3 mAbs, TGN1412-stimulation leads to formation of microclusters in the plasma membrane containing the central Ca2+ regulating molecule SLP-76. However, the TGN1412-induced SLP-76-clusters remain immobile within the T cell membrane and do not show the centripetal movement, which rapidly occurs upon CD3/TCR-stimulation. The altered dynamics of the SLP-76-microcluster can be explained by a lack of TGN1412 to induce F-actin formation, a process that is essential for microcluster mobility. Hence, it appears that SLP-76 cannot be removed from the signaling machinery upon TGN1412 stimulation. This could represent one mechanism underlying the sustained Ca2+-signaling of TGN1412 stimulated human T cells.

To identify the regions within CD28 that mediate TGN1412 signaling, mutations were introduced into the cytoplasmic domain of human CD28. The CD28-mutants were expressed in the CD28-deficient Jurkat cell line JCH7C17 (which was also reconstituted with PTEN) followed by Ca2+ flux measurements. Surprisingly, these experiments revealed that, in addition to the YXNM-motif, TGN1412 signaling depends on the integrity of a signaling motif directly downstream of YXNM. Importantly, this motif has been reported to be dispensable for CD28 superagonistic IL2-production in rodents.

Conclusion:
Our data clearly show that CD28 superagonistic signaling is differentially organized in human vs. rodent T cells. In line with this assumption we observed that TGN1412 stimulation is absolutely dependent on the PI3K-pathways which has been reported to be dispensable for CD28 superagonistic signaling in mice and rats.
**Introduction:**
Signaling via the Ras-Erk cascade presides to the regulation of a variety of cellular processes such as proliferation, differentiation, migration or cell death. Recent progress has shown that variations in the duration of Erk activation determine the specificity of the signaling output and the consequent cellular outcome. In fact, in PC12 cells, transient Erk activation correlates with proliferation, whereas sustained Erk signals parallel with differentiation. We have recently shown that, in primary human T cells, transient Erk activity correlates with an anergic-like state, whereas sustained Erk signaling corresponds with proliferation. Upon ligation of the TCR, the activation of Ras is thought to be mediated via the action of two GEFs, RasGRP1 and Sos. Studies based on lymphoid cell lines and in silico simulations have indeed shown that Ras activation depends on the coordinated action of RasGRP1 and Sos. The current model postulates that the unusual interplay of RasGRP1 and Sos regulates full Ras-Erk activation and provides an intriguing mechanistic explanation on how rare antigenic peptide/MHC complexes induce productive T-cell responses. However, whether this model is valid in primary human T cells and how RasGRP1 and Sos contribute to transient vs. sustained Erk activation is not yet clear.

**Objectives:**
We have assessed the contribution of RasGRP1 and Sos to Ras-Erk activation upon TCR triggering in primary human T cells.

**Materials & methods:**
We used two different stimuli, CD3 mAbs cross-linked in solution (sAbs) or immobilized on microbeads (iAbs). sAbs induce a transient Erk signaling and unresponsive state, whereas iAbs induce sustained Erk activation and T-cell proliferation. In order to evaluate the role of Sos and RasGRP1 in transient vs. sustained Erk activation, we suppressed Sos1, Sos2 or RasGRP1 expression using RNA interference.

**Results:**
We found that suppression of RasGRP1 expression in human primary T cells led to attenuated Erk phosphorylation during both transient and sustained TCR-mediated signaling. Moreover, we demonstrated that suppression of RasGRP1 expression impaired T-cell activation and proliferation.

In contrast to RasGRP1, Sos1 is dispensable for the regulation of transient Erk activation. Conversely, we found that sustained Erk signaling depends also on Sos1. We further demonstrated that Sos1 is required for productive T-cell responses, as in addition to TCR-mediated Erk activation, Sos1 also regulates IL-2-mediated Erk phosphorylation and T-cell proliferation.

Additionally, we have shown that Sos2 is dispensable for T-cell activation.

**Conclusion:**
In summary, we have found that transient Erk activation does not depend on Sos1 but requires RasGRP1. Conversely, sustained Erk signaling and T-cell proliferation depend on both Sos1 and RasGRP1. Therefore, T cells appear to be endowed with a sophisticated set of GEFs for the fine-tuning of Erk dynamics, which in turn regulate cell-fate decisions.
T cell death: role of common gamma chain cytokines and Stat5/6 signaling in the regulation of Bcl-2-family proteins

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T cell death has been studied intensively over the past years. T cell death is driven pro- and anti-apoptotic Bcl-2-family proteins but the molecular regulation of this process remains elusive. Cytokines signaling through the common gamma chain (e.g. IL-2, -7, -15) are known to play a pivotal role in lymphocyte homeostasis. In this study, we investigated the mechanism of regulation of Bcl-2-family proteins by common gamma chain cytokines. Stimulation of naive T cells with these cytokines resulted in the induction of the main pro-apoptotic protein Bim. Quantitative RT-PCR data indicated significant induction of Bim on the mRNA level suggesting transcriptional regulation. Computer based analysis of the murine Bim promoter indicated 3 Stat5/6 binding sites. Chromatin immunoprecipitation assay revealed Bim as a transcriptional target of Stat5 in T cells. While common gamma chain cytokines thus induced the main pro-apoptotic protein, IL-15 stimulation also resulted in a striking increase in the levels of anti-apoptotic protein Mcl-1 and a moderate increase in Bcl-XL levels. Treatment with inhibitors of JAK/STAT- and PI3-kinase signaling resulted in blockade of Mcl-1 induction. Analysis of Mcl1 mRNA expression levels indicated that Mcl-1 regulation by IL-15 probably did not occur via-transcriptional or post-translational mechanisms. Preliminary data indicates a potential role of translational regulation in Mcl-1 up-regulation by IL-15. We propose a novel mechanism by which common gamma chain cytokines cause the up-regulation of both Bim and Mcl1 whereas the removal of these survival factors results in concomitant loss of Mcl-1, followed by Bim-mediated death of T cells.
T cell receptor-induced Warburg effect-like metabolic shift is linked to the generation of mitochondrial oxidative signal: an unexpected eukaryotic role of ADP-dependent glucokinase, an archaeal glycolytic enzyme

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Recently, we and others have shown that mitochondria-derived oxidative signals are crucial for T cell activation-induced gene expression [1-4]. Here, we report a novel molecular pathway connecting T cell receptor (TCR)-induced metabolic shift (closely resembling the Warburg effect) with increased mitochondrial reactive oxygen species (ROS) levels and enhanced NF-κB signaling [5].

We show that TCR-triggered activation of ADP-dependent glucokinase (ADPGK), a typically archaeal glycolytic enzyme, controls generation of the T cell activation-induced oxidative signal. Our data indicate, that TCR stimulation activates human ADPGK, an endoplasmic reticulum membrane-bound protein with an active site protruding towards the cytosol. In turn, the ADPGK-driven increase in glycolytic flux coincides with TCR-induced glucose uptake, downregulation of mitochondrial respiration, and re-direction of glycolysis towards mitochondrial glycerol-3-phosphate dehydrogenase (GPD) shuttle; i.e. the Warburg effect-like metabolic shift. TCR-induced increase in activity of mitochondrial respiratory-chain-associated GPD2 leads to hyperreduction of ubiquinone and, consequently, ROS release from mitochondria. This is paralleled by changes in mitochondrial bioenergetics and ultrastructure. Since the TCR-induced mitochondrial oxidative signal is indispensible for NF-κB-mediated gene expression, sh/siRNA-mediated downregulation of ADPGK and GPD2 abundance or ADPGK over-expression results in respective inhibition or enhancement of NF-κB induction and NF-κB-dependent gene expression.

In conclusion, we provide experimental evidence for an unexpected role for ADPGK as a novel regulator of T cell activation and describe an interplay of signaling and metabolic pathways resulting in mitochondrial ROS release and subsequent NF-κB-dependent gene expression.

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The classical concept of chemokine receptor signaling via PTX-sensitive heterotrimeric G proteins has recently been challenged by alternative models which propose a major role for either chemokine receptor homodimerization or binding of the adaptor protein ß-arrestin. In order to bypass ligand-mediated G protein activation we took advantage of a chemically inducible homo- and heterodimerization system by fusing FKBP12 (DmrA) and FRB (DmrC) binding domains to the C-terminus of either CCR5/CXCR4 or ß-arrestin2. Forced arrestin-receptor binding by the heterodimerizer AP21967 resulted in receptor desensitization, ERK1/2 activation and receptor internalization in the absence of chemokine stimulation. Interestingly, chemical-induced internalization and recycling routes of CCR5- and CXCR4-DmrA fusion proteins in HEK-293 cells differed significantly and resembled those of the two receptors after CCL5/CXCL12 stimulation, respectively. In contrast, forced homodimerization of CCR5 with AP20187 had no detectable functional consequences on its own, but facilitated CCL5-induced receptor endocytosis. These studies establish ß-arrestin as a major regulator of CCR5/CXCR4 signaling and endocytosis independent of heterotrimeric G proteins, while the functional significance of CCR5/CXCR4 receptor homodimerization is less clear.
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T cell activation induces local conformational changes of the SRC-family protein tyrosine kinase \( p56^{\delta{\alpha}} \) to induce its enzymatic activity

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Introduction:
The lymphocyte-specific Src-family protein tyrosine kinase \( p56^{\delta{\alpha}} \) is essential for T cell development and activation and hence adaptive immune responses. The mechanisms by which \( Lck \) induces phosphorylation of intracellular substrates upon activation via the T Cell Receptor (TCR) remain elusive.

Objectives:
We aimed at resolving the temporal and spatial conformational dynamics of \( p56^{\delta{\alpha}} \) within the activated T cell using a novel strategy of live cell imaging and the functional consequences for its enzymatic activities.

Materials and Methods:
We used widefield Fluorescence Lifetime Imaging Microscopy (FLIM) to assess activation-dependent spatio-temporal changes of \( p56^{\delta{\alpha}} \) conformation in living human T cells.

Results:
Kinetic analysis of fluorescence lifetime of a CFP- and YFP- tagged Lck-biosensors allowed us to directly visualize dynamic local opening of a 20% fraction of total \( Lck \) following \( \alpha CD3 \) - or superantigen-mediated activation of T cells. Parallel biochemical analysis of TCR-complexes revealed that the conformational changes of \( Lck \) correlate with induction of \( Lck \) enzymatic activity.

Conclusion:
Our data record for the first time a dynamic, local activation of the Src family kinase \( Lck \) at sites of TCR-engagement.
Semaphorin 3A increases the immunogenicity of tumour cells in an allogeneic and autologous setting

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Class II transactivator (CIITA) is the master regulator of HLA class II expression. Previously, we have shown that semaphorin 3A (Sema3A) increases the expression of CIITA in several cell types. Now we investigated if this Sema3A-mediated effect can be applied to increase the immunogenicity of tumour cells. For this purpose, Sema3A-stimulated tumour cell lines (KE-37 or K562 cells) and primary tumour cells derived from human multiple myeloma patients were used to stimulate T-cells in an allogeneic and autologous setting, respectively. Non-stimulated cells were used as control. In addition, T-cell cytotoxic assays were performed using Sema3A-stimulated versus non-stimulated tumour target cells. Also, the levels of Granzyme B transcripts produced by T-cells were measured. T-cell cytotoxicity rates were correlated to the Sema3A-induced CIITA and HLA-DR expression levels.

Sema3A stimulated KE-37 showed a significant increase by up to 2.3-fold in CIITA mRNA levels, which led to a significant increase on HLA-DR protein expression by up to 27% in comparison to non-stimulated KE-37 cells. HLA-DR upregulation caused an increase in T-cell mediated cytotoxicity against primary tumour cells (increase of 130%, p<0.001 for CD3 T cells and 80%, p<0.001 for CD4 T cells). A similar increase in Granzyme B mRNA level was observed. Also, a 2.5-fold increase in T-cell cytotoxicity when Sema3A-stimulated K562 cells were used as targets cells was observed. We observed a 2.8-fold (p<0.001) increase in the expression of HLA-DR on primary multiple myeloma cells upon Sema3A stimulation and obtained an increase of 2.6-fold increase in cytotoxicity (p<0.05) when using autologous CD3 T cells.

This data suggests that Sema3A is capable of increasing the immunogenicity of tumour cells in an allogeneic and autologous setting. This mechanism might be used to identify new tumour associated antigens useful for targeted cellular therapies for hematologic disorders.
Octamer-dependent transcription in T cells is mediated by NFAT and NF-κB

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The transcriptional co-activator BOB.1/OBF.1 was originally identified in B cells and is constitutively expressed throughout B cell development. BOB.1/OBF.1 associates with the transcription factors Oct1 and Oct2, thereby enhancing octamer-dependent transcription. In contrast, in T cells, BOB.1/OBF.1 expression is inducible by treatment of cells with PMA/Ionomycin or by antigen receptor engagement, indicating a marked difference in the regulation of BOB.1/OBF.1 expression in B versus T cells. The molecular mechanisms underlying the differential expression of BOB.1/OBF.1 in T and B cells remain largely unknown. Therefore, our present study focuses on mechanisms controlling the transcriptional regulation of BOB.1/OBF.1 and Oct2 in T cells. We show that both calcineurin- and NF-κB-inhibitors efficiently attenuate the expression of BOB.1/OBF.1 and Oct2 in T cells. In silico analyses of the BOB.1/OBF.1 promoter revealed the presence of previously unappreciated combined NFAT/NF-κB sites. An array of genetic and biochemical analyses illustrates the involvement of the Ca²⁺/calmodulin-dependent phosphatase calcineurin as well as NFAT and NF-κB transcription factors in the transcriptional regulation of octamer-dependent transcription in T cells. Conclusively, impaired expression of BOB.1/OBF.1 and Oct2 and therefore a hampered octamer-dependent transcription may participate in T cell-mediated immunodeficiency caused by the deletion of NFAT or NF-κB transcription factors.
Skewing of SAG mediated therapy for a predominant Th1 during Visceral Leishmaniasis on triggering CD2 epitope

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Background:
Visceral leishmaniasis is a macrophage associated disorder which is linked with a profound decrease in the immunotherapeutic potential of the infected subjects leading to a marked reduction in the CD4 linked Th1 protective immune response. It greatly affects the liver leading to abnormal levels of SGPT and SGOT. Also the patients suffering from VL have been reported to be coinfected with Hepatitis C during some circumstances. Simultaneously the patients in Bihar are showing unresponsiveness towards SAG which is still a first line of drug in many countries around the world against Visceral Leishmaniasis. We have previously reported down regulation of CD2 co receptor on the surface of CD4 cells in patients suffering from Visceral Leishmaniasis. Stimulation of CD2 epitope with antiCD2 antibody has led to a remarkable increase in the Protein kinase C alpha mediated phosphorylation on CD2 co receptor on CD4 T cells, induction of IFN-γ led Th1 dominated immune response, a substantial increase in the lymphoblast population and this response remained Th1 dominated even in the presence of Th2 predominant conditions signified with rIL4. Studies in the 1980s showed that biological immunomodulators such as interferon (IFN)-γ can provide a missing signal and enhance the activity of antimonials in the treatment of VL and CL.

Methodology/Principal:
Findings In the present part of the study we have tried to evaluate the use of CD2 antibody as an immunotherapeutic agent along with SAG in ensuring treatment of BALB/c mice induced with experimental Visceral leishmaniasis. It has been found in the present set of studies that stimulation of CD2 co receptor along with along with therapeutic dose of SAG has led to the enhancement in the release of IFN-gamma which leads to the release of TNF-alpha and activates the macrophages. An increase in the NO mediated killing further observed by the activated macrophages leading to the reduction in the parasitic load.

Conclusions/Significance:
The results indicate that enhancing the immune potential of a VL patient will help in the better response of Sodium Antimony Gluconate which is the first line of drug against VL in many countries.
Chemokine receptor CCR7 on CD4+ T cells plays a crucial role in the induction of experimental autoimmune encephalomyelitis

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Purpose/Objective: Experimental autoimmune encephalomyelitis (EAE) is the animal model for the human disease multiple sclerosis. EAE is mediated by myelin-specific CD4+ helper T cells. The chemokine receptor CCR7 is an important factor for immune cell trafficking and recirculation not only in the secondary lymphoid organs, but also within target organs of an inflammatory attack. Previous data suggested that CCR7 deficiency influenced clinical disease by altering dendritic cell biology.

Materials and Methods: Using different animal models for multiple sclerosis and in vivo imaging technique within the CNS, we investigated the role of T cells in animals which lack the CCR7 on T cells.

Results: We demonstrate here that CD4+ T cell-specific constitutive deletion of CCR7 led to an impaired clinical course in EAE. Lymphopenic RAG-/-yc-/- mice were replenished with CD4 T cells of CCR7-deficient or wildtype donor mice. Active EAE was induced by subcutaneous immunization with MOG35-55 in complete Freund’s adjuvant. CCR7 deficiency on CD4+ cells led to a delayed disease onset in active EAE. Within the CNS CD4+ cells lacking CCR7 showed a lower potential to produce inflammatory cytokines. Two-photon laser scanning microscopy revealed a lower number of CCR7-deficient CD4+ T cells with antigen recognition motility compared to wild-type CD4+ T cells in inflammatory CNS lesions. Thus, deficiency of CCR7 on CD4+ T cells results in lower numbers of encephalitogenic T cells. This could be attributed to impaired T cell priming in secondary lymphoid organs, which most likely resulted from reduced lymph node homing potential. In adoptive transfer EAE, mice receiving CCR7-/- myelin antigen T cell receptor transgenic 2d2 TH17 cells showed similar to slightly earlier disease onset compared to mice adoptively transferred with CCR7+/+ 2d2 TH17 cells. Thus CCR7-deficiency on TH17 cells did not reduce encephalitogenicity in adoptive transfer EAE. We monitored the trafficking of CCR7-/- and CCR7+/+ 2d2 TH17 cells within the CNS by two-photon laser scanning microscopy in living anaesthetized mice and found similar activation motility of CCR7 deficient and wildtype CD4+ T cells.

Conclusions: Taken together these findings underline a crucial role of CCR7 in neuroinflammation in particular in the secondary lymphoid organs during the priming of autoimmune CD4+ T cells.

No conflict of interest
A reducing milieu renders cofilin insensitive to PIP$_2$ inhibition

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Question:
Oxidative stress can lead to T-cell hyporesponsiveness. A reducing micromilieu (e.g. provided by dendritic cells) can rescue T-cells from such oxidant-induced dysfunction. However, reducing effects on proteins leading to restored T-cell activation remained unknown. One key molecule of T-cell activation is the actin-remodeling protein cofilin, which is essential for formation of mature immune synapses between T-cells and antigen-presenting cells. Cofilin is spatio-temporally regulated. At the plasma membrane it can be inhibited by PIP2. Here, we asked whether the structure and function of cofilin are influenced by a reducing milieu.

Methods:
NMR spectroscopy - Actin depolymerization assay - In-flow microscopy - flow cytometry

Results:
We show by NMR spectroscopy that a reducing milieu leads to structural changes in the cofilin molecule predominantly located on the protein surface. They overlap with the PIP2, but not the actin-binding sites. Accordingly, reduction of cofilin has no effect on its F-actin binding and depolymerization. However, it does prevent inhibition of cofilin activity through PIP2. Thereby, a reducing milieu may generate an additional pool of active cofilin at the plasma membrane. Consistently, in-flow-microscopy revealed increased actin dynamics in the immune synapse of untransformed human T-cells under reducing conditions.

Conclusions:
We introduce a novel mechanism of redox regulation: Reduction of the actin remodeling protein cofilin renders it insensitive to PIP2 inhibition resulting in enhanced actin dynamics.
Development of a bispecific antibody for targeted immunotherapy of solid tumors expressing the tight-junction protein CLDN6

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Question:
Recombinant bispecific antibodies are potent anti-cancer drugs that redirect effector cells to specifically lyse tumor cells. The validity of this approach and its underlying bispecific molecular format has been shown in various proof-of-concept studies up to clinical phase II trials. Despite of impressive advances in the past, there is still a high unmet medical need for the treatment of solid cancers. Amongst others this is due to the only small number of addressable tumor specific markers which might enable the efficient and site-directed eradication of the malignant tissue. To overcome this problem, we selected CLDN6 as a target molecule for our studies. The expression of this antigen is highly tumor-specific, except for the expression in human placenta and during embryogenesis. CLDN6 positive tumor entities comprise ovarian, bladder, lung, gastric, pancreatic, breast, hepatic and other solid cancers.

Methods:
We constructed a bispecific single chain molecule or short bi-(scFv), recognizing simultaneously the T cell antigen CD3 and the tumor antigen CLDN6. The potency of the bi-(scFv), was assessed by co-incubating CLDN6-expressing tumor cells with human T cells. Tumor cell lysis, T cell activation, proliferation and cytokine release were monitored by in vitro assays. Further, we tested the therapeutic efficacy of the CLDN6-specific bi-(scFv), in a tumor mouse model. Therefore, the bi-(scFv), was administered to immunodeficient mice engrafted with human PBMCs and carrying a subcutaneous tumor generated by human carcinoma cells that endogenously expressed CLDN6.

Results:
EC50 values in the picomolar range could be determined for the CLDN6-specific bi-(scFv), namely 6PHU3. Significant T cell activation started at a concentration of 0.1 ng/ml 6PHU3 and a total T cell activation of up to 75 % was achieved with 1000 ng/ml 6PHU3 after 48 hours of assay incubation. In the presence of target cells and 1 ng/ml 6PHU3, 80 % of T cells responded with proliferation when incubated for 96 hours. Moreover, the T cell cytokine release mediated by 6PHU3 could be shown to be strictly target dependent. In the xenograft mouse model mice were treated daily or 3-times per week with 5 µg 6PHU3 per intraperitoneal injection. Tumors were eradicated or growth arrested by 6PHU3 treatment and survival of mice was significantly prolonged. Redirection of T cells to the tumor was shown to be selectively mediated by 6PHU3.

Conclusions:
Our results show the in vitro and in vivo potency of the CLDN6-specific bi-(scFv),. Combination of the bispecific antibody concept with the selective targeting of this oncofetal tumor marker appears to be a promising strategy for the therapeutic treatment of solid tumors.
Quantification and immune sensing of 8-Hydroxy-guanosine in RNA

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Introduction:
RNA sequence, structure or nucleoside modifications are crucial features for RNA's to be recognized via PRR's. Over 100 different RNA modifications have been identified and exist in all three kingdoms of life. RNA modifications may occur by oxidation through reactive oxygen species (ROS). Over 20 different purine and pyrimidine modifications formed by ROS are known, however 8-hydroxyguanosine (8-OHG) is the most prominent modification. This modification in mRNA leads to reduced protein levels and altered protein function due to ribosome stalling. Interestingly, age-associated oxidative damage to RNA has been demonstrated in neurons and may play a role in neurodegeneration and other diseases. The innate immune system has many different functions which in general can be divided into two major tasks. On the one hand is the primary effector defense line in fighting pathogens whereas on the other hand it initiates the immunological response of the adaptive effector part of the immune system. Pattern recognition receptors (PRR's) are crucial for the activation of lymphocytes. For the detection of patterns in the RNA world the endosomal TLR 3, 7 or 8 and the cytoplasmatic receptors RIG-I, MDA5 as well as the inflammasome are relevant receptors. The influence of ribonucleoside modification on immune activation is important for understanding mechanisms of RNA-induced activation or immune evasion. Therefore we established methods to insert 8-OHG modifications into RNA based on Fenton reaction or in vitro transcription and developed 8-OHG quantitative detection protocols using HPLC and ELISA.

Objectives:
In this study we developed suitable quantification methods of 8-OHG and specified the immunogenity of oxidized guanosines within naturally occurring RNA sequences.

Materials and Methods:
For cell stimulation we used synthetic RNA40 carrying an 8-OHG either at position 1 or 9 and in vitro synthesized RNA's with random 8-OHG modifications by adding 8-OHG triphosphate to the reaction mixture. To introduce oxidized guanosines within existing RNA's we used fenton chemistry. RNA40 with phosphodiester bond was used as positive control whereas RNA41 with phosphothioate backbone served as negative control. RNA was complexed with DOTAP prior to stimulation. Human PBMC's and monocytes derived from buffy coats as well as murine macrophages and Flt-3 differentiated DC's derived from bone marrow of wt, TLR 3, -7 and 3, 7 double ko Bl-6 mice were used for stimulation. The cytokine release was measured by IFNa, IL-6 and IL-1ß ELISA.

Results:
Electrophoresis showed unaffected RNA integrity of the modified oligos. Introduction of 8-OHG could be successfully determined by HPLC and ELISA. Oxidized guanosines in naturally occurring RNA sequences lowered IL-6 and IL-1ß production in human PBMC's to 50%. No differences were detected in human monocytes and in murine immune cells.

Conclusions:
Oxidized RNA can inhibit an inflammatory reaction. The responsible receptors for this effect may be TLR8 or the NALP3-Inflammasome. Furthermore other yet unknown receptors or orphan receptors (e.g. TLR10) may be responsible for this effect. Further research is necessary to investigate the involved receptors and pathways and the biological relevance in disorders like Alzheimer or liver diseases.
CD4+CD25 T cell differentiation towards regulatory T cells by demethylating agents 5-Aza-2'-deoxycytidine and EGCG

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Introduction:
CD4+ regulatory T cells (Treg cells) are essential players in the adaptive immune system. Adoptive transfer of in vitro generated Treg cells might be a therapeutic option in the future, e.g. in Graft versus Host disease. Besides differentiating into Treg cells, naive CD4+CD25 T cells can differentiate into TH1-, TH2- and TH17 T cells. The different T cells subsets are characterized by the expression of specific master transcription factors, which is Foxp3 for Treg cells, Tbet for TH1- cells, GATA3 for TH2-cells and RORγT for TH17 cells. Plasticity between the different T cell subsets has been discussed during the last years. For stability of Treg cells, epigenetic status of DNA methylation within FOXP3-TSDR (Treg specific demethylated region) has been shown to be essential.

Objectives:
The two DNA methyltransferase (DNMT) inhibitors, 5-Aza-2'-deoxycytidine (5-AZA), which is used for the treatment of myelodysplastic syndrome, and epigallocatechin-3-gallate (EGCG), a main component of green tea, were analyzed for their potency to differentiate CD4+CD25 T cells into regulatory T cells.

Materials & Methods:
FACS sorted human CD4+CD25- cells were activated with anti-CD3/CD28 antibodies and IL-2 and cultured in RPMI1640 Medium with 5-AZA or EGCG for up to seven days. Cells were characterized by flow cytometry for expression of Treg cell specific proteins or were subjected to DNA- and RNA-extraction for RNA-expression (qRT-PCR & gene array) or DNA-methylation analysis.

Results:
5-AZA and EGCG both induce a global DNA demethylation in the cultured cells, but only 5-AZA induces significant DNA demethylation of FOXP3 TSDR. A significant up-regulation of Foxp3 expression on RNA- and protein level was detected only in 5-Aza cultured cells. Other specific Treg cell molecules were also up-regulated by 5-AZA in contrast to EGCG. 5-AZA cultured cells showed lower proliferative capacity compared to untreated or EGCG cultured cells. Nevertheless, cultivation with 5-AZA did not induce complete Treg cell phenotype, as revealed by RNA-microarray and RNA expression analysis by quantitative RT-PCR.

Conclusion:
Although 5-AZA promotes differentiation of CD4+CD25 T cells towards a Treg cell phenotype regarding the epigenetic methylation profile of FOXP3-TSDR, expression of its master transcription factor Foxp3 and other Treg specific proteins, further manipulations are necessary for generating Treg cells before being used for adoptive transfer therapy.
Enhanced-Evasion Cytomegalovirus Reveals a Major Contribution of Direct Antigen Presentation to Antiviral CD8 T-Cell Priming

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The control of cytomegalovirus (CMV) infection by CD8 T cells depends on an efficient presentation of viral peptides at the cell surface. Virus-encoded immunoevasins subvert recognition of infected cells by inhibiting the cell surface transport of peptide-loaded MHC-I (pMHC-I) complexes. Yet, it is current opinion that antiviral CD8 T-cell priming occurs predominantly through cross-presentation of antigenic material endocytosed by uninfected professional antigen-presenting cells (profAPC, mainly CD8⁺DCs) and is therefore independent of viral immunoevasins.

We present here data demonstrating that murine CMV (mCMV) induces a broad antiviral CD8 T-cell response also in mice deficient in the canonical cross-presentation pathway, which indicated direct antigen presentation is possible in infected profAPC despite the expression of immunoevasins. To evaluate the primary mode of antigen presentation in mice competent for both presentation pathways, we studied the CD8 T-cell response against a recombinant ‘enhanced-evasion’ mCMV that expresses the most efficient mCMV-encoded immunoevasin m152/gp40 with accelerated kinetics, resulting in a strong inhibition of pMHC-I presentation. Notably, the enhanced immune evasion resulted in a reduced CD8 T-cell priming in spite of increased viral gene expression in the draining regional lymph node (RLN), compared to mCMV-wildtype infection. This finding clearly identified direct antigen presentation as the primary mode of CD8 T-cell priming. Paradoxically, however, enhanced direct antigen presentation after infection with the m152 gene deletion mutant mCMV-Δm152 was also associated with a curtailed CD8 T-cell priming. This ‘immune evasion paradox’ is explained by a negative feedback regulation exerted by early-primed CD8 T cells that restrict gene expression and spread of the mutant virus in the RLN, thus limiting the number of profAPC available for direct presentation. We conclude that inverse mechanisms can lead to an inhibition of CD8 T-cell priming, either diminished direct antigen presentation by enhanced expression of immunoevasins or diminished viral gene expression and spread caused by an early CD8 T-cell control in absence of immunoevasins. In summary, we show here that antiviral CD8 T-cell priming in the RLN is predominantly driven by direct antigen presentation despite the presence of viral immune evasion molecules.
The activation-induced expression of human cationic amino acid transporter 1 (hCAT-1) is regulated by signaling via PI3K, Akt and PKC in human T lymphocytes.

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Introduction:
Arginine uptake is dramatically increased upon T cell stimulation via the T cell receptor + costimulation and availability of the amino acid arginine is crucial for full activation of human T lymphocytes. Arginine becomes a limiting nutrient factor under conditions of cancer inflammation in the tumor microenvironment due to myeloid cell arginase-mediated arginine hydrolysis. Human T cells specifically upregulate the human cationic amino acid transporter 1 (hCAT-1) in order to mediate the activation-induced increase of transmembranous arginine uptake.

Objectives:
Here, we wanted to clarify the intracellular signaling pathways that mediate hCAT-1 induction upon activation of human T lymphocytes.

Materials & methods:
Human primary T cells were highly purified by negative selection from healthy blood donors and stimulated with anti-CD3 / anti-CD28 coupled microbeads. Expression of hCAT-1 mRNA was determined by quantitative Real Time PCR and hCAT-1 proteins as well as different signal transduction proteins by Western Blot. Phosphorylation-dependent activation of the various signaling proteins was determined by phospho-specific antibodies. T cell proliferation was determined by [³H]thymidine incorporation, IFN-γ secretion was measured by ELISA. The following pharmacological inhibitors were used to suppress different signal transduction pathways upon T cell activation: Ly294002 and wortmannin (phosphatidylinositol-3-kinase, PI3K), Akt inhibitor VIII (protein kinase B / Akt), rapamycin (mammalian target of rapamycin complex 1, mTORC1), OSI-027 (mTORC1/mTORC2), CGP57380 (Mnk1), Gö6976 and Gö6983 (protein kinase C, PKC).

Results:
Direct pharmacological inhibition of Akt by the specific Akt inhibitor VIII not only completely inhibits T cell proliferation and IFN-γ secretion, but also leads to a total suppression of inducible hCAT-1 induction in human T cells. The upstream PI3K inhibitors Ly294002 and wortmannin diminish the phosphorylation of Akt on Ser473 and Thr308 and suppress T cell proliferation and cytokine synthesis efficiently. Concurrently, this is associated with a partial reduction of hCAT-1 expression upon T cell activation. Similarly, pharmacological PKC inhibition leads to a partial loss of hCAT-1 induction, associated with a profound functional T cell inhibition. In contrast, inhibition of the ERK-Mnk1 pathway or the mTORC1 +/- mTORC2 signaling modules significantly suppresses T cell proliferation and / or cytokine synthesis while no influence on activation-induced hCAT-1 mRNA or protein was detectable.

Conclusion:
Activation-triggered induction of the amino acid transporter hCAT-1 in human T lymphocytes is mediated by PI3K, Akt and PKC. While efficient upregulation of hCAT-1 seems to be a prerequisite for full functional T cell activation, this is clearly not an all-or-none process and certain functional aspects of T cell activation are associated with specific signaling modules.
Somatic or germline mutations in \textit{PTPN11}, which encodes the nonreceptor tyrosine phosphatase SHP2, are the most frequent cause of juvenile myelomonocytic leukemia or the Noonan syndrome. Since both disorders are characterized by autonomous cell proliferation, we intended to analyze T cell activation and termination in the presence of a point mutation that can be detected in both disorders. Therefore, we crossed knock in mice that conditionally express the mutant \textit{PTPN11}^{D61Y} with CD4Cre mice, generating mice that express the mutant \textit{PTPN11}^{D61Y} only in CD4+ T cells. Our results show a pronounced splenomegaly with increased CD4+ T cell numbers in the spleen of \textit{PTPN11}^{D61Y} mice compared to control mice. Interestingly, autonomous proliferating cells can be separated from spleens of \textit{PTPN11}^{D61Y} mice which display adherent growth and are positive for the cellular markers CD3, CD4 and CD44. Furthermore, tumors next to the kidney and the intestine could be detected in aged mice (> one year) bearing the \textit{PTPN11}^{D61Y} mutation. Analyzing signalling of T cells derived from spleens of \textit{PTPN11}^{D61Y} mice revealed a decreased phosphorylation of the extracellular signal-regulated kinase (ERK1/2) after stimulation with CD3/CD28 compared to control mice. We are currently investigating alternative TCR-dependent activation cascades, which might be responsible for the activation and proliferation of CD4+ T cells in the presence of the \textit{PTPN11}^{D61Y} mutation.
Selective elimination of CLDN18.2 positive carcinoma cells by a T cell activating bispecific antibody

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Question:
Multiple immune evasion mechanisms of cancer cells hinder conventional cancer immunotherapies to completely eradicate resisting malignant cells. Therefore, improved approaches are required to overcome the limitations of existing immunotherapies. Bispecific tandem single-chain antibodies (bsAb) are potent anti-cancer drugs enabling the patient’s immune system to fight cancer cells. The potency of this approach has already been confirmed in clinical trials (Boder und Jiang 2011). Therefore we developed a bsAb targeting the tumor-associated antigen CLDN18.2. The efficacy of this molecule was investigated in in vitro and in vivo experiments.

Method:
The bispecific antibody used in this proof of concept study - termed 1BiMAB - is a tailor-made recombinant antibody fragment, which consists of two fused single-chain variable fragments recognizing different antigens. Within 1BiMAB, these two individual binding sites are arranged in a tandem-wise manner on a single peptide chain and are separated by a linker. The format comprises a T cell-specific scFv targeting the CD3 epsilon chain of the T cell receptor complex, whereas the second specificity binds to the tumor-associated antigen CLDN18.2. It has been shown that CLDN18.2 is presented almost exclusively on the surface of 70 % of defined entities of cancer e.g. gastric, esophageal, pancreatic, lung, non-small cell lung cancer (NSCLC), breast, ovarian, colon, hepatic, head-neck cancer as well as on cancer of the gallbladder (Sahin et al. 2008).

Results:
The main goal of this approach is the specific recruitment of effector T cells to the tumor tissue. A physical linkage of T cells to TAA-expressing tumor cells is hereby mediated by the bsAb. Simultaneous binding of the bsAb to CLDN18.2 and CD3 induces then the clustering of CD3-receptor complexes on the surface of T cells resulting in their activation. Consequently, T cells up-regulate and release their cytotoxic enzymes thereby mediating lysis of the tumor cell. In our in vitro study we could indeed show that 1BiMAB redirects the effector functions of the T cells towards the tumor cells. This could be demonstrated by strong induction of T cell activation and proliferation as well as up-regulation of cytolytic proteins only in the presence of CLDN18.2 expressing tumor cells and 1BiMAB in a concentration dependent manner. Effective tumor cell lysis with EC50 values in the picomolar ranges could be reached. Furthermore, the therapeutic efficacy of 1BiMAB was investigated in a xenograft tumor mouse model. Therefore, immunodeficient mice engrafted with human PBMCs and carrying a subcutaneous tumor generated by HEK293 cells stably expressing CLDN18.2 were treated daily with 5 µg 1BiMAB for 22 days. Consequently, mice treated with 1BiMAB and engrafted T cells showed a significantly higher survival (p< 0.0022) than the control groups.

Conclusion:
Taken together, our results demonstrate that simultaneous targeting of CLDN18.2 and CD3 induces potent T cell-mediated tumor cell elimination. 1BiMAB is able to activate T cells independent of signals, which are usually involved in the T cell recognition process such as antigen presentation, MHC restriction, and co-stimulatory effector molecules (Travers et al. 2008). Therefore, 1BiMAB will potentially enable to overcome several immune evasion mechanisms of tumor cells consequently supporting current solid cancer therapies.
Isolation of CD4+ T lymphocytes for the unbiased analysis of their gene expression profiles

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Biomarkers are important tools for diagnosis of immune-mediated diseases, their prognosis and the prediction of response to therapy. Peripheral blood has been pursued as a minimally invasive and inexpensive source for cells, whose gene expression profile is expected to reflect systemic and local pathogenesis. Microarray analysis of cell-type specific transcriptomes, however, requires isolation of defined cells to high purity, without affecting their gene expression. Here, we analyzed the influence of different preparatory techniques prior to cytometric cell sorting on gene expression of human peripheral CD3+CD4+CD45RO+ T lymphocytes.

Procedures included the use of erythrocyte lysis buffer, density gradient centrifugation, direct and indirect enrichment by magnetic bead assay as well as the addition of the transcriptional blocker ActinomycinD. Additionally, one sample was intentionally activated, using ionomycin and phorbol myristate acetate (PMA). Total RNA was extracted from cells after cytometric high purity cell sort and microarray analysis of the transcriptomes was performed using Affymetrix Human Genome U133 Plus arrays.

The transcriptom analysis revealed the gene expression pattern of CD4+CD45RO+ T helper to be severely impacted by the duration and conditions of storage of the samples between venipuncture and cell isolation. When cells were isolated from fresh blood, the gene expression pattern was maintained through the isolation procedures tested, allowing for flexibility where peripheral blood is used as a reverence for diseased or otherwise abnormal tissue.
The soluble cytoplasmic tail of CD45 (ct-CD45) induces a non-canonical form of anergy in human T cells

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Question:
The cytoplasmic tail of CD45 (ct-CD45) is proteolytically cleaved and released upon activation of human phagocytes. The soluble ct-CD45 was found to act on T cells as an inhibitory, cytokine-like factor that reduces T cell proliferation. In this study, we aimed to elucidate the molecular mechanisms acting within T cells, upon ct-CD45 binding.

Methods:
Human primary T cells were stimulated in vitro via plate-bound antibodies in the presence of immobilized ct-CD45. The cells were analyzed for their cytokine release and proliferative capacity. Gene expression profiling was performed via microarrays and qPCR.

Results:
Here, we demonstrate that ct-CD45 induces a novel form of anergy in human peripheral blood T cells. Ct-CD45 inhibited the proliferation of purified CD4+ as well as of CD8+ T cells, the cytokine production (IL-4, IFN-γ, IL-10, IL-17, IL-13) and the induction of typical T cell activation markers (CD25, CD69). Co-stimulation via CD28 or CD63 failed to prevent this inhibitory signal. Moreover, we found that T cells activated via CD3/CD28 or CD3/CD63 in the presence of ct-CD45 failed to proliferate in response to restimulation which was reversible by the addition of exogenous IL-2 or IL-7. Recent studies have clearly demonstrated that such a hypo-proliferative or anergic state in T cells is not a simple loss of signaling molecules, but an active process where “anergy factors” are being induced and synthesized to establish and maintain the unresponsive state. Classical anergy-associated genes encode, among other proteins, transcription factors (early growth response protein 2 and 3, EGR2, EGR3), E3 ubiquitin ligases (e.g. Casitas B cell lymphoma b, CBL-b), and diacylglycerol kinases (e.g. DGK-α). However, when we analyzed the gene expression profile of ct-CD45-induced anergic T cells we did not observe induction of any of these anergy factors. Characterizing the expression patterns of cell cycle regulatory factors, we found inhibition in the induction of cyclin D1 while other cyclins were unaltered.

Conclusions:
Ct-CD45 triggers an anergy program in T cells which is reversible by exogenous IL-2, acting independently of classical anergy factors. From our data, the inhibition of cyclin D1 suggests a cell cycle arrest in the early G1 phase, thus making it distinct from canonical T cell anergy.
The cold-shock protein YB-1 controls T cell proliferation

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The cold-shock protein YB-1 is an oncogenic transcription/translation factor highly expressed in tumor cells of breast, ovarian, and lung cancer. It correlates with their increased cell survival, proliferation, and migration. Its enhanced expression of mRNA and its localization within the nucleus has been shown to correlate with poor prognosis for breast cancer patients.

Although YB-1 plays a central role in tumor etiopathogenesis its role in T cell leukemia and T cell responses is not understood, yet. In this study we analyzed the function of YB-1 in peripheral and malignant CD4\textsuperscript{+} T cells, and its contribution to the pathogenesis of T cell leukemia.

CD4\textsuperscript{+} T cells isolated from PBMCs or the T-ALL cell line Jurkat were stimulated with either anti-CD3 and isotype or anti-CD3 and anti-CD28 coupled sulfate latex beads at a ratio of 1:1. Subsequently, subcellular fractions were analyzed by Western blot, mRNA by real-time PCR, and expression of surface receptors, intracellular and nuclear located molecules by flow cytometry. YB-1 was silenced by lentiviral transduction of YB-1-shRNA or siRNA transfection. Nuclear YB-1 content of T cells isolated from bone marrow biopsies and blood samples from T-ALL patients were analyzed by flow cytometry and fluorescence microscopy.

Here we demonstrate that the YB-1 protein was unambiguously expressed in naive and effector/memory human CD4\textsuperscript{+} T cells. In contrast to CD4\textsuperscript{+} T cells, the T-ALL cell line Jurkat showed an enhanced localization of YB-1 in the nucleus independently of stimulation. Upon stimulation of human CD4\textsuperscript{+} T cells with anti-CD3 and anti-CD28, YB-1 protein was highly expressed in the cytoplasm and the nucleus of T cells 16h after initial stimulation. The accumulation of YB-1 within the nucleus was inhibited in stimulated CD4\textsuperscript{+} T cells when RSK1/2 was inactivated with a specific inhibitor and to a lesser extent when Akt and PKCa were inactivated. YB-1 S\textsuperscript{102} phosphorylation mediated by the MEK-ERK-RSK signalling pathway was identified as a prerequisite for YB-1 translocation into the nucleus. ShRNA/siRNA-mediated knockdown of YB-1 resulted in abrogated proliferation and increased apoptosis of CD4\textsuperscript{+} T cells and Jurkat cells that was not rescued by IL-2. Analysis of nuclear YB-1 content in isolated nuclei of T cells from T-ALL patients was enhanced compared to healthy controls and to T-ALL Jurkat cells.

Altogether, our data demonstrate that YB-1 is tightly controlled in T cells by co-stimulation and is centrally involved in cell cycle progression of T cells.

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Cytotoxic T lymphocyte-associated molecule-4 (CTLA-4, CD152) is shown to be the primary attenuator of T cell responses. CTLA-4 is expressed 1-2 days after beginning of the T cell stimulation, when the primary activation program is already initiated. This implies that CTLA-4 mediated down-regulation of T cell responses is an active process rather than a sole ablation of augmenting signals. The mechanisms of CTLA-4 mediated signaling still remain incompletely understood.

Compared to CD4+ T cells, the CD8+ Cytotoxic T lymphocyte compartment reacts with a fast and strong immune response accompanied by a much higher expression of CTLA-4, implicating a critical role for CTLA-4 in the regulation of CD8+ T cell differentiation and effector functions. CTLA-4 can also act as a profound mean to protect against host damage, since CD8+ T cells directly target MHC-I presenting cells. Furthermore, the neutralization of CTLA-4 promisingly enhances anti-tumor immunity in mice and men, however, its side effects such as dramatic autoimmune-symptoms ask for more precise targets in anti-tumor therapy. Reasonably, the CD8+ T cells provide an important and applicable research object to analyze the signaling effects of CTLA-4 during T cell differentiation.

To identify signaling pathways, which CTLA-4 induces to attenuate T-cell activation and effector functions, we investigated its impact during the differentiation of CD8+ T cells. By inducing agonistic CTLA-4 signals or not in CD8+ T cells during stimulation under inflammatory conditions, we performed a comparison of the phosphoproteomes using iTRAQ mass spectrometry.

The analysis revealed a differentiated pattern of phosphorylated proteins. Among the identified proteins were 18% signaling molecules, 17% transcription factors, 15% cell cycle control elements, 3% proteins of apoptosis, 6% proteins of RNA processing, 6% proteins for trafficking, 4% translational regulators, 4% proteins of the cytoskeleton, 6% enzymes for metabolism, 3% proteins of DNA assembly, and 8% proteins with unknown function. Interestingly, inhibitory molecules such as CHK1, BRCA-1, and PDCD4 showed to be higher phosphorylated under CTLA-4 signaling.

These results exhibit novel regulatory mechanisms in controlling the CD8+ T cell differentiation and effector functions giving the opportunity to have novel targets to enhance anti-tumor responses.
Deletion of immunodominant epitopes of murine cytomegalovirus favors the expansion of high-avidity CD8 T cells specific for subdominant epitopes

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Immune control of murine cytomegalovirus (mCMV) infection is mediated by CD8 T cells. Four specificities defined by antigenic peptides derived from proteins IE1, m164, M105, and m145 dominate the immune response in BALB/c mice. To investigate the contribution of these specificities to the control of infection we functionally deleted the respective immunodominant epitopes (IDEs) by replacement of their C-terminal amino acids with alanines. Against expectation, quantitation of naturally processed mCMV-encoded peptides in infected cells and organs indicated no influence of the IDEs on the amounts of the subdominant peptides. Accordingly, presentation of subdominant peptides in mCMV-Δ4IDE infected fibroblasts did not stimulate higher numbers of cells from the corresponding epitope-specific CTL lines compared to mCMV-wildtype infected cells. Nevertheless, frequencies of ex vivo CD8 T cells of the corresponding subdominant specificities increased after deletion of the dominant specificities, especially in the clinically relevant situation after hematopoietic cell transplantation but also in the memory phase of immunocompetent infected animals. However, using an mCMV-specific ORF library screening assay which covers all open reading frames (ORF) of mCMV, we could show that the total number of antiviral CD8 T cells in immunocompetent animals infected with the epitope mutant mCMV-Δ4IDE did not reach the level after wildtype infection. Surprisingly, adoptive transfer of CD8 T cells derived from mCMV-Δ4IDE infected animals proved to be highly protective against mCMV-wildtype infection. This implies (i) that the protective potential of the subdominant specificities suffices for controlling infection despite a low number of subdominant peptides presented in vivo and (ii) that immunodominant specificities are dispensable for the control. Analyzing the avidity distribution of the subdominant specificities revealed a shift towards higher avidities in the absence of the dominant specificities. This remarkable finding could explain the high protective potential of subdominant specificities primed in the absence of the IDEs.
Nematode infection interferes with antibody response to vaccination against T cell-dependent model antigens

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Worldwide more than two billion people are infected with helminths. To promote their longevity and to limit pathology, helminths have developed several strategies to suppress the immune response of their host. As this immune suppression spills over to unrelated antigens (Ags), a pre-existing helminth infection may interfere with vaccinations against other pathogens. We use natural infections of fully-permissive BALB/c mice with the filarial nematode *Litomosoides sigmodontis* to analyze the impact of different nematode life stages on vaccination efficacy. Here we show that presence of fourth-stage larvae, young adults, and mature adults that had reproduced and released microfilariae (MF) resulted in reduced antibody response to vaccination with the thymus-dependent (TD) Ag DNP-KLH. Thereby both DNP-specific Th1-associated IgG2 and Th2-associated IgG1 were suppressed. Interestingly, MF that were injected in the absence of adults led to an increased Th1-associated TD Ag-specific antibody response. Thus isolated MF delivered a pro-inflammatory stimulus that was overruled by the anti-inflammatory capacity of the mature adults in the natural situation. Interference with vaccination efficiency in nematode-infected mice increased with infection time and was observed even 4 month after natural clearance of infection. Neither application of Ag in Complete Freund’s Adjuvant nor the introduction of an Ag/alum boost immunization restored vaccination efficacy. However, immunization with polyvalent TI-2 Ags (NIP-Ficoll) that do not require T cell help resulted in comparable antibody responses in naive and nematode-infected mice. This strongly suggests that not B cells but T helper cells are targeted by helminth-mediated suppression. Indeed first results indicate that *L. sigmodontis* infection suppresses the germinal center reaction as infected mice recorded reduced numbers and frequencies of vaccination-induced follicular T helper cells in the draining lymph node. Currently we compare the phenotype of follicular T helper cells and Ag-specific B cells elicited in response to DNP-KLH vaccination in naive and nematode-infected mice.
Expression analysis of inhibitory T-cell receptors on CD28−CD27− CD4 T-cells allows stimulation independent assessment of CMV specific immunity and prediction of viremic episodes in transplant recipients

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Expression of the inhibitory receptors PD-1 and CTLA-4 on CMV-specific CD4 T-cells defines an anergic phenotype associated with CMV-viremia in transplant-recipients. Moreover, CD28−CD27− double-negativity is known as a typical phenotype of CMV-specific CD4 T-cells. Therefore, the co-expression of inhibitory receptors on CD28−CD27− CD4 T-cells was assessed as a rapid, stimulation-independent parameter for monitoring CMV-complications after transplantation.

93 controls, 67 hemodialysis-patients and 81 renal transplant-recipients were recruited in a cross-sectional and longitudinal manner. CMV-specific CD4 T-cell levels quantified after stimulation were compared to levels of CD28−CD27− CD4 T-cells. PD-1 and CTLA-4 expression on CD28−CD27− CD4 T-cells were related to viremia.

A percentage ≥0.44% CD28−CD27− CD4 T-cells defined CMV-seropositivity (93.3% sensitivity, 97.1% specificity), and their frequencies correlated strongly with CMV-specific CD4 T-cell levels after stimulation (r=0.73; p<0.0001). Highest PD-1 expression levels on CD28−CD27− CD4 T-cells were observed in patients with primary CMV-viremia and -reactivation (p=0.0001), whereas CTLA-4 expression was only elevated during primary CMV-viremia (p<0.05). Longitudinal analysis showed a significant increase in PD-1 expression in relation to viremia (p<0.001), whereas changes in non-viremic patients were non-significant.

In conclusion, increased PD-1 expression on CD28−CD27− CD4 T-cells correlates with CMV-viremia in transplant-recipients and may serve as a specific, stimulation-independent parameter to guide duration of antiviral therapy.
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Phenotype and function of schistosome induced Treg cells on suppressing allergic airway inflammation

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Development, antigen-specificity and the differentiation of nTreg and iTreg are prime topics currently addressed in regulatory T cell (Treg) research. With regards to tropical diseases, CD4+CD25+Foxp3+ Treg during the chronic helminth infection Schistosoma mansoni have been shown to be beneficial for both the host and parasite. Our studies have proven that without Treg, the immunopathology of the host is significantly elevated and that Treg isolated from infected mice differentially express genes which are solely up-regulated indicating that Treg contribute to immunosuppression rather by change of phenotype and mode of action than simply by expansion. Furthermore, we have shown that schistosome-induced Treg suppress responses to bystander antigens using an OVA-induced asthma model.

In this study we further investigate the phenotype of Treg in order to differentiate iTreg from nTreg and to test their suppressive capacity on bystander immune responses. We could not observe differences in Treg frequency during infection in general, however applying new markers like Neuropilin-1 (Nrp-1) and Helios we were able to demonstrate that the distribution within the Treg population changed. Compared to naïve mice, the proportion of Nrp-1-negative iTreg increases within the CD4+ Foxp3+ compartment, whereas frequencies of Nrp-1-positive nTreg decrease. In contrast, using another marker implicated to define iTreg, namely the transcription factor Helios, we found an expansion of Helios-positive nTreg during infection. These results imply that the surface marker Nrp-1 is a more reliable marker to discriminate between n/iTreg during chronic helminth infection and can be applied to sort these cells for functional suppression assays.
One key aspect of infection immunity is how a host recognizes pathogens. Traditional concepts on pathogen recognition by the host’s innate immune system are largely based on the idea that pathogen associated molecular patterns (PAMPS) are recognized by corresponding receptors (PRR). However, molecules of microbial origin recognized by TLRs, NODs and other PRR are not confined to pathogens. Therefore, the term MAMPs (microorganism-associated molecular patterns) has been recently coined, still leaving unsolved, however, the issue whether and how pathogens are sensed in non-sterile compartments. Studies on cellular responses to pore forming toxins point to the possibility that damage inflicted by microbial products rather than microbial products per se might serve to discriminate pathogens from non-pathogens on colonized epithelia in animals. The results also suggest a role of cell autonomous defence as an integral part of innate immune effector mechanisms. Here, we summarize recent data on the role of membrane repair and autophagy for defence against the small pore forming S. aureus alpha-toxin, and discuss them in the context of the emerging concept of effector-triggered innate immunity (1).

Altered phenotype and functionality of varicella zoster virus-specific cellular immunity in immunocompetent and immunocompromised patients with acute herpes zoster

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Varicella zoster virus (VZV) establishes lifelong persistence and may frequently reactivate in immunocompromised patients such as hemodialysis patients (HD) and transplant recipients (Tx). To assess correlates of protection and to identify immunologic parameters associated with acute VZV reactivation, T-cell immunity and IgG responses towards VZV were characterized in 42 immunocompetent and immunocompromised patients with acute herpes zoster and compared with those of 90 patients and controls without VZV reactivation (11 healthy controls, 29 HD and 50 Tx patients).

VZV-specific CD4 T cells were analyzed after whole blood stimulation with VZV-lysate using intracellular staining for IFNγ, IL-2, and TNFα. CD127, CTLA-4 and PD-1 were chosen as markers for functional anergy. Stimulation with VZV-free control-lyslates and the superantigen Staphylococcus Enterotoxin-B (SEB) served as negative and positive controls, respectively. IgG titers were assessed using standard ELISA.

VZV-seropositive non-symptomatic immunocompetent controls had median frequencies of VZV-specific CD4 T cells of 0.15% (0.03-0.29%). The VZV-specific T-cell profile showed multifunctional characteristics, marked by a predominant expression of all three cytokines (median 54.0%, IQR 9.8%; IFNγ-single positive cells: median 5.2%, IQR 4.0%), but low expression of the inhibitory receptors CTLA-4 (MFI 1081±654) and PD-1 (MFI 215±94). Moreover, nearly all cells were positive for CD127 (96.0±6.2%). Non-symptomatic immunocompromised patients had similar T-cell properties showing only slightly lower median frequencies (p=0.02) and percentages of multifunctional (IFNγ, IL-2, and TNFα positive) VZV-specific cells (p=0.15). In contrast, elevated IgG titers (median 7064 IU/L, IQR 1896 IU/L) as well as frequencies of VZV-specific CD4 T-cells (median 0.48%, IQR 0.65%) were found in both immunocompetent and immunocompromised patients with acute herpes zoster. The cytokine-profile of VZV-specific T cells was shifted towards IFNγ-single positive cells (25.5%). Furthermore, these T-cells showed a significant increase in CTLA-4 and PD-1 expression, and a concomitant decrease in CD127 expression, whereas there was no effect on polyclonally stimulated T-cells. Interestingly, VZV-specific T cells analysed >3 months after acute reactivation reverted back to the phenotype observed in non-symptomatic individuals.

Conclusion: VZV-specific CD4 T cells in patients with acute herpes zoster are elevated in frequencies and bear typical features of anergic cells such as increased expression of CTLA-4 and PD-1, decreased expression of CD127, and restricted functionality, marked by an increased percentage of IFNγ-single positive cells. This dynamic phenotype may be applied for monitoring infectious complications in patients at risk.
“Enforced virus replication” is an immunological strategy to activate innate and adaptive immune responses

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The innate immune system suppresses virus replication by interferon type I (IFN-I) and also induces presentation of viral antigens to adaptive immune cells. Here, we analyzed how the innate immune system manages to inhibit virus propagation but still allows the presentation of antigen to adaptive immune cells. We found that the expression of Usp18 in CD169⁺ macrophages and dendritic cells reduces their IFN-I responsiveness, thereby allowing locally restricted replication of virus. This early virus replication is essential for the induction of sufficient antiviral innate and adaptive immune responses. Therefore this mechanism was essential to inhibit the spread of virus to peripheral tissue and to prevent fatal disease. In conclusion, we found that “enforced” virus replication within marginal zone macrophages is an immunological mechanism that ensures the production of sufficient antigen for effective adaptive immune activation.

figure 1
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Foxp3\(^{+}\) regulatory T cells delay expulsion of intestinal nematodes by suppression of IL-9 driven mast cell activation in BALB/c but not in C57BL/6 mice

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Pathogenic nematodes exploit regulatory pathways that are intrinsic parts of the mammalian immune system to dampen the immune response directed against them. Foxp3\(^{+}\) regulatory T cells (Treg) represent one central mediator of immunological homeostasis. Here we demonstrate a different role for Treg in nematode induced immune evasion in BALB/c and C57BL/6 mice. Transient Treg depletion reduced parasite burden selectively in *Strongyloides ratti* infected BALB/c but not in C57BL/6 mice. Treg function was apparent in both mouse strains as Treg depletion increased nematode specific production of IL-4, IL-13, IL-3 and IL-10 and elevated serum concentration of IgE and IgM in BALB/c and C57BL/6 mice to the same extent. Neither depletion of granulocytes that attack migrating larvae nor neutralization of IL-13 abrogated improved resistance in Treg depleted BALB/c mice. Improved resistance was accompanied by increased production of IL-9 and by accelerated degranulation of mast cells in Treg depleted BALB/c mice while these parameters remained unchanged in Treg depleted C57BL/6 mice. By *in vivo* neutralization of IL-9 at different time points we directly demonstrate that specifically this increased production of IL-9 during the first days of infection accelerated mast cell degranulation and promoted rapid expulsion of *S. ratti* adults from the small intestine of Treg depleted BALB/c mice. Interestingly, IL-9 driven mast cell degranulation is a central mechanism of *S. ratti* expulsion in both BALB/c and C57BL/6 mice, because IL-9 injection reduced and IL-9 neutralization increased parasite burden in the presence of Treg in both strains. Therefore our results suggest that Foxp3\(^{+}\) Treg regulate endogenous IL-9 production during nematode infection in a non-redundant manner in BALB/c mice whereas other regulatory elements contribute to the control of these pathways in C57BL/6 mice.
Alveolar type II epithelial cells inhibit alveolar macrophage function following viral stimulation and rapidly react to *in vivo* influenza A virus infection in a partly TLR7-dependent fashion

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Influenza A virus (IAV) infections often predispose patients for severe disease through secondary bacterial pathogens. This effect has been shown to be mediated through modulation of anti-bacterial immune responses by the viral infection. Here, a hallmark feature is the inhibition of the alveolar macrophage phagocytic function, which we have shown to be attenuated in TLR7-deficient hosts. Up to now, the underlying triggering events as well as the mediators of this inhibition of macrophage function are incompletely understood. Alveolar type II epithelial cells (AECII) are primary targets for IAV replication and can at the same time be considered as non-lymphoid immune cells of the respiratory tract. Therefore we are analyzing the AECII-response to *in vivo* IAV infection with respect to the modulation of anti-bacterial defense mechanisms, also with regard to TLR7. To this end we have treated MLE-15 cells with poly(I:C) and inactivated IAV and assessed the effect of secreted mediators on alveolar macrophage function. Furthermore we have performed gene expression analyses of primary murine AECII isolated from IAV infected wild-type and TLR7ko mice on the first three days following infection.

In response to stimulation with either poly(I:C) or inactivated IAV, MLE-15 cells secreted substantial amounts of IL-6 and type I interferon. Conditioned medium from these stimulated MLE-15 cells was used to pre-treat primary alveolar macrophages before assessing their phagocytic potential. Alveolar macrophages treated with medium from virally stimulated MLE-15 cells showed significantly inhibited phagocytic function, which was similar to the inhibition found in alveolar macrophages isolated from IAV-infected mice. Microarray analyses of primary alveolar type II epithelial cells isolated from IAV-infected mice on the first three days following infection revealed broad anti-viral and inflammatory responses. The comparison with gene expression analyses of whole lungs revealed a strong contribution of AECII to the early respiratory anti-IAV immune response. Regulation of gene expression in response to IAV was attenuated in AECII isolated from TLR7ko mice with regard to the number of regulated genes as well as the fold-change induction of a wide number of cytokines and chemokines over baseline expression in the respective controls.

With these analyses we show that AECII rapidly and strongly react to *in vivo* IAV infection in a partially TLR7-dependent manner. Furthermore, AECII secreted factors are potent modulators of the phagocytic function of alveolar macrophages. Altogether this gives new insight into the role of AECII not only for anti-influenza immune reactions but also for the processes underlying enhanced susceptibility to secondary bacterial infections following IAV.
Impact of chronic autoimmune-mediated lung inflammation on *Streptococcus pneumoniae* infection

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**Question:**
Chronic respiratory diseases (CRDs) affect hundreds of millions of people of all ages worldwide thus constituting a considerable part among non-communicable diseases. In this context community-acquired pneumonia (CAP) caused by the Gram-positive bacterium *Streptococcus pneumoniae* is known as a major comorbidity in CRDs. Despite different etiologies and clinical manifestations inflammatory airway disorders like chronic obstructive pulmonary disease (COPD), asthma or chronic bronchitis commonly implicate a fatal outcome of respiratory tract infections in humans. Therefore, we aimed at clarifying the impact of chronic pulmonary inflammation on the shape of innate antibacterial immunity during pneumococcal infection in a mouse model. In the past we have established transgenic mouse models for autoimmune-mediated CRD that are based on the expression of the influenza A hemagglutinin (HA) neo-self-model-antigen in alveolar epithelium (SPC-HA mouse). Breeding of the SPC-HA mouse with mice, that produce high numbers of HA-specific CD4+ T cells (TCR-HA mice), results in severe pulmonary inflammation in double-transgenic progeny (SPC-HA x TCR-HA mice). In the present study we investigate the impact of CRD on early immune responses towards *S. pneumoniae* in the SPC-HA x TCR-HA transgenic mouse model.

**Methods:**
In order to identify hallmarks of chronic autoimmune-mediated pulmonary inflammation with possible implications for pneumococcal infection, we performed whole lung gene expression analyses of uninfected SPC-HA and SPC-HA x TCR-HA mice. In the next step, healthy SPC-HA control mice and diseased double-transgenic SPC-HA x TCR-HA mice were infected with a low dose of *S. pneumoniae* and pro-inflammatory cytokine levels and bacterial burden in lung tissue and bronchoalveolar lavage (BALF) were determined.

**Results:**
We identified several hundreds of differentially regulated genes in lungs of SPC-HA x TCR-HA mice. Pathway analyses revealed an overrepresentation of genes involved in cytokine-cytokine receptor interaction and chemokine signaling pathways. Additionally, in comparison to SPC-HA mice- in SPC-HA x TCR-HA mice significantly elevated levels of TNF-alpha (BALF, lung) and interleukin-6 (BALF) were determined 24 h post infection with *S. pneumoniae*. No marked differences between SPC-HA and SPC-HA x TCR-HA mice could be observed with regard to bacterial burden during early pneumococcal infection.

**Conclusions:**
With these analyses we show that CRD in our model is associated with marked changes in whole lung gene regulation thus elucidating the autoimmune-induced inflammatory framework of chronically diseased lungs. Within this altered inflammatory framework we could show a significantly increased pro-inflammatory cytokine response towards the respiratory pathogen *S. pneumoniae*. These findings point out a definite impact of chronic pulmonary inflammation on the shape of innate inflammatory immune responses during pneumococcal pneumonia.
CD38 controls the innate immune response against *Listeria monocytogenes*

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CD38, adenosine-5'-diphosphate-ribosyl cyclase 1, is a multifunctional enzyme, expressed on a wide variety of cell types. CD38 has been assigned diverse functions including generation of calcium-mobilizing metabolites, cell activation, and chemotaxis. Using a murine *Listeria monocytogenes*-infection model, we found that CD38 KO mice were highly susceptible to infection. Enhanced susceptibility was already evident within three days of infection suggesting a function of CD38 in the innate immune response. CD38 was expressed on neutrophils and inflammatory monocytes, and especially inflammatory monocytes further upregulated CD38 during infection. Absence of CD38 caused alterations of the migration pattern of both cell types to sites of infection. We observed impaired accumulation of cells in the spleen, but surprisingly similar or even higher accumulation of cells in the liver. CD38 KO and WT mice showed similar changes in the composition of neutrophils and inflammatory monocytes in blood and bone marrow, indicating that mobilization of these cells from the bone marrow was CD38-independent. Dendritic cells also displayed enhanced CD38 expression following infection. However, absence of CD38 did not impair the priming of CD8+ T cells against *L. monocytogenes*. In conclusion, our results demonstrate an essential role for CD38 in the innate immune response against *L. monocytogenes*. 
Clinical Presentation Of Potts Disease Of The Spine In Adult Sudanese Patients

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Tuberculosis (TB) of the spine (Pott’s disease) is the commonest and most dangerous form of skeletal TB. Delay in establishing diagnosis and management can cause spinal cord compression and spinal deformity resulting in serious neurological deficit and bad prognosis. This was a prospective hospital-based study investigating the data on hundred cases of Pott’s disease presented to Khartoum Teaching Hospital during the period from 2008 to 2010. 60 patients were females and 40 were males. The mean age of our patients was ± 41. The course of the disease was progressive and of gradual onset in the majority of the cases. 76% of our study group was presented with neurological deficits ranging from lower limb anaesthesia, numbness, trunk weakness, root pain, muscle pain and flexion spasm.
Adoptive T-cell immunotherapy with T cells from seropositive stem cell and third-party donors: establishment of a T-cell donor registry

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Adoptive immunotherapy with virus-specific T cells can efficiently reconstitute antiviral immunity against CMV, EBV and ADV without causing acute toxicity or increasing GvHD. Recent studies showed that during G-CSF mobilization the functional activity of antiviral memory T cells is impaired, suggesting that even stem cell donors may not be the best source of T cells. Further, in conditions, where patients receiving a cord blood or a transplant from a virus-seronegative donor and since donor blood is generally not available for solid organ recipients, allogeneic third party T-cell donors would offer an alternative option.

To gain more insight into virus-specific memory T-cell pools in healthy subjects and to identify the most efficient antigens for immunotherapy, we assessed the frequencies of CMV-, EBV- and ADV-specific T cells in 204 HLA-typed healthy donors by T-cell receptor staining and IFN-γ ELISpot using peptides and peptide pools available in GMP quality. Specific T cells were detected in 100% of CMV-seropositive donors, 73% of EBV-seropositive donors and 73% of ADV-seropositive donors with highly significant differences in frequencies. Among the tested antigens frequencies for CMV_pp65 and EBV_BZLF1 peptide pool were highest. Overall frequencies of peptidespecific T cells detected by T-cell receptor staining were lower than those of the corresponding peptide pools. Short-term in vitro peptide stimulation assays revealed that, in the case of ADV and EBV, a donor response to a certain peptide may not be determined with prior stimulation. A modified granzyme B ELISpot was established and used to detect T-cell specificity and alloreactivity. Moreover, confirmatory testing for CMV serology using western blot technique revealed 19/143 (13%) false-positive results, possibly impacting future analysis and selection of potential stem cell and T-cell donors. An allogeneic T-cell donor registry, which will document each donor’s HLA type, virus serology, virus-specific T-cell frequencies, best T-cell detection method, and results from functional and alloreactivity assays is under development.
A strongly increased activation threshold focuses the T cell repertoire in secondary or cross-reactive immune responses

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In adults many infections are caused by pathogens that are similar but not identical to previously encountered viruses, bacteria, or vaccines. In such infections some antigens are recognized by memory T cells while at the same time new antigens activate naïve T cells. How T cells reacting to earlier experienced antigens impact the repertoire of T cells specific to new antigens remained so far unknown. We will demonstrate that antigen overlap severely narrows the diversity of antigen-specific T cells and that broad T cell repertoires emerge only in the absence of antigen overlap or cross-reactivity. Diverse T cell repertoires are needed to effectively handle ongoing and subsequent infections involving pathogens expressing mutated epitopes. Such repertoire diversity is mainly achieved by expanding lower affinity T cells which suboptimally respond to antigen along with high affinity cells. We will show that only one epitope overlap between two infections drastically increases the T cell activation threshold. In this case T cells with slightly suboptimal antigen reactivity are excluded from the response. Our observations highlight how strongly the infectious history can leave its mark on the emerging repertoire of antigen-specific T cells and we will demonstrate the implications of our findings for vaccine design.
A spatially-organized multicellular innate immune response in lymph nodes limits systemic pathogen spread.

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The lymphatic network that transports interstitial fluid and antigens to lymph nodes constitutes a conduit system that can be hijacked by invading pathogens to achieve systemic spread unless dissemination is blocked in the lymph node itself. Here, we show that a network of diverse lymphoid cells (natural killer cells, γδ T cells, natural killer T cells, and innate-like CD8+ T cells) are spatially prepositioned close to lymphatic sinus-lining sentinel macrophages where they can rapidly and efficiently receive inflammasome-generated IL-18 and additional cytokine signals from the pathogen-sensing phagocytes. This leads to rapid IFNγ secretion by the strategically positioned innate lymphocytes, fostering antimicrobial resistance in the macrophage population. Interference with this innate immune response loop allows systemic spread of lymph-borne bacteria. These findings extend our understanding of the functional significance of cellular positioning and local intercellular communication within lymph nodes while emphasizing the role of these organs as highly active locations of innate host defense.

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Suppressed proliferation of OVA-specific CD4+ T cells in helminth-infected mice: unraveling the chain of events

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Human filarial parasites cause chronic infections with high morbidity. In order to prevent their expulsion helminths suppress the immune response of their host. This immunosuppression might impair the immune response to unrelated antigens such as vaccines or allergens. We have shown before that concurrent infection with the filarial nematode *Litomosoides sigmodontis* suppressed the humoral response in model antigen vaccinations by interference with T helper cell function. To analyze nematode-induced T cell suppression we measured the proliferation of CD4+ T cell receptor transgenic T cells after adoptive transfer in infected and non-infected C57BL/6 mice. OVA-specific OT-II T cell proliferation was reduced upon antigen-specific stimulation within *L. sigmodontis* infected mice compared to stimulation within non-infected mice. *L. sigmodontis* failed to suppress OT-II T cell proliferation in RAG ko mice. Reconstitution of RAG ko mice with B and T cells but not with T cells alone restored *L. sigmodontis*-induced suppression of OT-II T cell proliferation. In line with this, suppression of OT-II T cell proliferation was abolished in *L. sigmodontis*-infected B cell deficient JHT mice. Neutralization of TGF-β at the onset of infection or at the time point of the adoptive transfer of OT-II T cells did not abrogate suppression of OT-II T cell proliferation in helminth-infected mice. In contrast, neutralization of IL-10 with an anti-IL10 receptor antibody resulted in a comparable proliferation of OT-II T cell in infected mice and non-infected mice. We are currently identifying the source and the primary target cell of IL-10 in infected mice.

Taken together, infection with the murine nematode *L. sigmodontis* interferes with OVA-specific T cell proliferation via induction of a regulatory network. Cross talk between different cell types and soluble factors such as IL-10 contribute to diminished bystander antigen responses in helminth-infected mice.
Interplay of bacteria and host-derived factors in the skin innate immune response

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Human skin, the primary interfaces between the body and environment, is constantly exposed to a myriad of potential pathogens, while at the same time they allow harmless, non-pathogenic microorganisms to survive and colonize the tissue. Therefore, it seems that skin integrity is maintained by a kind of immune homeostasis in which the extent of skin immune response is controlled by active defence mechanisms and tolerogenic signals. We show that human keratinocytes in the epidermal layer of skin actively participate in the innate immune response towards pathogens by production of several cytokines and chemokines and antimicrobial peptides or proteins (AMPs) able to attract immune cells into the skin or kill directly the pathogens. We show that resident skin commensal bacteria create a protective environment by immune conditioning of epithelial surfaces. Interestingly, commensal bacteria are able to amplify the innate immune response of human keratinocytes to pathogens by activation of different signaling pathways acting in a synergistic way with pathogen induced pathways. Furthermore, our data indicate that there is an interplay between host and bacterial derived membrane-active peptides, which enhances clearance of pathogens. Furthermore, we investigated how murine skin responds to Staphylococcus aureus skin colonization in a physiologic setting using an epicutaneous skin infection model. We show, that the efficiency of skin colonization correlated with the induction level of proinflammatory cytokines and AMPs. Our study suggests that skin barrier defects promote S. aureus skin colonization and that prolonged colonization is associated with profound cutaneous inflammation. Our data indicate that there is a crosstalk between immunomodulatory factors derived from pathogens and the host as well as between commensal, pathogen and host-derived peptides during bacterial infection of the skin. By this, keratinocytes as innate immune sensors are able to sense signals from the environment and initiate differential immune responses to harmless commensals or harmful pathogens, respectively.
Mass spectrometric identification of potential antigenic vaccine candidates against *Leishmania major*

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In human cutaneous leishmaniasis (CL), both antigen-specific CD4+ and CD8+ T cells promote healing in *Leishmania (L.)* major-infected immunocompetent hosts by releasing interferon (IFN)-γ. However, currently no vaccine against this human pathogenic parasite exists. To identify antigenic proteins that could serve as vaccine candidates, we isolated *L. major*-specific soluble proteins (SP) from highly immunogenic parasite lysate (soluble *Leishmania* antigen, SLA) by differential centrifugation. Next, C57BL/6 mice were immunized intradermally in one ear with different amounts of SP ranging from 1 µg up to 100 µg + CpG as adjuvant in a prime/boost/boost approach. One week afterwards, infections with live parasites were initiated in the alternate ear. Interestingly, almost no lesion progression at the site of infection was revealed in groups immunized with the lowest amount of SP + adjuvant used. In contrast, immunization with doses of 10 µg, 50 µg or 100 µg of SP combined with CpG led to lesion volumes similar to those of CpG-treated controls. Subsequently, we aimed to identify and characterize individual immunogenic antigens from SP. Toward this purpose, we fractionated SP by two-step anion exchange chromatography. Eluted fractions were tested in vitro in restimulation assays using draining lymph node (dLN) T cells from *L. major*-infected C57BL/6 mice. dLN cells restimulated with SP showed a dominant Th1/Tc1 cytokine profile similar to total SLA. Surprisingly, only few reactive fractions from SP were found, which induced high IFN-γ levels compared to unstimulated controls. Using label-free quantitative mass spectrometry, we identified several parasite-specific proteins in reactive fractions from SP. Currently, selected proteins were recombinantly expressed in *E. coli* and will be investigated in future immunization studies *in vivo*. These antigenic vaccine candidates may serve as potential sources for protective T cell epitopes and may aid a better understanding of the underlying T cell-mediated healing processes in infected individuals or those at risk.
Mitochondria play a central role in a novel innate immune sensing mechanism of viral infection.

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Many viruses escape the immune response by suppressing antigen presentation in the infected cell or avoid recognition by innate receptors. We identified a new CTL (cytotoxic T lymphocytes) effector mechanism against viral infections of the liver that is independent of antigen presentation on target cells and classical innate immune recognition pathways. This effector function is mediated by TNF that is secreted by CTLs after cross-presentation of viral antigens by non-infected cells and selectively eliminates virally infected hepatocytes, whereas uninfected cells survive.\textsuperscript{1}

Here we investigate the molecular mechanism(s) underlying the sensitization of infected hepatocytes towards the death-inducing effect of TNF: Mice are infected with hepatotropic adenovirus and challenged with recombinant TNF to analyze key events in survival and apoptosis signaling.

We found dramatic changes in mitochondria after viral infection: While anti-apoptotic Bcl2 family proteins remained unchanged, the protein levels of the pro-apoptotic members Bax and Bim were elevated upon infection. Bcl2 family proteins regulate apoptosis by influencing ER and mitochondrial integrity. In line with this, mitochondrial intermembrane space proteins, like smac and HtrA2, were increased in cytoplasm of infected hepatocytes. This came along with an enhanced sensitivity of mitochondria towards apoptotic stimuli like activated caspase 8, which is the link between TNFR stimulation and mitochondrial apoptotic signaling.

Additionally, viral infection targets early events at the TNFR complex: A downregulation of cIAP-1 permitted the activation of caspase 8 and the execution of subsequent events in the apoptotic cascade such as Bid cleavage, activation of caspase 9 and effector caspase 3 that could be detected exclusively upon infection and TNF stimulation.

Taken together, our results demonstrate that viral infection sensitizes hepatocytes towards TNF induced apoptosis by changing the composition of the TNFR complex and by interfering with mitochondrial integrity. We hypothesize that the release of smac and HtrA2, inhibitors of IAPs, from mitochondria would account for the reduction in cIAP-1 levels and therefore also for the switch in TNFR signaling towards initiator caspase activation.

In conclusion we identified a novel innate immune sensing mechanism in which mitochondria and regulation of Bcl2 family proteins play a key role and that may overcome viral immune escape in infected cells.

\textsuperscript{1}Wohlleber et al., TNF-Induced Target Cell Killing by CTL Activated through Cross-Presentation, Cell Reports (2012)2(3):478-87
Gene expression profiling of IFNβ-producing plasmacytoid dendritic cells defines them as a novel functional DC subset

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Introduction:
Type I interferons (IFN) consisting of multiple IFNαs and one single IFNβ are crucial mediators of protective immune responses predominantly against viral infections. Initial binding of IFNβ to the type I IFN receptor (IFNAR) induces a positive autocrine and paracrine feedback loop leading to the expression of IFN-stimulated genes. Our previous studies using a bicistronic IFNβ/YFP reporter-knockin mouse model (IFNβmob/mob) revealed that IFNβ expression after TLR9 stimulation is restricted to a minor population of plasmacytoid dendritic cells (pDCs), both in vitro and in vivo.

Objectives:
We want to determine whether IFNβ-producing pDCs harbor a specialized gene expression profile as compared to pDCs not producing IFNβ.

Materials & Methods:
CpG1668 was used for the in vitro stimulation and injection into IFNβmob/mob or IFNAR−/−xIFNβmob/mob mice, respectively. Splenic IFNβ/YFP+ pDCs and IFNβ/YFP− pDCs on WT and IFNAR-deficient background were isolated by FACS sorting and comparative ex vivo mRNA Microarray and qRT-PCR expression analyses were performed. Localization of IFNβ/YFP+ pDCs and IFNβ/YFP− pDCs was determined by confocal immunofluorescent microscopy.

Results:
In vitro and in vivo, pDCs are the major source of IFNβ in the spleen after TLR9 stimulation. While in response to TLR9 stimulation all pDCs upregulate the expression of activation markers, such as CD86, only up to 10% of all pDCs produce IFNβ. Intriguingly, sorted IFNβ/YFP+ pDCs from CpG stimulated in vitro Flt3L cultured BM-DCs were not able the produce the cytokine even after additional CpG stimulation. This indicates a restriction of the potential to produce IFNβ to a specific pDC subtype. To test this hypothesis we performed a microarray based transcriptome analysis of ex vivo isolated IFNβ-producing vs. non-producing pDC 6h after CpG stimulation. This revealed that more than 1500 genes were differentially expressed between these two populations. IFNβ/YFP+ pDCs exhibited a higher expression of genes involved in T and NK cell modulation, e.g. certain cytokines (e.g. IL-12) and chemokines (e.g. CCL3, CCL5). Of note, we observed a higher expression level of the chemokine receptor 7 (CCR7) in IFNβ/YFP+ vs. IFNβ/YFP− pDCs. Accordingly, IFNβ/YFP+ pDCs displayed a different localization within the spleen as compared to IFNβ/YFP− pDCs. In CpG stimulated mice IFNβ/YFP+ pDCs can be found at the interface of the B and T cell areas while the vast majority of pDCs are located around the marginal zone. qRT-PCR analyses in CpG stimulated IFNAR−/− mice revealed that the development of this IFNβ-pDCs specific gene expression profile is independent of IFNAR-mediated signaling.

Conclusions:
IFNβ expression defines a subpopulation of splenic pDCs, which is unique not only with regard to the expression of IFNβ and its response genes, but also differs from pDCs not producing IFNβ in the expression of other immune-relevant genes. Intriguingly, signaling via the IFNAR plays no essential role in the expression of selected genes involved in T and NK cell activation and migration. Taken together, the differential expression of genes involved in immune modulation contradicts the current hypothesis of IFNβ induction being a stochastic event and provides a molecular mechanism for e.g. the selective recruitment of IFNβ-producing pDCs into the T cell zone and the induction of inflammatory immune responses mediated by these specialized pDCs.
Combining regulatory T cell depletion and inhibitory receptor blockade improves reactivation of exhausted virus-specific CD8$^+$ T cells and efficiently reduces chronic retroviral load

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Cytotoxic T cells facilitate control of acute viremia in many viral infections, including HCV and retroviruses like HIV or HTLV. However, most viruses that establish chronic infections evade destruction by CD8$^+$ T cells. These chronic infections with viruses such as HIV and HCV have been associated with functional exhaustion of CD8$^+$ T cells. Two main mechanisms have been described that mediate this exhaustion; expression of inhibitory receptors on CD8$^+$ T cells and expansion of regulatory T cells (Tregs) that suppress CD8$^+$ T cell activity. Several studies showed that blockage of inhibitory receptor/ligand interactions results in reactivation of CD8$^+$ T cells and partial reduction in chronic viral loads. Furthermore, using transgenic mice in which Tregs can be selectively ablated, we recently showed that viral loads can be significantly reduced by transient depletion of Tregs during a chronic retroviral infection. Depletion was associated with the reappearance of virus-specific CD8$^+$ T cells that showed an improved functionality and displayed a significantly improved virus-specific cytolytic activity in vivo. This demonstrates the important role of both mechanisms for the control of CD8$^+$ T cell responses and the maintenance of chronic viral infection, indicating that modulating Treg function and blocking inhibitory pathways should be an important part of therapeutic interventions.

Therefore, we used blocking antibodies against PD-1 ligand and Tim-3 and transgenic mice in which Tregs can be selectively ablated, we compared these two treatment strategies and combined them for the first time in a model of chronic retrovirus infection. Blocking inhibitory receptors was more efficient than transient depletion of Tregs in reactivating exhausted CD8$^+$ T cells and reducing viral set points. However, a combination therapy was superior to any single treatment and further augmented CD8$^+$ T cell responses and reduced chronic viral loads. These results demonstrate that Tregs and inhibitory receptors are non-overlapping factors in the maintenance of chronic viral infections and that immunotherapies targeting both pathways may be a promising strategy to treat chronic infectious diseases.
Exploring molecular mechanisms of apoptosis in the protozoan parasite *Leishmania major*

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Apoptosis has been shown to occur in different phyla of protists including the eukaryotic parasite *Leishmania major* (*L. major*). Interestingly, many hallmarks of metazoan apoptosis, such as phosphatidylserine (PS) externalization, destabilization of the mitochondrion, reactive oxygen species (ROS) dysregulation, cytochrome c release and DNA fragmentation, are also described for *L. major*. However, the protein machinery mediating apoptosis in mammalian cells like caspases or members of the BCL-2 family are completely absent in *Leishmania*. In this study we want to identify the mechanisms and the proteins that are involved in the protozoan apoptotic program.

To investigate the process of apoptosis, we use an *in vitro* promastigote culture as well as a new culture method to generate axenic amastigotes of *L. major* and induced apoptosis using staurosporine or miltefosine treatment. Apoptosis induction with staurosporine resulted in ROS-dependent apoptosis in promastigotes, whereas miltefosine induced a ROS-independent apoptosis. To investigate the different apoptosis pathways in more detail we used mass-spectrometry analysis and characterized the most abundant proteins that were either up or down regulated after apoptosis induction. We quantified a total of 707 leishmanial proteins. Furthermore, we could identify the enzyme calpain like cystein peptidase (CLCP) to be specifically up regulated in apoptotic promastigotes and the dipeptidyl peptidase III (DPP III) to be specifically down regulated in apoptotic amastigotes. To investigate the role of CLCP and DPP III during apoptosis we modified their function using specific inhibitory compounds. Upon apoptosis induction, treatment with the inhibitor for CLCP resulted in a reduced apoptosis, whereas treatment with the inhibitor for DPP III caused an even higher apoptosis, suggesting these proteins to be involved in the apoptosis machinery of *L. major*.

Focusing on CLCP and DPP III we are currently analyzing overexpression parasites for these two candidate proteins to elucidate their role as new potential apoptosis modulating enzymes in *Leishmania* parasites.
Apoptotic *Leishmania* induce “LC3 associated phagocytosis” in human primary macrophages leading to a reduced lymphoproliferative response in unexposed individuals

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Virulent *Leishmania major* (*L. major*) parasites, consisting out of apoptotic and viable promastigotes, use human primary macrophages (MF) as host cell. In addition, MF are known as professional phagocytes which clear pathogens and apoptotic cells, of which the latter one also plays an active role in modulating immunity. The effective clearance is achieved by both phagosomal maturation and the autophagy machinery, leading to antigen presentation to T lymphocytes. As described, *Leishmania* spp. are able to induce an *in vitro* proliferation of peripheral blood mononuclear cells (PBMCs) from non-exposed individuals. However, the role of apoptotic parasites during *L. major* infection and their effect on T lymphocytes is still unclear. In this study, we hypothesize that the apoptotic promastigotes use the MFs’ autophagy machinery to modulate MF antigen presentation, hereby reducing T cell activation.

Upon *L. major* infection of human primary MF, we observed MF to internalize *L. major* promastigotes in dissimilar compartments. In contrast to viable promastigotes, we found apoptotic promastigotes to enter a compartment positive for the autophagy marker LC3. Upon examining the ultrastructure of these compartments, we found all apoptotic promastigotes to reside in LC3+ compartments consisting out of a single bilayer, favoring the process of LC3 associated phagocytosis (LAP). This LC3+ compartment matured over time and became LAMP positive. After 24 h, the compartment resolved after efficient parasite degradation. When co-incubated with autologous PBMCs, MF infected with viable promastigotes induced a strong CD4+ MHCII restricted T cell response. Moreover, T cell proliferation could be associated with a reduced MF infection rate and parasite load. Interestingly compared to viable parasites, a significantly lower T cell reactivity was observed in response to MF inoculated with only apoptotic or a mixture of apoptotic and viable parasites. Furthermore, upon chemically inducing autophagy during *L. major* infection, using GDC-0941, PI-103 or rapamycin, T cell proliferation could be reduced even more.

In conclusion, we found apoptotic *Leishmania* to reside in a single membrane compartment being LC3+, suggesting LAP. Moreover, *L. major* infected macrophages induced a naïve T cell response, being CD4+ MHCII restricted. However, upon LAP induction by apoptotic *L. major*, we found a reduced T cell proliferation, suggesting a novel pathogenic silencing mechanism in human primary macrophages.
Inhibitor of Apoptosis Proteins are required for effective T cell expansion/survival during T cell mediated anti-viral immunity in mice

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Inhibitor of apoptosis proteins (IAPs) were originally described to regulate apoptosis by direct binding to caspases. More recently, IAPs have been identified as important modulators of canonical and non-canonical NF-κB signalling via their ubiquitin-E3 ligase activity. IAPs are therefore not only gatekeepers of cell death but probably also involved in the regulation of inflammation as well as innate and adaptive immunity. Here analyse the role of IAPs in T cell immunity during LCMV infection by pharmacological targeting with an IAP antagonists/Smac mimetic. Expansion of virus-specific CD8 T cells was drastically reduced in LCMV-infected mice exposed to IAP antagonist. Accordingly, virus control was substantially impaired, indicated by high virus titres in the spleen and spread of LCMV to peripheral organs. The profound negative effect of IAP antagonists on T cell immunity was partially linked to TNF-mediated cell death of activated T cells. Thus, IAPs play an important role in T cell expansion and survival in the context of a highly inflammatory environment such as a virus infection, and IAP-antagonists may interfere with immune responses.
Malaria-induced CTLA-4Foxp3− T cells inhibit T effector cell function in trans

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During the course of an infection with Plasmodium ssp. the parasites develop via two stages: The first replication step in the liver is followed by replication in the blood. In the first phase only few hepatocytes are infected and the antigenic load is low, but in the subsequent blood stage many erythrocytes are infected which leads to a vast amount of antigenic material delivered to the immune system. This induces a strong activation of T cells followed by the production of proinflammatory cytokines that contribute to the severe complication of malaria, the cerebral malaria (CM). Thus the immune response has to be tightly regulated to achieve on the one hand protection against the parasite and on the other hand prevent immunopathology. CTLA-4 is implicated in the inhibition of T cell function and is expressed constitutively on regulatory T cells (Treg) and after activation on T effector cells (Ter). We have previously shown that blockade of CTLA-4 with antibodies leads to an exacerbation of the disease. However Treg play only a limited role in the development of CM. Thus we investigated the role of CTLA-4 on the evolving T effector cells.

To this end C57Bl/6 mice were infected with Plasmodium berghei ANKA infected erythrocytes. During the development of the disease the expression of CTLA-4 on T cells and their function was investigated. CTLA-4 expression is strongly induced on CD4+ T cells. On day 6 of infection up to 50% of CD4+ cells express CTLA-4 but remained Foxp3−. Expression of CTLA-4 on Treg is also increased, but no expansion of Treg was seen. We observed rather a decline in the ratio of Treg to Ter.

After restimulation almost all IFN-γ producing cells are CTLA-4+. In contrast TNF-α is produced predominantly by CTLA-4− cells. By means of an IFN-γ secretion assay we isolated IFN-γ+ CTLA-4− cells and tested them in an in vitro suppression assay. These CTLA-4− Foxp3+ cells inhibit proliferation and IL-2 secretion as effective as conventional Treg. The high number of CTLA-4− cells suggested a bystander activation of CD4+ T cells. To proof this hypothesis we made use of transgenic P. berghei ANKA parasites expressing Ova323-339. Transfer of CFSE labelled OT-2 cells into mice infected with Ova323-339 transgenic or wildtype parasites resulted only in division and CTLA-4 expression in mice infected with the Ova-transgenic parasites which indicates that these CTLA-4 Foxp3+ cells are conventionally activated antigen-specific T cells.

These results demonstrate a strong induction of CTLA-4 on activated CD4+ T cells rather than on Treg during the course of murine malaria. These cells show no anergic phenotype since they produce IFN-γ but have acquired a suppressive phenotype. The exact mechanism of this suppression remains to be solved.
Analysis of the immunoregulatory function of ADAR1

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Introduction:
Adenosine-to-inosine (A-to-I) editing of primary transcripts in the nucleus recodes exonic information and can lead to structural and functional changes in the encoded protein. In vertebrates three members of the adenosine deaminase acting on RNA (ADAR) family are known: ADAR1, ADAR2 and ADAR3. ADAR1 has several features that distinguish it from the others. These features comprise two putative Z-DNA-binding domains, a third dsRNA-binding region, more widespread expression and transcription originating from at least two promoters. One promoter directs transcripts inducible by type I and type II interferon (IFN) that encode full-length ADAR1 protein (p150). The other promoter provides for constitutive expression of an amino-terminally truncated ADAR1 protein (p110) which localizes to the nucleus, consistent with involvement in editing of pre-mRNA, whereas p150 is found in both the nucleus and cytoplasm.

Objectives:
Goal of this study is to characterize the immunological role of ADAR1 in the context of contact with various Pattern-recognition receptors (PRR) ligands and viruses.

Methods:
We established ADAR1 deficient macrophages and dendritic cells using the Cre/loxP recombination system (ADAR1fl7-9/LysMcre or CD11cCre). For the human system we developed an ADAR1 knockdown in human monocytes by using short inhibitory RNAs (siRNAs).

Results:
ADAR1 deficient cells show an equal expression of corresponding differentiation markers and viability in comparison to ADAR1 competent cells, while expression of certain activation markers is increased. In all analyzed cell systems ADAR1 deficiency strongly modified the release of type I IFN upon stimulation with various PRR ligands and viruses. Especially in murine macrophages ADAR1 deficiency leads to an abolished release of IFNα and a 50% reduction in IFNβ secretion after virus infection or stimulation with viral PAMPs. LPS stimulation increased IFNβ production significantly in ADAR1 deficient cells, indicating a differential role of ADAR1 in type I IFN production. Of note, interleukin-6 (IL-6) secretion was mainly unaffected. Further investigations of signalling molecules, important for the induction of type I IFNs, revealed a remarkable pronounced type I IFN signature at early time points in ADAR1 deficient macrophages upon stimulation with LPS consistent with the increased IFNβ level. Surprisingly even in context of virus infection ADAR1 deficiency increased the type I IFN signature at later time points, however secretion of IFNβ was decreased and in case of IFNα completely abrogated. Notably IRF3 as well as the activated phosphorylated form were increased upon stimulation with LPS or virus. Immunofluorescence data suggest that in case of virus infection despite elevated cytosolic IRF3 levels the nuclear translocation of IRF3 is inhibited in ADAR1 deficient macrophages, however LPS stimulation leads to IRF3 translocalisation.

Conclusions:
Our findings indicate that ADAR1 is an important modulator of type I IFN responses to PRR ligands and viral infections.
The role of the Aryl hydrocarbon Receptor Repressor (AhRR) in innate immunity and infection

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Introduction:
The Aryl hydrocarbon Receptor (AhR) is known to be a ligand activated transcription factor and is involved in the detoxification of xenobiotics. The AhR is regulated by the Aryl hydrocarbon Receptor Repressor (AhRR) through a negative feedback mechanism. The AhRR acts by competing with AhR for dimerization with Aryl hydrocarbon receptor nuclear translocator (ARNT) and binding to xenobiotic responsive element (XRE) sequences in the promoter regions of its target genes (e.g. CYP1A1, cytokines), to regulate their expression.

Objectives:
The aim of this study is to examine the expression and function of the AhRR in innate immunity and infection.

Methods:
To determine the expression and function of AhRR, we generated AhRR/EGFP-reporter (AhRR+/+) and knockout mice (AhRR−/−). The impact of AhR and AhRR signaling was analysed upon TLR and inflammasome stimulation in BMDC and BMMΦ cultures from these mice. Additionally, we used a murine LPS induced septic shock model and performed oral infections with Citrobacter rodentium to specify the role of AhRR in vivo.

Results:
Analysis of naive AhRR+/+ mice revealed constitutive AhRR expression, mainly in immune cells of barrier organs. TLR stimulation led to enhanced AhRR expression in dendritic cells in vitro and in vivo. Contrary, in BMMΦ an increase of AhRR expression was detected upon stimulation with the AhR ligand 3MC.

Further, AhRR−/− mice are protected from LPS induced septic shock and are also protected from infections with Citrobacter rodentium. Using the LPS induced septic shock model we demonstrated that AhRR−/− mice show reduced levels of cytokines such as TNFα, IFNγ, IL-12p70, IL-10 and IL-1β systemically as well as locally in lung and liver. Thioglycolate elicited macrophages of AhRR−/− mice exhibited diminished levels of IL-1β as well. Moreover, a reduced apoptosis rate upon LPS injection was detectable in the livers of AhRR−/− mice. However, in response to LPS, or a typical inflammasome inducer such as alum crystals, BMMΦ of AhRR−/− mice showed an enhanced expression and secretion of IL-1β.

Conclusion:
AhRR expression enhances the immune response to LPS, presumably by feedback inhibition of the AhR. Our findings suggest a cross talk between AhR/AhRR activation and TLR-induced signaling and the inflammasome.
Computer-based algorithm for pre-selection of vaccine-candidates against murine cutaneous leishmaniasis

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Cutaneous leishmaniasis is caused by the protozoan Leishmania (L) major. Healing of L. major infections is based on IFNg secretion by CD8+ and CD4+ T cells. Currently, no vaccine against this human pathogen exists. To expedite the generation of a vaccine, we first identified the most abundant proteins of both life-forms - infectious-stage promastigotes and intracellular amastigotes - of L. major by quantitative mass spectrometry. Next, we chose 300 Kb/Db peptides based on their predicted immunoreactivity using the computer-based algorithm SYFPEITHI. In a next step, for pre-selection of possible vaccine-candidates, we compared four different algorithms (SYFPEITHI, BIMAS, IEDB and MAPPP): this revealed p54 as highly interesting vaccine-candidate. However, to confirm the in silico pre-selection, all 300 peptides were tested in vitro experiments. Peptides were co-cultured with dendritic cells and primed CD8+ T cells - both extracted from C57BL/6 mice - for 48 hrs. Supernatants were assayed for the amounts of IFNg, IL-17A, IL-4 and IL-10. To examine vaccination efficacy in vivo experiments, we tested 15 peptides for their ability to protect mice against L. major infection using a prime/boost (P/B) (10 µg peptide twice) approach. Here, p54 was not able to reduce lesion volumes. Upon a change in the immunization strategy, where mice were immunized in a prime/boost/boost (P/B/B) (20 µg followed by 2x 10 µg peptide) approach, p54 significantly reduced lesion volumes compared to control. p54 is part of a L. major housekeeping protein, and can thereby possibly be found in other Leishmania subspecies as well. In summary, we identified one peptide which protected C57BL/6 mice against challenge with L. major, thereby providing the basis for establishing a long desired vaccine. For further studies, using different algorithms for pre-selection of possible vaccine-candidates is a useful tool and will therefore aid the development of a vaccine against this important human pathogen.
TLR9 stimulation via CpG ODN enables T cell-independent class switch recombination in human B lymphocytes

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Question:
Class switch recombination (CSR) is a prerequisite for the formation of B cell memory which is required for a long term protection from infection. In the past it was undisputable that this type of B cell response requires T cell help. However, this view point has lately been challenged, raising the question whether immunoglobulin gene rearrangement might also be possible in the absence of T cells.

Methods:
CSR in human peripheral blood IgM+ B cells stimulated with CpG ODN was assessed by flow cytometry, AID expression, detection of immunoglobulin (Ig) and switch circle transcripts.

Results:
We previously described that the TLR9 ligand CpG ODN, which is often considered a polyclonal B cell mitogen primarily targets IgM-expressing B cells and not, as suspected, switch memory B cells (Ref. 1). These results were further corroborated by the finding that B cells stimulated with CpG ODN produce high amounts of IgM but only negligible amounts of IgG when compared to unstimulated controls. Furthermore, relevant IgA production was only observed when B cells were challenged with BAFF additionally to CpG ODN. Further analysis revealed that IgM, IgG and IgA production were derived from CD27+ memory B cells. We, therefore asked whether secreted IgG or IgA could originate from CD27+ IgM memory B cells.

B cells stimulated with CpG ODN expressed activation induced cytidine deaminase (AID) and elevated levels of PCNA, two molecules participating in CSR. We, next, provided evidence for CSR by flow cytometry and detection of switch circle and Ig transcripts. Our results showed that, indeed, IgM+ B cells stimulated with CpG ODN initially expressed IgM and only later acquired IgG expression. This was not seen with purified naïve CD27- B cells. Detection of switch circle transcripts Iggammaγ1/2-Cm, Iggammaγ3-Cm, Iggammaγ4-Cm and absence of IgG transcripts in the unstimulated IgM+ B cells but detection of IgG and IgA transcripts after stimulation corroborated these findings. Our data further highlighted the requirement of BAFF in human serum for the induction of CSR by CpG ODN, because neutralization of BAFF impeded CSR.

Conclusions:
Altogether, our data provide evidence for T cell-independent CSR in human peripheral blood B cells stimulated with CpG ODN. Our data further suggest that CSR is induced in CD27+ IgM+ B cells. We further demonstrate that BAFF is a prerequisite for T cell-independent TLR9-triggered CSR. In vivo analyses will have to clarify whether CpG ODN-triggered CSR could be exploited in vaccine design.

Reference:
Non-redundant functions of IFN-β within the central nervous system protect against virus infection

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Previous studies showed that type I interferon receptor (IFNAR) triggering is critically required to control many different virus infections. In mice one IFN-β and 14 different IFN-α isotypes interact with IFNAR. Infection experiments with peripherally challenged IFN-β−/− mice did not reveal any overt phenotype suggesting that IFN-α and IFN-β play a redundant role under such conditions. Here we readdressed IFN-β function under conditions of intranasal (i.n.) virus infection. Under such conditions IFN-β−/− mice showed a significantly enhanced vulnerability to lethal vesicular stomatitis virus (VSV) infection. A detailed analysis revealed that upon i.n. VSV instillation of C57BL/6 mice the virus readily infected olfactory sensory neurons and moved along axons into the glomerular layer of the olfactory bulb where it was arrested in an IFNAR-dependent manner. After i.v. as well as i.n. VSV infection IFN-α, but no IFN-β serum responses were induced. Analysis of IFN-β reporter mice revealed that within the central nervous system protective IFN-β was triggered primarily in neuronal cells of the olfactory bulb. Interestingly, locally produced IFN-β was active also in distal brain areas as evidenced by induction of GFP expression in the cerebrum of i.n. infected ISRE-eGFP reporter mice. Of note ISRE-eGFP mice intercrossed with IFN-β−/− show strongly reduced local IFNAR signaling within the CNS. This is in accordance with the observation that mainly IFN-β mRNA is upregulated in the brain of VSV infected animals, whereas the various IFN-α isotypes play only a minor role. In conclusion, our experiments are in accordance with the model that upon virus infection of the CNS IFN-β responses are selectively induced within the olfactory bulb that stimulate also distal brain areas, whereas IFN-α subtypes are primarily induced in the periphery and there account for protection of peripheral tissues.
Absence of Siglec-H in MCMV infection elevates Interferon alpha production by plasmacytoid dendritic cells but does not enhance viral clearance

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Introduction:
Plasmacytoid dendritic cells (pDCs) express the C-type lectin receptor Siglec-H and produce IFN-α, a critical anti-viral cytokine during the acute phase of MCMV infection. Siglec-H has been postulated to have possible pattern recognition function to bind to viruses however the in vivo ligands and biological functions of the receptor still remain incompletely defined.

Objectives:
Hence, to uncover the functional properties of Siglec-H in the context of in vivo MCMV infection, we generated a novel mouse strain to better understand this putative interaction Here we generated a novel bacterial artificial chromosome (BAC)-transgenic “pDCre” mouse which expresses Cre recombinase under the control of the Siglec-H promoter. By crossing these mice with a Rosa26 reporter strain, a representative fraction of Siglec-H+ pDCs are terminally labelled with tandem-dimer red fluorescent protein (td-RFP). To further understand the importance of Siglec-H in viral clearance, we lethally irradiated CD45.1+ wt mice and reconstituted them with wt or Siglec-H−/− BM.

Methods:
In vitro infection of sorted pDCs:
To study the direct interaction and mechanism underlying the effects of MCMV infection on Siglec-H, we first in vitro sorted pDCs from Flt3-L mixed BMDC cultures and infected them with MCMV-GFP, CpG-A treated or mock infected with PBS.

In vivo infection of pDCre and Siglec-H−/− mice:
For our in vivo studies, pDCre mice and Siglec-H−/− mice were infected intra-peritoneally with 5 x 10⁴ pfu of wt Smith strain of MCMV.

Results:
Interestingly, systemic MCMV infection causes downregulation of Siglec-H expression while this decline occurs in a TLR9- and MyD88- dependent manner. To elucidate the functional role of Siglec-H during MCMV infection, we utilized a novel Siglec-H-deficient mouse strain. In the absence of Siglec-H, the low infection rate of pDCs remained unchanged, and pDC activation was still intact. Strikingly, Siglec-H deficiency induced a significant increase in serum IFN-α levels following systemic MCMV infection. Although Siglec-H modulates pDC activation and anti-viral IFN-α, the control of viral replication remained unchanged in vivo.

Conclusion:
Overall, our study examines some key questions to provide a better insight underlying the functional role of Siglec-H in an in vivo viral infection model. Additionally, our novel mouse models perceive as valuable tools to shed further light on pDC biology in future studies as well as can be adopted in other viral models where pDC function determines the outcome of disease.
A novel function for BPI: BPI influences the course of murine listeriosis


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Introduction:
Antimicrobial proteins have an important function in the innate immune system. The bactericidal/permeability-increasing protein (BPI) is an antimicrobial protein expressed in neutrophil granulocytes, dendritic cells and epithelial cells. It has been shown that human BPI displays a strong antimicrobial activity against gram-negative bacteria. The function of BPI in vivo is largely unknown. Furthermore, the antimicrobial potential of BPI against gram-positive bacteria is only superficially analysed.

Listeria monocytogenes is an aerob, gram-positive bacterium which is employed for a well established murine listeriosis model. During the innate immune response this pathogen is controlled by three different cell types: macrophages, dendritic cells and neutrophil granulocytes. Dependent of the infection dose and the functionality of these cells, the mice are able to control the bacteria or develop a severe infection.

Objectives:
Our aim was analyzing the effect of BPI against gram-positive bacteria in a murine listeriosis model.

Material & methods:
L. monocytogenes strain EGD were analysed in an in vitro killing assay with BPI. Thereafter, WT and BPI-Knock out mice (provided by Prof. Gessner, Regensburg) were infected intragastrally and intravenously with L. monocytogenes. The bacterial load in various organs was analyzed kinetically. Furthermore the inflammatory response in the serum of the infected mice was determined via TNF and IL-6 ELISA.

In order to determine which cells are responsible for the observed phenotype of the BPI-KO mice bone marrow-derived dendritic cells, macrophages and neutrophil granulocytes of WT and BPI-KO mice were infected or stimulated with listeria in vitro. The interaction of the cells with the listeria was determined by analyzing the phagocytosis and killing rate of intracellular listeria by gentamicin protection assays and CFSE-labeled Listeria. TNF and IL-6 production and the activity of iNOS were measured.

Results:
Surprisingly BPI has direct antimicrobial activity against L. monocytogenes. Consequently BPI KO mice suffer from a more severe infection as compared to WT mice. Therefore BPI-KO mice showed higher bacterial load in the spleen after infection and the mice produced more IL-6 and TNF in the serum.

In vitro neither macrophages nor dendritic cells were responsible for the observed phenotype of the BPI-deficient mice. These cells controlled intracellular Listeria with the same potency as cells derived from WT mice. However neutrophils isolated from BPI-deficient mice displayed a more robust response after stimulation with inactivated listeria when compared to neutrophils from WT mice. This could be determined by higher production of TNF in the supernatant and a stronger iNOS activity.

Conclusion:
BPI displays antimicrobial activity against Listeria in vitro. Moreover BPI expressed by neutrophil granulocytes is an important mechanism in controlling Listeria in the early phase of the immune response.
A new role for mesenchymal stem cells as sensors and immune regulators of infection

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Background:
The anti-inflammatory immunoregulatory properties of mesenchymal stem cells (MSC) during cellular therapy are well documented. However, recent studies demonstrated the expression of toll-like receptors (TLRs) on MSCs, which opens the possibility for a role of MSCs as sensors and immune regulators in infections. In this project we investigate the response of MSCs of the nasal mucosa to viral TLR ligands.

Materials and Methods:
Human nasal mucosal MSC (nmMSCs) were isolated from nasal concha and showed classic hallmarks of MSC phenotype, function and tri-lineage differentiation. TLR expression and expression of cytokines in response to different TLR agonists were assessed by quantitative PCR. The release of cytokines by nmMSC was analysed by ELISA and multiplex assays. Characterisation of the signalling mechanisms was performed by immunofluorescence and western blot. Paracrine effects were examined by collecting the soluble factors released after Poly(I:C) stimulation of nmMSC and incubating these factors with resting nmMSC from the same donor.

Results:
Our studies showed that nmMSC expressed a wide repertoire of TLRs (1-10) to sense and respond to pathogens. Among different TLR ligands tested, TLR3 ligand Poly(I:C) gave the greatest response followed by TLR4 agonist LPS. nmMSC constitutively secreted inflammatory cytokines and type I interferon; stimulation with Poly(I:C) subsequently elicited p38 MAPK, NFκB and IRF3 signalling and enhanced the production of IL-6, IL-8 and type I interferon. Finally these induced soluble mediators acted in a paracrine manner causing an amplification loop which primed other nmMSC increasing their sensitivity to viral PAMPs.

Conclusions:
These data shed new light on the response of MSC to pathogenic stimulation. Their ability to sense viral PAMPs and induce a pro-inflammatory and anti-viral response identifies tissue-specific MSC of the nasal mucosa as a potential new regulator in anti-viral cellular immunity.
The C-type lectin receptor DCIR is involved in the genesis of cerebral malaria

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Malaria is one of the main global causes of death from infectious diseases, resulting in more than 200 million clinical cases per year. The most severe complication of an infection with Plasmodium falciparum is cerebral malaria (CM) mainly affecting children below the age of five in malaria-endemic areas. The murine Plasmodium berghei ANKA (PbA) infection model shares some characteristics with the human CM, thus it can be used to identify crucial players in CM pathogenesis. While adaptive immunity during the course of malaria has been intensively investigated, little is known about the role of innate immunity in CM induction. C-type lectin receptors (CLRs) are a family of pattern-recognition receptors in innate immunity. They are predominantly expressed by antigen-presenting cells and bind to glycan structures on pathogens, but also self-antigens. Currently, it is unknown if CLRs contribute to immunity during malaria infection or if they may even be involved in immune pathology such as CM development.

In this study, we focused on the role of the CLR Dendritic cell immunoreceptor (DCIR) in CM genesis. DCIR has an extracellular carbohydrate recognition domain and an intracellular tyrosine-based inhibitory motif. To assess whether DCIR is involved in immune pathology during the course of malaria, the murine PbA infection model was employed.

Using the murine PbA infection, we show here that DCIR indeed contributes to CM induction. While PbA infection led to 80% CM in wild-type C57BL/6 mice, DCIR-deficient mice were highly protected from CM. In agreement with the reduced CM incidence, brain inflammation and T cell sequestration in the brain were markedly reduced in PbA-infected DCIR-deficient mice. The reduced T cell sequestration in the brain was caused by a decreased serum TNF-α level as well as a modulated splenic T cell activation in PbA-infected DCIR-deficient mice.

In conclusion, this study indicates a critical role for DCIR in CM induction, thus highlighting the importance of this CLR in innate immunity during malaria.
RNA and Imidazoquinolines are sensed by distinct TLR7/8 ectodomain sites resulting in functionally disparate signaling events

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Toll-like receptors (TLR) 7 and 8 are pattern recognition receptors controlling antiviral host defense or autoimmunity. Apart from foreign and host RNA, synthetic RNA oligoribonucleotides (ORN) or small molecules of the imidazoquinoline family activate TLR7 and 8 and are being developed as therapeutic agonists. The structure-function relationships for RNA ORN and imidazoquinoline sensing and consequent downstream signaling by human TLR7 and TLR8 are unknown. Here we report that TLR7 and 8 ectodomains discriminate between RNA ORN and imidazoquinolines by overlapping and non-overlapping recognition sites to which murine loss-of-function mutations and human naturally occurring hyporesponsive polymorphisms map. Proteome- and genome-wide analyses in primary human monocyte-derived dendritic cells show that TLR8 sensing of RNA ORN vs. imidazoquinoline translates to ligand-specific differential phosphorylation and transcriptional events. Thus TLR7 and TLR8 signal in two different ‘modes’ depending on the class of ligand. Considering RNA ORN and imidazoquinolines are regarded as functionally interchangeable, our study highlights important functional incongruities whose understanding will be important for developing TLR7 or 8 therapeutics with desirable effector and safety profiles for in vivo application.
The context of peptide localization within an MCMV protein critically defines the immunodominance and type of CD8 immune response

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Defined cytomegalovirus (CMV) antigens induce immunodominant responses which contract upon the clearance of lytical infection and assume a central memory (CM) phenotype (CD62L+CD127+). Other peptides induce a response that inflates over time and stabilizes at high levels of effector memory (EM) CD8 cells, a phenomenon termed memory inflation. While the reasons for this difference remain incompletely understood, we showed recently that the context of gene expression defines the kinetic of the immunodominance hierarchy of murine CMV (MCMV) genes. The insertion of the K\textsuperscript{b}-restricted SSIEFARL peptide at the 3' end of the MCMV \textit{ie}2 gene induced inflationary responses in mice infected with the mutant virus, but the MCMV expressing the same peptide in the context of the M45 gene induced a contracting response. Since the peptide was the same, the difference in the response size and kinetic had to be caused by differences in the gene expression context (Dekhtiarenko et al. J. Immunol 2013).

We extended this study to investigate the phenotype of responding cells and the importance of the peptide localization within the same protein. Surprisingly, the SSIEFARLL-specific CD8 T-cells showed sustained EM phenotypes in mice infected with the recombinant virus carrying the peptide at the 3' end of the M45 gene. Even more remarkably, the M45 gene encodes an endogenous immunodominant peptide (HGIRNASFI), which induced CD8 T-cell responses that promptly switched to CM phenotypes. To test if this difference in phenotypes was due to differences between peptide sequences, or in their localization within the M45 gene, we inserted a sequence encoding the HGIRNASFI peptide at the 3' end of the M45 gene, thus generating the MCMV\textit{dM45} recombinant. The kinetic of HGIRNASFI-specific CD8 T-cells in mice infected with MCMV WT or MCMV\textit{dM45} was similar, but the initial response was 8 fold stronger when HGIRNASFI was present at the 3' end of the gene and almost 40-fold stronger by day 120 post infection. Interestingly, about 40% of HGIRNASFI-specific CD8 T-cells preserved the EM phenotype in MCMV\textit{dM45} infection, whereas almost all of these cells switched to the CM phenotype in mice infected with MCMV WT.

Therefore, our data show that the location of an antigenic peptide within a CMV protein may result in drastic differences in the magnitude of the CD8 response, in the size of the long-term memory pool and in the phenotype of responding cells. Our data may be explained either by differences in proteasomal processing and/or in peptide loading on MHC molecules and bear huge relevance for the rational design of CMV-based vaccine vectors.
Impaired anti-bacterial immunity in mice with cutaneous RANKL overexpression

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Infectious diseases frequently occur in humans and Staphylococcus aureus (S. aureus) represents the bacterial pathogen causing the majority of cutaneous infections. Bacterial infections are controlled by the innate as well as adaptive immune system and in this context pathogenic microorganisms need to suppress host anti-microbial immune responses. For the suppression of cellular anti-microbial immunity, regulatory T cells (Treg) play a crucial role since Treg are able to inhibit MHC class I- and MHC class II-restricted immunity. Previously, by using transgenic (tg) mice over-expressing RANKL (CD254) in the epidermis (K14-RANKL tg mice) we have demonstrated that RANK-RANKL signaling plays an important role for the peripheral expansion of Treg. As RANK and RANKL are up-regulated in inflammatory skin lesions we investigated the relevance of RANK-RANKL interactions for the regulation of cutaneous anti-bacterial immunity. Therefore, K14-RANKL tg mice and wildtype (wt) controls were intradermally infected with 2 x 10^7 colony forming units (CFU) of the S. aureus strain SH1000. Notably, tg mice developed larger skin lesions compared to wt controls, which was paralleled by altered levels of bacteria in lesional skin of tg versus wt mice as evidenced by Gram staining and quantitative real-time PCR. FACS analyses of cell subsets in lymph nodes draining cutaneous lesions revealed up-regulated numbers of Treg in S. aureus infected K14-RANKL tg mice compared to wt controls suggesting that the expansion of Treg might result in the inhibition of anti-bacterial immune responses in K14-RANKL tg mice. Thus, we quantified the numbers of effector cells known to play a role in anti-bacterial immunity, such as neutrophils, macrophages, Th1 or Th17 cells in cutaneous lesions and in regional lymph nodes. Interestingly, we could show that the numbers of macrophages and the neutrophil count was reduced in cutaneous lesions from tg compared to wt mice. Moreover, in lymph nodes draining lesional skin from K14-RANKL tg mice we detected decreased levels of CD4+ T cells expressing transcription factors, surface markers as well as pro-inflammatory cytokines specific for Th1 or Th17 cells suggesting that Treg might indeed have suppressed primary anti-bacterial immune responses finally leading to larger skin lesions. To analyze whether RANK-RANKL signaling also affects memory responses wt and K14-RANKL tg mice were re-infected with 4 x 10^7 CFU of the S. aureus strain SH1000 10 weeks after the primary infection. Similar to primary infected mice, re-infected K14-RANKL tg mice developed larger skin lesions, whereas the difference in skin lesion size between wt and tg mice was comparable after the first and second bacterial challenge suggesting insignificant effects of RANK-RANKL signaling on anti-bacterial memory. In support of this, we observed similar numbers of central and effector memory cells in re-infected wt and tg mice as evidenced by CD62L and CCR7 staining as well as comparable levels of S. aureus specific antibodies in the serum of wt and tg mice indicating a rather minor role of the RANK-RANKL pathway in the regulation of memory anti-bacterial immune responses. Together, these data indicate that RANK-RANKL interactions are critically involved in the suppression of primary innate and adaptive anti-bacterial immunity but most likely do not modulate memory responses.
Enhanced susceptibility to opportunistic infection with *Pseudomonas aeruginosa* during polymicrobial sepsis is associated with an impaired function of Natural killer cells

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Polymicrobial sepsis, a systemic mostly bacterial infection, results in the development of immunosuppression that leads to an increased susceptibility to secondary infections. The opportunistic gram-negative pathogen *Pseudomonas aeruginosa* frequently induces pneumonia in septic patients. An efficient immune defense against *P. aeruginosa* is based on the activation of functional Natural Killer cells (NK cells) that produce IFN-gamma in response to IL-12 released by accessory cells as macrophages and dendritic cells (DC) in the lung. The increased susceptibility to *P. aeruginosa* after polymicrobial sepsis is associated with a suppressed formation of IFN-gamma in the lung and points to an involvement of NK cells. We investigated the impact of murine polymicrobial sepsis on NK cell function in the lung in response to secondary infection with *P. aeruginosa*. Sepsis was induced by cecal ligation and puncture (CLP) in female BALB/c mice and after four days the function of lung-derived leukocytes was analysed *in vivo* and *in vitro*. Four days after sepsis induction mice were more susceptible to *P. aeruginosa* infection *in vivo* compared to sham operated mice. At this time point CLP mice showed decreased numbers of NK cells and DC in the lung. The production of proinflammatory cytokines from lung leukocytes, especially IFN-gamma and IL-12, was suppressed whereas the IL-10 level increased after *in vitro* stimulation with *P. aeruginosa*. *In vivo* infection with *P. aeruginosa* led to infiltrating DC into the lung but the NK cell number remained constant. Depletion experiments showed that CD11c+ DC and alveolar macrophages were necessary for an effective IFN-gamma production by NK cells. However, CD11c+ cells from CLP mice were inferior to cells from sham mice in NK cell stimulation. Replacement of CD11c+ cells by naïve CD11c+ cells increased the IFN-gamma production from sham leukocytes but not from CLP leukocytes. IFN-gamma production from NK cells of CLP mice could be partly restored by addition of recombinant IL-12 whereas neutralization of IL-10 was ineffective. These findings suggest that a higher susceptibility to pulmonary infections after polymicrobial sepsis is mediated by a dysfunction of NK cells in the lung possibly caused by a disturbed interaction with accessory cells.
Essential role of CD40L expressed by CD8+ T cells in cellular immunity

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CD40L is a highly specific marker for activated CD4+ helper T cells and its interaction with CD40 on different target cells are thought to be responsible for a manifold variety of functions in the immune system. It is well accepted that CD40L-CD40 interactions link the activation of cellular immunity with the maturation of professional antigen-presenting cells as well as with isotype class switching and B cell differentiation. However, the role of CD40L-CD40 axis signaling for the induction and maintenance of stable immunological memory is less understood.

We report here on a new distinct and large subset of memory CD8+ T cells able to express CD40L that can be detected in various human and murine immune responses against pathogens such as CMV, Influenza, Listeria monocytogenes (L.m.), LCMV, and immune responses against tumor antigens such as SV40 large T antigen (Tag). In in vivo infection models such as LCMV and L.m., mice lacking CD40L exclusively in CD8 T cells demonstrated a reduced expansion capacity of CD8+ T cells at the peak of response as compared to wild type mice. This individual defect in cellular immunity cannot be compensated by the fact that CD40L competent CD4+ T cells are present in the analyzed mice. Moreover, during the primary acute immune response in LCMV the vast majority of CD40L is provided by CD8+ T cells rather than by CD4+ T-cells.

We hypothesize that CD40L+ CD8+ T cells can provide supportive immunological helper functions essential for a competent primary cellular immune response. The result that high frequencies of CD40L competent memory CD8+ T cells are generated in many pathogen-specific immune responses suggests that immunological help as provided by CD8+ T cells through CD40L-CD40 signaling maybe crucial for secondary immune responses in infection as well.
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Effector versus regulatory role of ICOS during influenza infection

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Inducible co-stimulator (ICOS) is one among the T cell co-stimulatory molecules, expressed on the activated T cells and mediates its function by binding to its ligand, ICOSL (1). ICOS has multiple roles on immune functions (2) (3) (4).

Aim:
Our major aim remains to elucidate if ICOS possesses an effector function in viral clearance or has any regulatory role to suppress the infection-mediated lung inflammation.

Methods:
For this purpose, BALB/c mice were infected intra nasal with HINI strain of influenza virus. Additionally, one group of infected mice received ICOS agonist antibody and the control group received no antibodies. On day 7 and 9 post-infection, i.e. during the peak phase of adaptive immunity, the mice were sacrificed. Both CD4+ and CD8+T cells from the lung, bronchial lymph nodes, and spleen were analyzed for the expression of effector T cell markers and their capacity to lyse the viral peptide loaded cells. Furthermore, viral loads were quantified. Moreover, bronchoalveolar lavage fluid and serum were analysed for IFN-gamma, TNF-alpha, IL-6 and IL-10 and for the immune-regulatory enzyme IDO, respectively. Parallely, expressions of regulatory related markers like FoxP3, neuropilin-1, were also analysed.

Results:
We found that ICOS signalling during influenza infection resulted in an overall reduction of CD8+ T cells, decreased number of effector T with subsequent delay in the viral clearance in ICOS treated group compared to its isotype control and the infected group alone. But, IL-6 levels were markedly increased in ICOS agonist group compared to isotype and infected group alone. Intriguingly, immunopathology was reduced and at the same time there were decreased Tregs and altered levels of IL-10 in ICOS treated group. Moreover, no significant difference in serum and BALF IDO activity between the groups was found.

Conclusion:
ICOS signalling neither have any prominent role in effector function or has any profoundly distinct role in immune regulation.

References:

Figure Legend:
Figure 1. Decreased lung lavage and lung expression of polyclonal CD8+T cells between ICOS agonist treated group and isotype group and infected PBS control, on day 7 and day 8, respectively.
Figure 2. Quantification of viral load in the lung samples of 3 groups of mice was represented. Delay in the viral clearance was clearly found with ICOS agonist treated group compared to its isotype and PBS control groups. Significant differences and tendencies were found between ICOS agonist treated, isotype and PBS control group on day 7, 8 and 9.
Figure 1

**BALF**

- p=0.0001
- p=0.0020
- p=ns

**LUNG**

- p=0.031
- p=0.035
- p=ns

Day 7

Day 8

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Figure 2

- p=0.022
- p=ns
- p=ns

Day 7

Day 8

Day 9

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Characterization of the murine pulmonary phagocytic network during *Aspergillus fumigatus* infection

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**Introduction:**
Several hundred spores of the saprophytic mold *Aspergillus fumigatus* are being inhaled by all humans daily. The innate immune system of an immunocompetent individual with a variety of phagocytic cell types encounters this pathogen in a way that it cannot establish a severe infection. However with the enlargement of immunosuppressed patients in the clinic, especially bone marrow and solid organ transplant recipients, this ubiquitous fungus has become a major reason for many fatal human infections, worldwide. Despite a broad range of research in this field the objective impact of all alveolar phagocytic cell types involved in fungal clearance remains unknown at present.

**Objective:**
In this study we introduce the phagocytic network of a murine lung upon *A. fumigates* infection. The main objective is the characterization of all phagocytes involved in the immunological fight against the fungal attack down to the molecular level. In contrast to the published studies which have analyzed different parameters of the whole lung cells, we aim at investigating all cell types individually. Therefore we first have to establish suitable cell isolation protocols which leave the target cell populations as untouched as possible. Having hands on these lung phagocytes we will assess their activation status, in vitro and in vivo phagocytic capacity, cytokine profile upon contact to *A. fumigatus* and the proteomic differences to cells isolated from uninfected lungs.

**Methods:**
To reach this goal we utilize two different approaches: 2D gel electrophoresis followed by MALDI-TOF/TOF analysis and direct mass spectrometry independent of 2D gel electrophoresis. In parallel we analyze the isolated cells for the fungal uptake rate by a time-lapse in vitro microscopy and by a flow cytometry approach. Histological lung images are also taken into account to verify our findings with the in situ situation.

**Results:**
Beside well known phagocytes such as macrophages, neutrophil granulocytes and dendritic cells preliminary experiments of our lab also suggest epithelial cells to be involved in phagocytic immune responses. As this cell type is the least investigated one in this context and therefore in our hands the most interesting immune cell, we first have focused on this entity. We succeeded in defining two protocols for isolating alveolar epithelial type I and type II cells with a purity of greater than 90 %, each. At the moment we are gathering experimental data dealing with the differentially regulated proteome of these cells isolated from infected and uninfected lungs.

**Discussion:**
Future work in this project will deal with the isolation and analysis of further phagocytic cell types. We will aim at identifying suitable surface molecules of important monocyte subsets such as alveolar macrophages, interstitial tissue resident macrophages and dendritic cells which can be used as markers for novel negative enrichment protocols. Further including experimental results of neutrophils the project will be brought down to a round figure which will demonstrate the differences in the alveolar phagocytic network upon fungal infection. We believe that this will dramatically increase our understanding of how the orchestration of immune cells is mediated in the setting of a fungal attack.
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**Lipopeptide specific T cells in the early immune response against mycobacteria – investigations in the guinea pig model**

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**Introduction:**

A rich and highly diverse lipid content is one of the most particular features of the mycobacterial cell wall. 10% of the mycobacterial genome is dedicated to genes involved in lipid and lipoprotein synthesis. Although the main focus in tuberculosis immunology and vaccinology is on protein antigens, the outstanding immunogenicity of lipid and lipopeptide antigens has been well known for decades. In this line, two recent publications [Bastian et al., J Immunol, 2008; Seshadri et al., J Immunol, 2013] have described a novel, highly immunogenic class of MHC-II restricted lipopeptide antigens.

**Objectives:**

Our hypothesis is that this currently uncharacterized lipopeptide fraction is important for the overall immunogenicity of mycobacteria of the tuberculosis complex. The aim of the project is to establish the relevance of this specific class of antigens and characterize its immunogenic function in vitro and in vivo.

**Materials & methods:**

As a small animal model we used guinea pigs because they are sensitive to mycobacterial infections and develop a disease pattern that is very similar to humans and cattle. To test for the immunogenicity of lipopeptides *ex vivo* we sensitized guinea pigs with BCG, the live attenuated mycobacterial vaccine strain. After 4 weeks we obtained blood, isolated PBMCs, stained with CFSE and stimulated with different mycobacterial chloroform:methanol extracts (CME) and lipopeptide-enriched subfractions. For comparison we used tuberculin (PPD) as a total mycobacterial antigen preparation. After 5 days antigen-specific T cell proliferation was determined by flow cytometry. The quantitative relevance of lipopeptide reactivity was tested in restimulation assays. To this end we stimulated PBMCs in a 1st round with PPD or CME. The antigen-specific T cells were then sorted and re-stimulated in a 2nd round with PPD, CME or individual lipopeptide enriched fractions.

**Results:**

In accordance with the cited publications we observed a vigorous antigen-specific cell proliferation of CD4+ guinea pig T cells in response to CME or the lipopeptide-enriched subfractions. The strength of the lipopeptide induced T cell proliferation was comparable to the response to PPD. The restimulation assay revealed that T cells that were antigen-specifically expanded by PPD-stimulation in the 1st round showed a substantial cross-reactivity to lipopeptide enriched antigen preparations (CME and its subfractions) in a 2nd round and likewise CME-expanded T cells proliferated in response to PPD.

**Conclusion:**

In summary, our data indicate that lipopeptide-specific T cells predominate in the early cellular immune response after BCG vaccination. This is in accordance with observations in humans and emphasizes the relevance of this novel class of immunogenic lipopeptides. Ongoing efforts are now aiming to identify individual immunogenic lipopeptides and to find out the role lipopeptide-specific T cells play in the immune...
Listeria monocytogenes infection leads to the onset of type 1 diabetes in predisposed mice

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Introduction:
Infections and toll-like receptor (TLR) mediated immune activation is believed to initiate or accelerate autoimmune diseases like type 1 diabetes in predisposed individuals. Moreover, microbial products have been demonstrated to interfere with the suppressive effect of CD4+CD25+ regulatory T cells (Tregs). To extend our knowledge on the impact of infections on diabetes onset we studied the outcome of infections on well-established self-tolerance in diabetes prone mice.

Methods:
Studies were performed using the INS-HAxTCR-HA mouse model for type 1 diabetes which is based on concomitant expression of the model self-antigen influenza hemagglutinin (HA) in the pancreas and HA-specific CD4+ T cells. Pancreatic self-antigen recognition by CD4+ T cells results in insulitis in all and diabetes development in about 40% of INS-HAxTCR-HA mice. Non-diabetic INS-HAxTCR-HA mice were infected with Listeria monocytogenes and diabetes induction was monitored by blood glucose measurement.

Results:
Upon infection of previously non-diabetic mice with Listeria monocytogenes tolerance to pancreatic self-antigen was broken and mice develop diabetes within 10–12 days. Loss of self-tolerance and diabetes onset were neither observed when non-diabetic INS-HAxTCR-HA mice were infected intra-nasally with influenza A virus nor when treated intravenously with heat-killed Listeria which are a well-known ligand of the TLR-2 or Listeria monocytogenes Δhly lacking the virulence factor listeriolysin needed to escape from the phagolysosome into the cytosol and for cell-to-cell spread. Interestingly, diabetes onset in INS-HAxTCR-HA mice infected with wild type Listeria correlates with decreased suppression capacity of CD4+CD25+ Tregs in infected mice and a robust type I interferon response.

Conclusion:
Since we have demonstrated before that regulatory T cells are critically involved in the protection of INS-HAxTCR-HA mice from progression of insulitis to diabetes we hypothesize that impaired function of Tregs during infection with Listeria monocytogenes may result in loss of T cell tolerance to pancreatic self-antigen and diabetes onset.
**Orientia tsutsugamushi induces apoptosis via TLR2 in non-infected bystander macrophages**

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**Introduction:**
*Orientia (O.) tsutsugamushi* is a mite-transmitted, obligate intracellular bacterium that is endemic in wide parts of Southeast Asia. It was shown before that *O. tsutsugamushi* elicits pro- and anti-apoptotic as well as pro-inflammatory pathways in its host cells. However, it remains unknown which components of the innate immune system play a role in tipping the balance between favouring bacterial growth and contributing to host defense. We previously identified Toll-like receptor (TLR)-2 as the most important pattern recognition receptor involved in innate signalling.

**Objectives:**
We investigated by which mechanisms TLR2-dependent signaling influences intracellular growth of *O. tsutsugamushi* in macrophages.

**Materials & methods:**
TLR2-competent and -deficient macrophages or bone marrow-derived dendritic cells were infected with *O. tsutsugamushi*. The production of pro-inflammatory cytokines such as TNF-α, interleukin-12 (IL-12) and IL-6 was measured by ELISA. Activation of the NF-κB signaling pathway was studied by immunofluorescence to assess the nuclear translocation of NF-κB. Intracellular growth of *O. tsutsugamushi* in both cell types was measured by quantitative real-time PCR. Induction of apoptosis in macrophages infected with different doses of *O. tsutsugamushi* was measured by FACS to determine the percentage of macrophages positive for Annexin V or TUNEL. To determine whether infected or non-infected macrophages become apoptotic, immunofluorescence staining for caspase-3 was used.

**Results:**
Within the first 24 hours post infection, the production of pro-inflammatory cytokines by BMDCs was dependent on TLR2-dependent signaling. The induction of the NF-κB pathway depended equally on TLR2. TLR2-deficient macrophages allowed a more efficient growth of *O. tsutsugamushi* compared to the wild type, which was measurable after 4 days post infection. In wild type macrophages, apoptosis was induced by *O. tsutsugamushi* infection in a dose-dependent fashion as measured by Annexin V and TUNEL positivity. In contrast, the induction of apoptosis was less efficient in TLR2-deficient macrophages and even decreased with higher infectious doses. When TLR2-competent macrophages were double-stained for *O. tsutsugamushi* and caspase-3, it became clear that the induction of apoptosis was confined to the non-infected macrophages, while infected macrophages remained negative for caspase-3.

**Conclusion:**
TLR2-dependent pathways restrict the intracellular growth of *O. tsutsugamushi*. While *O. tsutsugamushi* is also known to inhibit apoptosis in infected macrophages, TLR2-dependent signals were shown to be required for the induction of apoptosis in non-infected bystander macrophages. Innate signaling via TLR2 thus contributes to restrict further pathogen growth by the induction of apoptosis.
Chemokines responses in Cameroonian children with *Plasmodium Falciparum* malaria

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**Background and Aims:**
Malaria is an important parasitic infection. It causes significant mortality with children being more vulnerable. Chemokines regulate the host immune response to a variety of infectious pathogens. For instance, chemokines receptor CXCR3 and its ligand CXCL10 has been implicated as a biomarker in cerebral malaria in Ghanaian children. There is dearth of information on chemokines responses in malaria among Cameroonian children.

**Methods:**
Chemokines in the serum of the 80 children with *P. falciparum* malaria and 16 control subjects were analysed by ELISA using the manufacturer's instruction (Abcam, UK). Also their haemoglobin was determined by an automated 5 Evolution Diana machine.

**Results:**
Of 309 children examined, 238 (77.0%) had *P. falciparum* in their peripheral blood. They had a mean malaria load is 5384.94 parasite/µl with a mean haemoglobin level of 10.8g/dl. The mean serum chemokines concentration of 80 *P. falciparum* infected children and their control subjects are chemokines 3 CX3CL 1.3±1.1 and 1.4±2.95 pg/ml, CXCL5 9618.8±2944.2 and 12013.3±4503.0, CXCL7 87.1±193.9 and 465.3±293.9; CXCL11 124.7±120.9 and 158.1±263.4; CCL28 88.9±217 and 1.7±6.71. The chemokines correlated negatively with the malarial loads (r = -0.23, -0.1 -0.04, -0.04, -0.1 and -0.2 respectively.

**Conclusion:**
Elevated chemokines CXCL7 and CCL28 attempt to protect the children against malaria. Depressed chemokines CX3CL1, CXCL5, CXCL9 and CXCL11 may be indicative of suppressed immunity, thereby influencing the pathogenesis of *P. falciparum* malaria in Cameroonian children.
We discovered a novel human immune dysregulation syndrome that is caused by homozygous mutations in the human LRBA gene (lipopolysaccharide responsive beige-like anchor protein) with loss of LRBA protein expression. Clinically, this syndrome is characterized by early-onset hypogammaglobulinemia, autoimmunity, inflammatory bowel disease, and recurrent infections. In addition, LRBA patients present defective T and B cell activation, reduced proliferation in response to PMA-ionomycin, reduced autophagy with accumulation of autophagosomes, and increased apoptosis under starvation. However, LRBA’s exact function and its association with the clinical phenotype is unknown. Our work provides a screening method to identify LRBA defects in candidate patients and insights about the biological function of LRBA. Patient’s suspected of having LRBA deficiency based on clinical grounds were screened for LRBA protein expression by Western Blot in PHA-stimulated PBMCs. Since January 2012, 36 patients with the diagnosis of CVID have been evaluated. Seventeen out the 37 patients tested, expressed normal levels of LRBA; one presented an over-expression, seven had clearly reduced LRBA expression; nine lacked LRBA expression; and two samples failed. We are currently performing mutation detection by Sanger sequencing on the nine patients unable to express LRBA, and so far we have found several heterozygous polymorphisms and one of the patient presented a homozygous mutation in the splice-acceptor site. In addition, we have performed biological studies: A Blue-native-gel has shown that LRBA is forming a complex of 880kDa. SILAC will be the next approach in order to identify the LRBA interactions partners. Finally, the mTOR signaling pathway has been evaluated by Western Blot in EBV cell lines from a healthy donor, an LRBA missense mutation patient and an LRBA stop codon mutation patient. These analyses have shown an increase of Akt phosphorylation at T308 (PDK1 site) and P70S6k in the stop codon of mutated cells and a phosphorylation increase of AMPK at T172 (eIF2a site and TORC1) in both patients cell lines. Whereas Akt-pS473, Akt total, mTOR total, PRAS40, 4EBP1 phosphorylated and total, IRS-pS636 and total IRS were all normal. Further analysis using inhibitors such as rapamycin, metformin and Torin 1 are currently running. In conclusion, the absence of LRBA protein expression in CVID patients is suggestive of LRBA deficiency. However mutation detection by sequencing is required to provide a final diagnosis. We suggest using this screening protocol to look for LRBA deficiency in childhood-onset CVID, patients with an AR form of CVID and with hypogammaglobulinemia, autoimmunity and lymphoproliferation. In addition, we suggest a primary role of LRBA in the regulation of mTOR signaling pathway upstream of TORC1 which could be related to the autoimmune manifestations presented in LRBA syndrome.
Brugia malayi peptic inhibitor homolog (rBm33) driven human macrophage function: in vitro study with THP1 cells

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Introduction:
Nematode parasites like Wuchereria bancrofti, Brugia malayi, Brugia timori inhabits the lymphatics and sub cutaneous tissues causing lymphatic filariasis in humans. Brugia malayi peptic inhibitor (rBm33), a secreted antigen expressed by all the stages of the filarial parasite attributes to the immune evasion strategy of the parasite to sustain in the hostile immune niche of the host. The infected individuals exhibit monocyte dysfunction which is one hypothesis to explain the lack of an antigen-specific T cell response. Hence, understanding rBm33 induced immunoregulatory network would help in unravelling the pathogenesis of the disease.

Objectives:
This study evaluates rBm-33 induced modulation of monocytes function and provides interesting insights in to the role of this aspartyl protease inhibitor mediated immune modulation in human hosts.

Methods:
In order to elucidate the effect of the rBm33 on monocyte function, THP-1 cells were chosen as an in vitro system. Phagocytosis assay and cytokine, iNOS gene expression assessment by RT PCR was performed. In addition, flowcytometric analysis using annexin V/PI and DNA fragmentation assay was performed using Endemic Normal (EN) Peripheral Blood Mononuclear Cells (PBMC).

Results:
THP-1 cells stimulated with rBm33 along with appropriate controls (Unstimulated cells, LPS) showed an enhanced level of expression of pro-inflammatory cytokines (IL-1β, TNF-α, IL-6) and diminished levels of anti-inflammatory cytokine (IL-10) expression comparatively suggesting that rBm33 as a potent stimulator of human monocytes. Further, phagocytosis was performed using Phorbol Myristate Acetate (PMA) treated THP-1 cells stimulated with rBm33 and subsequent incubation with GFP expressing E.coli for 2 h showed an enhancement in the uptake of E. coli. However, rBm33 stimulated culture supernatants did not show significant levels of nitric oxide (NO). The flowcytometric analysis using Annexin V/PI staining revealed that rBm33 did not induce cell death in both monocytes and lymphocyte populations of the isolated PBMCs.

Conclusion:
Thus, it is evident that rBm33 induced monocyte/macrophage responses would influence the cascade of events of T-cell responses to filarial antigens and would further help to tailor a therapeutic approach for lymphatic filariasis.

References:
Anti-MSP1 Antibody responses in Malaria positive pregnant women in south western NG

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Background:
Malaria is one of the deadly diseases caused by Plasmodium spp. Children and pregnant women are more susceptible than any other group. Pregnancy suppresses immunity, but the maternal immune system still continues to respond to malaria infection by producing antibodies. IgG has been reported to play significant role in immune response against *P. falciparum*. Anti-MSP-1\(_{19}\) antibody has been shown to be protective against malaria infection in children. This work therefore studies the response of anti-MSP-1\(_{19}\) antibody in pregnant women in wet and dry seasons.

Methods:
Blood sample was collected in dry and wet seasons. *P. falciparum* infection was determined by microscopy. Anti MSP-1\(_{19}\) IgG level was determined using ELISA.

Results:
Parasitaemia was significantly higher in wet than in dry season, and its level decreases with the increase gravidity. There was significant increase in anti MSP-1\(_{19}\) IgG level in the dry season than in the wet season. Anti MSP-1\(_{19}\) IgG was significantly higher in *P. falciparum* positive primigravidae than *P. falciparum* negative primigravidae in both wet and dry seasons. In wet season ant MSP-1\(_{19}\) IgG level was significantly increased in *P. falciparum* positive multigravidae than *P. falciparum* negative multigravidae. The anti MSP-1\(_{19}\) IgG was significantly higher in malaria positive multigravidae than primigravidae.

Conclusion:
The study suggests that IgG response is positively associated with the presence of malaria infection.
Cross-talk between sentinel and helper macrophages optimizes the antibacterial neutrophil response

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The phagocytes of the innate immune system, macrophages and neutrophils, contribute to antibacterial defense, but their functional specialization and cooperation remains unclear. Here we report that distinct phagocyte subsets play highly coordinated roles in bacterial urinary tract infection. Ly6C⁻ macrophages acted as tissue-resident sentinels that attracted circulating neutrophils and Ly6C⁺ macrophages. The neutrophils cleared the bacteria by phagocytosis and the Ly6C⁺ macrophages played an innate helper role: once recruited, they produced the cytokine TNF, which caused Ly6C⁻ macrophages to secrete CXCL2. This chemokine activated matrix metalloproteinase-9 in neutrophils, allowing their entry into the uroepithelium to combat the bacteria. Thus, the sentinel macrophages optimizes the positioning of neutrophils after confirmation by the helper macrophages. Such positioning prevents relapsing infections and may serve to prevent collateral damage.
Type I IFN signaling triggers immunopathology by modulating lung phagocyte kinetics in tuberculosis susceptible hosts


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Type I interferons (IFN-I) are instrumental in defense against viral infections. However, the role of IFN-I in bacterial diseases is not clearly understood. Bacterial pathogens, including Mycobacterium tuberculosis (Mtb), induce release of IFN-I and thus a thorough understanding of the role of IFN-I in pathogenesis of tuberculosis (TB) is needed. To address this question we used the murine aerogenic infection model. We found that TB-susceptible mice lacking the receptor for IFN I (IFNAR1) were protected from death following Mtb infection. Increased survival was accompanied by reduced bacterial load and ameliorated lung pathology as well as diminished production of proinflammatory cytokines. IFNAR1 signaling apparently did not affect T cell responses, but markedly augmented the frequency of inflammatory phagocytes in the infected lung. This phenotype was driven by expression of IFNAR1 in both immune and tissue-resident radioresistant cells. IFNAR1 signaling triggered early CXCL5/CXCL1-dependent accumulation of neutrophils into alveoli and induced a distinct compartmentalization of Mtb in lung phagocytes. We conclude that IFN I alters early innate events at the site of Mtb invasion with consequences for the ensuing inflammation.
ISG15 modulates IFN-γ immunity in a mouse model of CoxsackievirusB3 myocarditis

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Introduction:
In immunocompetent hosts viral infections are often self-limited. However, in some cases, chronic progression evolves and the combination of viral persistence and ongoing inflammation may lead to lifetime limiting organ destruction. Ongoing research focuses on determinants, which are responsible for the establishment of a sufficient anti-viral immune response leading to successful virus clearance.

In this study, we investigated the impact of the type I interferon stimulated gene 15 (ISG15) on the progression of murine enterovirus myocarditis. ISG15 was found to be secreted as a free protein by monocytes and lymphocytes and the cytokine function of ISG15, which promotes NK and T cell immunity. Moreover, as an ubiquitin-like modifier (ULM) ISG15 can be conjugated to both viral and host cell proteins, thereby exhibiting antiviral properties. During the acute stage of CoxsackievirusB3 (CVB3)-induced myocarditis, ISG15-deficient mice suffered from a more distinctive cardiac injury with severely depressed cardiac function compared to wild type littermates. In this host, chronic inflammation and viral persistence resulted in end-stage dilative cardiomyopathy. Thus, ISG15 is essential to prevent chronic CVB3 disease.

Objective:
Here, we report on the impact of ISG15 in cellular and humoral immunity in CVB3-infection.

Results:
Natural killer cell (NK cells) demonstrated efficient induction of activating ligands and maturation markers at d3 p.i., being accompanied with increased NK cell cytotoxicity in both ISG15-deficient and wild type mice. However, ISG15-deficient NK cells produced remarkably smaller amounts of IFN-γ. Moreover, T cell differentiation and activation were addressed. Compared to ISG15 competent controls, ISG15-deficient CD8+ and CD4+ T cells generated significantly lower amounts of IFN-γ. Beyond that, up-regulation of the early activation marker CD69 (d3 p.i.) as well as the induction of activated CD137+ T cells (d10 p.i.) was observed in both hosts. Also, iTreg/Th17 ratios were within the same range. B cell function as determined by CVB3-directed IgGs was not altered in ISG15-deficient mice. Altogether, these results point towards a specific upstream effect of ISG15 on the IFN-γ signaling pathway.

To distinguish if IFN-γ immunity was affected by free ISG15 or by protein conjugation with ISG15, NK and T cell activation was also studied in mice deficient for the ISG15-E1-activating enzyme UBE1L. In CVB3-infection, these mice possessed large amounts of free ISG15, but were incapable to conjugate target proteins with ISG15. UBE1L-deficient NK and T cells responded to CVB3 infection or in vitro stimulation with an efficient induction of IFN-γ with levels comparable to those of wild type controls. There appears to be a marginal of at all present pathophysiological impact of impaired IFN-γ immunity as observed here in ISG15-deficient mice. Despite the intact IFN-γ immunity in UBE1L/-/- mice, this host developed overwhelming viral and immune-mediated tissue damage closely resembling the phenotype of ISG15-deficient mice upon CVB3-infection.

Conclusion:
Free ISG15 affects IFN-γ immunity in CVB3-infection. Our findings suggest that alternative ISG15-protein conjugation dependent processes determine disease progression in CVB3-myocarditis.
Leishmania infection and persistence in the absence of parasite arginase and host cell arginase 1

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Control of experimental cutaneous Leishmania (L.) major infections requires a T helper 1 (Th1)-response and the expression of inducible nitric oxide synthase (iNOS) by infected macrophages. iNOS expression is induced by interferon (IFN)-γ in the presence of tumor necrosis factor (TNF). We and others previously showed that the activity as well as protein expression of iNOS, which generates leishmanicidal nitric oxide (NO) from L-arginine, can be impeded by arginase (Arg) that converts arginine into ornithine. In mouse macrophages (Mφ), Arg1 is upregulated by Th2 cytokines, whereas Arg2 is constitutively expressed at low levels. Ornithine is the precursor for polyamines that are critical for the replication of both Leishmania and immune cells.

Based on the use of isoform-non-selective arginase inhibitors and the analysis of T cell proliferation under arginine-limiting conditions in the L. major BALB/c mouse model it was recently suggested that arginase expression impairs the host immune response and promotes parasite survival. To unequivocally clarify whether Arg1 indeed accounts for the detrimental outcome of L. major infections, we investigated mice with a Tie2 Cre-recombinase-mediated deletion of Arg1 on a BALB/c background. We observed a long-lasting healing phenotype in Tie2creArg1fl/fl mice compared to wildtype (WT)-controls upon L. major infection. Unexpectedly, the parasite load in infected tissues of Tie2creArg1fl/fl mice was not dramatically reduced and the expression of iNOS, the Th1/2-cytokine pattern or the expansion of T-cells in vivo were not significantly altered in the absence of Arg1. To elucidate whether the leishmanial arginase in addition to the host cell arginase might be critical for parasite survival and expansion in vivo, we resorted to the L. mexicana mouse model, where we compared infections with WT or Arg-deficient L. mexicana parasites in WT or Tie2creArg1fl/fl BALB/c mice. Both the parasite arginase and the host cell Arg1 turned out to contribute to disease development, but were non-essential for long-term parasite persistence in vivo. Taken together, our data indicate that the host cell arginase is able to influence the outcome of CL, but the mode of action appear to differ from what was previously proposed.
Viral infection reprograms pattern recognition receptor signaling to augment T\(^{H1}\) cell priming

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Professional antigen presenting cells are equipped with several pattern-recognition receptors (PRR) sensing nucleic acids of bacterial and viral origin. Much progress has been made in recent years in identifying positive and negative regulators of nucleic-acid recognition signaling. Detailed studies on activation of those receptors in a sequential fashion and their relevance for infection and adaptive immunity are however still lacking.

We show here that viral infection or pretreatment by the dsRNA poly(I:C) modulated the response to subsequent stimulation of RIG-like helicases (RLH) and endosomal Toll-like receptors (TLR) in an opposite manner: Pretreatment of immune cells blocked subsequent IFN-\(\alpha\) production upon stimulation via MDA-5 and RIG-I but enhanced pro-inflammatory cytokine secretion upon endosomal TLR7 stimulation. This opposite modulation of TLR and RLR signaling pathways was equally observed after in vivo pretreatment of mice. The reprogramming effect was seen following pretreatment by synthetic ligands as well as direct viral infection or viral replication intermediates from infected cells. Reprogramming was dependent on the TBK/IKK-IFN-\(\beta\)-IFNaR axis and the receptor MDA-5 during pretreatment. Furthermore, we demonstrated that the sensitivity of intracellular signaling pathways rather than expression levels of receptors for secondary stimuli was altered by pretreatment with dsRNA.

Since dsRNA pretreatment increased IL-12p70 levels after secondary TLR7 stimulation, we speculated that reprogramming by virus-associated innate immune activation could improve adaptive immunity. In accordance with this hypothesis we demonstrated that dsRNA-pretreated and TLR7-restimulated DCs show higher T\(^{H1}\) cell polarizing activity than DC without pretreatment. We propose that a two-step spatio-temporally separated activation of RLH and TLR during virus infection optimally induces T\(^{H1}\) cell priming. PRR reprogramming by viral infection thus may be a key event in the generation of adaptive immunity against viruses that trigger RLH and endosomal TLRs.
Serum ANGPTL4 and ANGPTL6 levels in patients with chronic hepatitis C

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Objectives:
The objectives of this study were to measure angiopoietin-like 4 (ANGPTL4) and angiopoietin-like 6 (ANGPTL6) levels in chronic hepatitis C (CHC) patients and to investigate potential associations between ANGPTL4 and ANGPTL6 levels and biochemical markers of disease progression.

Materials and Methods:
40 patients (32 men and 8 women) positive for HCV-RNA by PCR were included in the study. Serum levels of ANGPTL4 and ANGPTL6 were determined by MILLIPLEX MAG Human Liver Protein (Cat. No. HLPPMAG-57K), Millipore Corporation, Billerica). HCV-RNA measurements were performed using real-time PCR, with the Cobas-Taqman-HCV Test (Roche Diagnostics, Rotkreuz, Switzerland). Serum aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP) and γ-glutamyltransferase (γ-GT) levels were measured on Siemens ADVIA 1800 fully automated analyzer (Siemens, Erlangen, Germany), by routine methods approved by IFCC.

Results:
ANGPTL4 levels ranged from 94.935 to 317.001 ng/ml (mean value 158.139±49.448) and ANGPTL6 levels ranged from 13.979 to 162.192 ng/ml (mean value 67.864±41.956). Interestingly, both ANGPTL4 and ANGPTL6 serum levels correlated positively with HCV-RNA (r=0.358 and 0.311 respectively). Moreover, ANGPTL4 levels showed strong positive correlation with γ-GT levels (r=0.918) and ANGPTL6 showed strong negative correlation with ALP levels (r=-0.974). Any type of correlation with AST or ALT levels was not observed.

Conclusions:
Chronic hepatitis C is a progressive disease characterized by the development of hepatocellular necrosis, inflammation, and fibrosis. In our study CHC patients showed increased serum levels of ANGPTL4 and ANGPTL6. Levels of both markers correlated with HCV-RNA, ANGPTL4 correlated with γ-GT and ANGPTL6 showed negative correlation with ALP. To our knowledge, such correlations are reported in very few studies and as a general remark we can say that they reflect the degree of activity of the inflammatory process in the liver.
Assessment of increased CD4+CD25+FoxP3 T-regulatory cell ratio in acute respiratory distress syndrome

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Question:
In the past, there have been numerous investigations studying the precise function of T-regulatory cells in human diseases. CD4+CD25+FoxP3+ T-regulatory lymphocytes cells (Tregs) efficiently migrate into inflammatory sites, and predominantly suppress peripheral inflammation. Up to now no data are available on the presence and function of Tregs in humans during acute respiratory distress syndrome (ARDS). With the hypothesis that Tregs decrease inflammatory process, we examined BAL and peripheral blood of 37 patients with ARDS and 8 control patients.

Methods:
Within 3 hours of BAL we performed FACS analysis using FACS-Calibur flow cytometer (Becton Dickinson, San Jose, CA) and Treg Detection Kit human (Miltenyi Biotec, Bergisch Gladbach, Germany). This kit contains FITC-conjugated CD4 antibody and PE-conjugated CD25 antibody for surface staining and APC-conjugated FoxP3 antibody for intracellular staining. First peripheral blood and BAL samples were incubated with CD4 FITC and CD25 PE antibodies for 10 minutes at 4°C than cells were fixed, permeabilized and stained with the anti-FoxP3-APC antibody for 30 minutes at 4°C. Gating was performed as follows: Autofluorescent debris was excluded using forward and side scatter. Gating of CD25 and FoxP3 positive cells was performed according to CD4 positive cell expression. Isotyp controls characterized the position of the quadrants. Dot plots show the percentage of CD25 and FoxP3 coexpressing CD4 positive cells.

Results:
We were able to detect Tregs in BAL in patients suffering from ARDS as well as in control patients without ARDS. The mean ration of Tregs to all CD4+ lymphocytes was 3 fold greater in ARDS non-survivors (16,5%; P= 0,025) and almost 2 fold greater in ARDS survivors (9%; p=0,015) compared to the control group (5,9%). Multivariate Cox-regression analysis revealed the ratio of CD4+CD25+FoxP3+ T-regulatory lymphocytes in the BAL to be an important and independent prognostic factor for 30-day survival (HR 6,5; 95% CI, 1,7-25; p=0,006). Tregs in blood samples did not show any differences between patients and controls.

Conclusion:
In contrast to our hypothesis, that Treg cells can suppress inflammation process and therefore contribute to lung repair, increased T-regulatory cell ration in the admission BAL of patients with ARDS is an important and independent risk factor for 30-day mortality. It seems, that very high levels of Treg cells led to suppressed function of T-effector cells via IL10. This was associated with increased disease severity and worse outcome.
IFN-γ induces rapid inhibition of IL-1β independent of nitric oxide

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Question:

IL-1β is a pro-inflammatory cytokine that is transcribed as an inactive precursor protein, pro-IL-1β, which is cleaved into its active form by caspase-1 within the inflammasome complex. It has been reported recently that IFN-γ inhibits production of active IL-1β in a nitric oxide (NO) dependent manner by interfering with inflammasome activation through S-nitrosylation of Nlrp3. However, generation of NO occurs only after prolonged stimulation with IFN-γ. We describe here an additional, very early suppression of bacterial RNA and LPS-induced IL-1β production independent of NO.

Methods:

Murine bone marrow derived macrophages (BMDM) and dendritic cells (DC) were stimulated with bacterial RNA or LPS in the presence or absence of IFN-γ. Secretion of active IL-1β as well as expression of pro-IL-1β on protein and mRNA level was evaluated. Binding of NF-κB p65 to the IL-1β promoter was assessed by Chromatin-IP (ChIP).

Results:

Co-stimulation of BMDM and DC with IFN-γ specifically suppressed bacterial RNA and LPS induced secretion of active IL-1β without affecting production of other pro-inflammatory cytokines like TNF, IL-6 and IL-12p40. Further analysis revealed that regulation occurred on the level of IL-1β transcription and was detectable as early as 30 min after stimulation. Of note, this rapid inhibition of IL-1β was independent of NO, as demonstrated using iNOS-deficient cells. Mechanistically, co-stimulation with IFN-γ attenuated binding of NF-κB p65 to the IL-1β promoter, thus representing a novel mechanism of IL-1β suppression by IFN-γ. In addition, IFN-γ dependent inhibition of IL-1β interfered with effector functions of this cytokine as demonstrated by impaired differentiation of Th17 cells in vitro and decreased production of neutrophil chemotactic factor CXCL1 in target cells.

Conclusion:

We describe here a novel, very rapid and NO-independent mechanism of IL-1β inhibition by IFN-γ. IFN-γ also impairs Th17 differentiation and production of neutrophil attractant CXCL1 in vitro by a mechanism involving suppression of IL-1β.
Impact of HIV co-infection on plasma level of cytokines and chemokines of pulmonary tuberculosis patients

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Background:
The immunologic environment during HIV/M.tuberculosis co-infection is characterized by cytokine and chemokine irregularities that have been shown to increase T cell activation, enhance HIV replication and result in a dysfunctional immune response. Materials and Methods:

Study subjects In this prospective study 33 subjects with active TB disease (17 HIV negative and 16 HIV positive) were recruited from selected health centers in Addis Ababa, Ethiopia. We used a 17plex kit (Epidermal Growth Factor (EGF), FRACtalkine, Granulocyte Macrophage Colony Stimulating Factor (GM CSF), IFN-g, IL-1, IL-10, IL-12, IL-17, IL-4, IL-7, IL-9, IFN-g inducible protein (IP-10/CXCL-10), Macrophage Chemoattractant Protein 1 (MCP-1/CXCL), MCP-3, Monocyte Inflammatory Protein 1 beta (MIP-1b), TNF and VEGF) from Millipore, Germany and multicytokine analysis was done using Luminex (Millipore, Germany) technology.

Result:
Effect of HIV on plasma level of cytokines and chemokines on TB patients
We found that the median plasma level of the cytokines and chemokines measured was not affected by HIV status although the HIV positive TB patients have a slightly higher level of most of the cytokines and chemokines. We also analysed the ratio of Th1 (IFN-g and IL-12(p40)) and Th2 (IL-4 and IL-10) hallmark cytokines before treatment, however, none of the ratios of IFN-g/IL4and IFN-g/IL 10 and IL-12(p40)/IL-4 and IL-12(p40)/IL-10 were significantly different between TB patients who are HIV positive and HIV negative.

Impact of anti TB treatment on plasma level of cytokines and chemokines
We also measured the plasma level of the cytokines and chemokines after 8 month of effective anti-TB therapy to see if any of these cytokines and chemokines showed a difference among HIV positive and HIV negative TB patients as a result of treatment. We found that the median level of IFN-g, IL-4, IP-10, MCP-3 and MIP-1b were statistically different (p<0.05). We have also compared the ratios of IFN-g/IL 4 and IFN-g/IL 10 and IL-12(p40)/IL-4 and IL-12(p40)/IL-10 before and after anti TB treatment in HIV positive and HIV negative TB patients and we found the plasma concentration of IFN-g/IL 4 and IL-12(p40)/IL 10 were statistically different (p<0.05) in HIV negative and HIV positive TB patients after treatment.

Conclusion:
HIV coinfected patients have a lower ratio of IFN-g/IL-4 and IFN-g/IL 10 after anti TB treatment and this might indicate increased disease severity in HIV positive TB patients and possibility of future development of TB in HIV-infected individuals.
Mechanisms of immune inhibition by modified bacterial RNA

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Question:
The innate immune system is important for recognition and clearance of pathogens. Their sensing is achieved by pattern recognition receptors which are able to recognise pathogen associated molecular pattern (PAMP). One of these PAMPs is bacterial RNA which is recognised in a cell-type specific manner by different endosomal Toll-like receptors (TLR). As RNA is also present in the host itself, it is important to distinguish between self and non-self RNA. Discrimination can be achieved by the introduction of posttranscriptional nucleotide modifications that differ in kind and extent between eukaryotic and bacterial RNA. Previous studies have shown that 2’-O-methylation of guanosine at position 18 in E. coli tRNA⁶⁰ leads to a lack of immunostimulation and is further able to dominantly inhibit TLR7-mediated IFN-alpha production in human pDCs by otherwise stimulatory RNA species, indicating a mechanism by which bacteria can escape immune recognition. The present study addressed the question how this inhibition is working.

Methods:
Human PBMCs isolated from voluntary healthy donors and murine bone-marrow derived dendritic cells (BMDC) and macrophages (BMDM) were transfected with stimulatory RNA alone or together with inhibitory RNA. The cell free supernatants were analysed for different cytokines by ELISAs. The stimulated cells were lysed and used for western blot analysis and quantitative real-time PCR.

Results:
We show that dominant inhibition of modified RNA is not specific for human TLR7 but that inhibitory RNA is likewise efficient in impairing production of pro-inflammatory cytokines including TNF and IL-12p40 in murine macrophages and dendritic cells that sense bacterial RNA via TLR13. Inhibition of cytokine production occurred without time lag and was associated with impaired activation of TLR downstream signalling pathways. Therefore the activation of different TLR downstream molecules was observed by western blotting. Stimulation with the activating RNA alone lead to strong induction of p-p38, p-p44/42 and p-JNK, while the addition of inhibitory RNA decreased the induction. Also, activation of IRF7, leading to type I interferon, showed a strong induction after stimulation with stimulatory RNA which could be decreased by adding inhibitory RNA species.

Conclusion:
We speculate that inhibitory RNA competes with stimulatory RNA species for TLR binding without inducing its activation. Co-IP experiments will be performed to confirm binding of inhibitory RNA to TLR7 and TLR13.
Formation of Herpes simplex virus 1 glycoprotein B induced giant vesicles depends on the presence of cytoplasmic sequence motifs of the viral protein

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Question:
Herpes simplex virus 1 (HSV-1) is a persistent human pathogen which prevents complete elimination of the virus from infected hosts due to viral evasion strategies. The HSV-1 encoded envelope glycoprotein B (gB) is a trimeric type 1 membrane protein which plays a central role in fusion of HSV-1 to the host cell membrane. gB consists of an extracellular domain containing a proline-rich sequence, a transmembrane region and a cytoplasmic tail with a putative Golgi-targeting motif, a di-leucine motif (sorting into the trans-Golgi network and internalization) and a tyrosine motif (internalization). Recently, we discovered that overexpression of gB yielded giant endocytic vesicles and showed impact on the MHC class II processing pathway. To examine the influence of gB’s cytoplasmic sorting motifs on vesicle formation we generated C-terminal deletion mutants.

Methods:
Four deletion mutants of gB were generated: One mutant contains all motifs, but lacks the last ten residues. The second deletion mutant lacks the tyrosine motif and the third mutant lacks di-leucine and tyrosine motifs. In the fourth mutant the whole cytoplasmic tail is deleted. Constructs were transiently transfected into IMRS cell, a human lung fibroblast cell line, and their expression was validated by western blot and FACS analysis. Vesicle formation was examined via immune fluorescence staining and interaction of deletion mutants with full length gB was investigated by co-immunoprecipitation.

Results:
The gB deletion construct, which contains all three sequence motifs, was still able to induce enlarged vesicles, while in absence of the tyrosine motif formation of gB-induced vesicles was abolished. Co-immunoprecipitation showed interaction of full length gB with all deletion constructs, which suggests that the deletion mutants are integrated into gB trimers. Coexpression of gB deletion mutants with full length gB revealed that the deletion constructs containing the putative Golgi-targeting motif are sorted to the limiting membrane and to the lumen of gB induced vesicles. The deletion construct lacking the complete cytoplasmic tail showed no surface expression, whereas gB mutants containing the putative Golgi-targeting motif showed expression on the cell membrane.

Conclusion:
By using HSV-1 glycoprotein B deletion mutants we show that the presence of the cytoplasmic tyrosine motif is important for sorting of the viral protein to endocytic vesicles and formation of giant vesicles.
DZIF Harmonized Biobanking Infrastructure

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Access to a comprehensive biobanking infrastructure is mandatory for multi-site infectious disease research and its translation into application. Within the German Center for Infectious Diseases (DZIF), a biobank coordination and technology platform will be established and during the starting phase (until 2012), the existing and complementary main biomaterial banks in Munich, Heidelberg, and Braunschweig will provide specific infection-related biomaterial collections for DZIF members.

Nowadays there are no existing harmonized biobanking structures for infectious diseases in Germany and also a lack of biobanking structures and expertise at several DZIF partner sites.

Biomaterial banking as a common DZIF resource will be based on existing banks, collectives, and their respective expertise, content, and documentation as initiated during the starting phase (until 2012). The DZIF biobank structure will be governed by a board consisting of the three applicants and the biobanking representatives of all other partner sites. The three applicants will be responsible for the three columns microbial pathogens and producers (Overmann), liquids (Wichmann), and tissues (Schirmacher). Central coordination is essential for further development, harmonization, and coordinative embedding (in- and outside DZIF) of DZIF biobanking. Wherever applicable, ELSI-, IT-, QM-, IP- and structural solutions will be harmonized between the three columns and with existing national (TMF, AG Tissue Banking) and international structures (BBMRI). Strengths of the project are to build up an early-on working platform by integrating pre-existing leading expertise (e.g. ELSI, QM, databases, project management) and technologies. Using these established and proofed structures adjusted to the DZIF allows a rapid generation of annotated collections and derivatives by integrating existing resources when possible. This platform will provide a structured ELSI-, QM-, and IT-frame for DZIF biobanking and respective solutions for other DZIF partner biobanks (roll-out). Another aim is to establish and characterise microbial pathogen and producer, as well as human biomaterial collectives and respective derivatives for DZIF and other partners. The harmonized biobanking infrastructure of the DZIF will rapidly provide researchers and outside collaborators access to high quality biomaterials in a sustainable manner, in order to support the development of DZIF and new research in the field of infectious diseases.
Mast cells are required for complete expulsion of the pathogenic nematode
*Strongyloides ratti* in mice

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Approximately one third of the human population is infected with parasitic worms, *Strongyloides ssp.* accounting for 30-100 million cases. We use *Strongyloides ratti* infection to study the protective immune response in mice. Infective third stage larvae penetrate the intact skin and migrate within 2 days to the nasofrontal region of the head, are swallowed and reach the small intestine. Larvae mold via a fourth larval stage to the parasitic adults that reproduce by day 6 post infection (p.i.). Mice clear the infection spontaneously within 2 to 3 weeks in the context of a Th2 response and remain semi-resistant to subsequent infections. Here, we employ a recently generated mast cell-deficient mouse strain, Cpa3<sup>CRE/Wt</sup> (Feyerabend et al., 2011, Immunity 35: 832) to analyze the role of mast cells during *S. ratti* infection. Mast cell deficient Cpa3<sup>CRE/Wt</sup> mice display an increased number of parasitic adults in the small intestine compared to their mast cell competent Cpa3<sup>Wt/Wt</sup> littermates. Mast cell deficiency also resulted in prolonged and increased output of *S. ratti* eggs and larvae as we measured by quantification of *S. ratti* DNA in the feces. Wild type mice cleared the infection around day 40 whereas mast cell deficient mice released *S. ratti* DNA in the feces at a low level for the entire time of our ongoing kinetic studies i.e. 105 days and 165 days, respectively. These findings strongly suggest that mast cells are required for final expulsion of *S. ratti* from the small intestine. Currently, we are comparing the antigen-specific cytokine production in the absence and presence of mast cells to analyze the contribution of mast cells to initiation and regulation of the nematode-specific immune response.
Role of Interferon-β in Host Defense Against *Mycobacterium avium* subspecies *paratuberculosis* Infection

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*Mycobacterium avium* ssp. *paratuberculosis* (MAP) is a slow growing mycobacterium. It is the causative agent of paratuberculosis (Johne’s disease; JD), a chronic transmural inflammation of the intestine in ruminants. The disease affects the productivity in cattle and causes morbidity and mortality in infected animals worldwide. MAP is also discussed as a possible etiological agent in Crohn’s disease (CD), a chronic inflammatory enteritis in humans.

In this study, we aim to dissect the differences and specificities of MAP infected macrophages by comparing infection of host macrophages with viable MAP(V) and heat killed MAP(HK) and viable non pathogenic *Mycobacterium smegmatis* (MSM(V)) and heat killed MSM(HK). Using qRT-PCR, we found that *ifnb* is weakly induced after infection of BMDM with MAP. In contrast to non-pathogenic bacteria MSM that showed high induction of *ifnb* mRNA expression at 5 hours post-infection. Moreover, induction of *ifnb* was depended on bacterial viability. Similar results were observed in the murine macrophage cell lines RAW264.7 and J774.A1. In agreement with qRT-PCR results, increase of luciferase expression which represents the IFNβ promoter activation was also detected in BMDM matured from IFNβ-luciferase reporter mice (ΔIFNβ-luc) infected with MSM(V). We further dissected the mechanism governing the *ifnb* expression by inhibition of bacterial internalization and phagosomal acidification using latrunculinB and bafilomycinA1 respectively. The results showed that pretreated RAW264.7 cells with either latrunculinB or bafilomycinA1 were able to abrogate MSM mediated increase of *ifnb* expression demonstrating that the internalization of MSM and phagosomal acidification are required for stimulating *ifnb* induction.

Overall, the results demonstrate that lower IFNβ response in host cells infected with MAP might be a crucial mechanism that enables MAP to circumvent host immune responses. The mechanism possibly contributes to chronic infection in ruminants and CD in human.
CYLD deficiency protects mice from severe lethal listeriosis

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Introduction:
The facultative intracellular bacterium Listeria monocytogenes (Lm) may cause severe infection in humans and livestock. Control of acute listeriosis is primarily dependent on innate immune responses, which are strongly regulated by NF-κB, and tissue protective factors including fibrin. However, molecular pathways connecting NF-κB and fibrin production are poorly described. CYLD is a deubiquitinating enzyme which plays a pivotal inhibitory role in immune responses. CYLD downregulates NF-κB activity by the proteolysis of K63-linked ubiquitin from various signal transducing molecules. In addition to NF-κB, CYLD is also known to inhibit MAPK and the tissue protective factor plasminogen activator inhibitor (PAI-1).

Objective:
We investigated whether the deubiquitinating enzyme CYLD regulated protective host responses in murine listeriosis.

Materials and Methods:
C57BL/6Cyld−/− and wildtype (WT) mice infected with Lm, Immunohistochemistry, Quantitative RT-PCR, Cytometric Bead Assay, Western Blot, Immunoprecipitation, Overexpression studies, Neutralization of fibrin by warfarin, IL-6 by anti-IL-6 antibody, STAT3 and CYLD by siRNA.

Results:
Upon high dose systemic infection, all C57BL/6 Cyld−/− mice survived, whereas 100% of wildtype mice succumbed to severe liver pathology with impaired pathogen control and hemorrhage within 6 days. Upon in vitro infection with Lm, CYLD reduced NF-κB-dependent production of reactive oxygen species, IL-6 secretion, and control of bacteria in macrophages. Furthermore, Western blot analyses showed that CYLD impaired STAT3-dependent fibrin production in cultivated hepatocytes. Immunoprecipitation experiments revealed that CYLD interacted with STAT3 in the cytoplasm and strongly reduced K63-ubiquitination of STAT3 in IL-6 stimulated hepatocytes. In addition, CYLD diminished IL-6-induced STAT3 activity by reducing nuclear accumulation of phosphorylated STAT3. In vivo, CYLD also reduced hepatic STAT3 K63-ubiquitination and activation, NF-κB activation, IL-6 and NOX2 mRNA production as well as fibrin production in murine listeriosis. In vivo neutralisation of IL-6 by anti-IL-6 antibody, STAT3 by siRNA, and fibrin by warfarin treatment, respectively, demonstrated that IL-6-induced, STAT3-mediated fibrin production significantly contributed to protection in Cyld−/− mice. In addition, in vivo Cyld siRNA treatment increased STAT3 phosphorylation, fibrin production, pathogen control and survival of Lm-infected WT mice illustrating that therapeutic inhibition of CYLD augments the protective NF-κB/IL-6/STAT3 pathway and fibrin production.

Conclusion:
Absence of CYLD is essential for survival during murine listeriosis. Our observation that neutralization of CYLD in WT mice by siRNA partially protected WT mice from lethal listerioses identifies CYLD as a potential therapeutic target in listeriosis.
PolyI:C sensitization induces a stabilized cytokine expression in dendritic cells with aggravation of septic peritonitis

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Introduction:
Most cases of sepsis are associated with an underlying preexisting illness. Specifically, viral infections are associated with increased susceptibility to bacterial super-infections and sepsis. IFNβ and IL-12p40 are key cytokines determining the outcome of sepsis in well-established mouse models of polymicrobial peritonitis. Here viral infections inducing type I IFN were shown to be detrimental. In contrast to that IL-12p40 is important in the induction of a protective immune response during sepsis as IL-12p40 deficiency leads to higher mortality and a delayed immune response. However, the specific contribution to sepsis of IFNβ which is the first type I IFN expressed after pathogen recognition remains unknown.

Objectives:
To define the functional role of IFNβ in the context of a virus infection mimicked by poly(I:C) stimulation on the outcome of the sepsis model of colon ascendens stent peritonitis (CASP) in WT, IFNβ−/− and IFNAR−/− mice was analyzed. Furthermore the IFNβ and IL-12p40 expressing cells after CASP surgery with prior poly(I:C) stimulation should be defined and followed after primary and secondary stimulation.

Materials & Methods:
Polymicrobial peritonitis was induced by the CASP surgery and the influence of poly(I:C) induced IFNβ on the outcome of disease was determined. We compared survival, bacterial counts, cellular recruitment and systemic cytokine production 12 h after surgery. Using the IFNβmob/mob×IL-12p40get40/get40 double knock-in reporter mouse model we analysed the simultaneous expression of IFNβ and IL-12p40 producing cells on a single cell level in a model of polymicrobial peritonitis.

Results:
IFNAR−/− mice showed significantly increased resistance compared to WT mice, while IFNβ−/− mice exhibited intermediate resistance in the colon ascendens stent peritonitis (CASP) model. Poly(I:C) prestimulation did not change the outcome in IFNAR−/− or IFNβ−/− mice but sensitized WT mice to CASP which was associated with a dysregulated cytokine profile and reduced peritoneal granulocyte numbers. Using the IFNβmob/mob×IL-12p40get40/get40 mice we showed that conventional DCs are responsible for the production of IFNβ and IL-12p40 after CASP surgery with or without poly(I:C) pretreatment. We recapitulated the viral followed by bacterial challenge in vitro and sorted poly(I:C) stimulated GM-CSF BM-DCs according to their reporter allele expression and restimulated with different TLR ligands. Poly(I:C) restimulated cells exhibited a fixed phenotype only expressing the cytokine they were sorted for initially, while CpG and LPS restimulated cells showed more plasticity. This fixed cytokine expression phenotype was confirmed in vivo by transferring sorted poly(I:C) stimulated cells into mice followed by CASP surgery.

Conclusion:
Taken together, we show for the first time that stimulation with poly(I:C), a synthetic viral analogue leads to a stabilized cytokine expression of IFNβ and IL-12p40 in cDCs and leaves these cells unable to adapt to secondary superinfections contributing to an overall detrimental dysregulation of the complex cytokine network that is associated with a high mortality in severe sepsis.
BPI-like proteins promote fimbrial adhesion of *Salmonella Typhimurium*

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**Introduction:**
Adhesion to biotic and abiotic surfaces such as epithelia is an important mechanism in the life cycle of many bacteria. *Salmonella Typhimurium* invades interstitial epithelial cells and is then transported to the underlying tissue where it replicates. Therefore the initial attachment to the gut epithelium is an essential step during an infection with this pathogen.

Antimicrobial peptides and proteins (AMPs) are part of the innate immune response in epithelia, where they provide a first line of defense against bacteria and other pathogens. Bactericidal/permeability-increasing protein (BPI) and the related palate, lung and nasal epithelium clone (PLUNCs) belong to the AMPs.

For BPI three different antimicrobial functions are known: 1) neutralization of LPS, 2) direct bactericidal activity against several gram-negative bacteria and 3) opsonization activity. Short and long PLUNC1, the most studied PLUNC proteins, are thought to have similar functions as BPI. In addition an influence of SPLUNC1 on the biofilm formation of *Pseudomonas aeruginosa* has been described, suggesting that BPI/PLUNC proteins may have a general function in the defense against bacteria by interfering with the bacterial adhesion and biofilm formation.

**Objectives:**
Our aim was to analyze the effect of BPI as well as short and long PLUNC1 on the adhesion of *Salmonella Typhimurium*.

**Materials & methods:**
Highly purified recombinant BPI, short PLUNC1 and long PLUNC1 were generated. These proteins were used to evaluate the bacterial adhesion via quantitative *in vitro* analysis. To determine specific *Salmonella* genes involved in the adhesion of the bacteria gene deletions via Lambda Red-recombination were introduced. By plasmid complementation the phenotype of the bacteria was reverted.

**Results:**
Surprisingly and unexpectedly several members of the BPI/PLUNC family of proteins, such as BPI, short and long PLUNC1, induce adhesion of *Salmonella Typhimurium* in a dose dependent manner when present in the medium. This induction can be inhibited by the addition mannose, a known inhibitor of type 1 fimbriae. Moreover adhesion is no longer induced by any of the proteins, if a *Salmonella Typhimurium* knockout strain is used that lacks *fimA*, the major structural component of type 1 fimbriae. The phenotype can be rescued by a plasmid carrying *fimA*.

**Conclusion:**
Our findings suggest that *Salmonella Typhimurium* responds to the presence of the AMP BPI as well as short and long PLUNC1 by adhesion. We interpret the data that *Salmonella* tries to escape the activity of these AMPs by enforced attachment.

To identify the components and pathways within the bacteria that lead to frimbriation upon contact with BPI and PLUNCS we have started a random mutagenesis screen.
Immunologic relevance of dense granule antigens of *Toxoplasma gondii* as target structures for B- and T- cell responses

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Protection against the parasite *Toxoplasma gondii* in infected individuals is realized by humoral and cellular immune responses. We here analyze the capacity of *Toxoplasma*-Lysat-Antigen (TLA) and of a range of different recombinant *Toxoplasma* proteins (GRA1, GRA2, GRA7, GRA9, MIC5, BAG1, SAG1) as targets for cellular and humoral immunity in infected and noninfected individuals of three different species.

Humoral response was evaluated by Western Blot analysis of human, murine and porcine sera, revealing the presence of specific antibodies against different recombinant *Toxoplasma* proteins. Cellular immunity was investigated by determining T- cell proliferation to TLA or different recombinant antigens in spleen cell cultures or isolated PBL cultures. Analyzing sera of seropositive human, swine and mice, antibodies against GRA2 could be found most frequently (100%), followed by GRA7 (90-100%), GRA9 (60-91%) and MIC5 (25-90%) and GRA1 (25-41%). Cells from all infected individuals proliferated to TLA whereas no response was observed in cultures of the uninfected control group. Response to the recombinant antigens was variable between individuals in all species and none of the analyzed antigens elicited proliferation in all cultures from seropositive individuals. Therefore we come to the conclusion that infection with *T. gondii* can be detected by T- cell proliferation in response to TLA whereas the tested recombinant antigens can be used diagnostically to detect the presence of specific antibodies against the parasite.
During chronic infection, pathogen-specific CD8+ T cells upregulate expression of molecules such as the inhibitory surface receptor PD-1, have diminished cytokine production and are thought to undergo terminal differentiation into exhausted cells. Here we found that T cells with memory-like properties were generated during chronic infection. After transfer into naive mice, these cells robustly proliferated and controlled a viral infection. The reexpanded T cell populations continued to have the exhausted phenotype they acquired during the chronic infection. Thus, the cells underwent a form of differentiation that was stably transmitted to daughter cells. We therefore propose that during persistent infection, effector T cells stably differentiate into a state that is optimized to limit viral replication without causing overwhelming immunological pathology.
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Indolamine-2,3-dioxygenase mediated degradation of tryptophan as antiparasitic effect in vivo

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Indoleamine-2,3-dioxygenase (IDO) has been identified as an important antimicrobial and immunoregulatory effector molecule due to its tryptophan (trp) degrading function. Toxoplasma gondii (T. gondii) is a trp-auxotroph apicomplexan parasite which causes toxoplasmosis and especially encephalitis in humans and mice. In addition a primary T. gondii infection during pregnancy can lead to connatal infection.

In vitro data show that T. gondii has a diminished growth rate depending on IDO-activity in the host cells. With the objective to verify these data in vivo we infected C57BL/6N and IDO−/− mice with T. gondii (ME49 strain). Compared to the wildtype IDO−/− mice show a higher mortality rate following intraperitoneal infection. Western Blot analysis was used to determine IDO protein expression in liver, brain, spleen and lungs. In order to verify IDO-enzyme activity tryptophan concentration in sera and organ lysates was determined by RP-HPLC. As consequence of T. gondii infection the trp-level decreases in sera of wildtype and IDO−/− mice. Trp-level decreases exclusively in lungs of wildtype mice, which nicely correlates with a dramatic up-regulation of IDO protein expression only in this tissue.

Additional data indicate that the liver is also affected during infection. Preliminary data show that T. gondii is replicating in liver tissue and increased levels of serum transaminases (GOT and GPT) suggest that an enhanced tissue damage occurs in the liver of IDO−/− mice. In our ongoing work we will determine the parasite load in organs of IDO deficient and wildtype mice. Furthermore Mouse Cytokine Arrays will be used to compare immune responses in T. gondii infected animals.
Leishmaniasis affects 12 million people in 89 countries, predominantly in tropical and subtropical regions. In BALB/c mice, infection with *L. major* triggers a Th2-mediated lethal manifestation, whereas in C57BL/6 and C3H mice, a Th1-mediated self-healing form takes place. Cysteine proteases of the papain family found in *Leishmania* have been described as essential for the parasite pathogenicity, virulence and survival, particularly cathepsin B (Cath B) and L (Cath L). Therefore, both cathepsins are promising targets for the development of new drugs. However, these enzymes are also expressed in mammals, and their role in immune response is currently under extensive research. Experiments with the Cath B inhibitor CA074 and a Cath L inhibitor CLIK148 showed that these compounds could direct the immune response of *L. major*-infected mice towards a Th1 or Th2 profile, respectively, however the mechanisms by which the polarization of naïve Th0 cells was modulated were not further investigated.

We therefore investigated the effect of these cathepsin inhibitors on the signals that dendritic cells use to instruct Th cell differentiation: signal 1 (antigen presentation via MHC class II molecules), signal 2 (costimulatory molecules such as CD86) and signal 3 (cytokine production). We generated bone marrow-derived dendritic cells (BMDC) from BALB/c mice, and stimulated the cells with LPS or CpG, as a pro-Th1 set up, or TNF-α and lysate from *L. major* promastigotes for pro-Th2 in the presence or absence of different cathepsin inhibitors. Altogether, our results indicate that these inhibitors alter the expression of MHC-II molecules (Fig.1) and cytokine production (Fig.2), particularly of IL-12, but not effect was found on the expression of co-stimulatory molecules. Future work will focus on the mechanisms of these effects and the role that they play in Th1/Th2 polarization.

References:

Figure legends:
Fig. 1: Effect of cathepsin inhibition on expression of MHC-II molecules in BMDCs stimulated with A) LPS, B) CpG, C)TNF-α and D) *L. major* lysate. Histograms and pooled MFI of CD11c+ gated cells. N>4 independent experiments, *p
Fig. 2: Effect of Cath L inhibition on IL-12 expression by BMDCs stimulated with LPS. N=3 independent experiments, *p
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Long-term impact of sepsis on pre-existing memory T cells and Plasma cells

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Introduction:
Severe Sepsis is accompanied by profound immunosuppression. It is not known whether this immunosuppression is completely resolved or long-lasting in sepsis survivors and could thus contribute to the high morbidity and mortality observed in patients at late time points after acute sepsis. Objective: We aimed to analyze the long term effects of sepsis on the number and function of memory T cells and Plasma cells in the bone marrow.

Materials and Methods:
We used a mouse model of severe polymicrobial sepsis based on i.p. injection of standardized fecal slurry. Memory cells were induced before sepsis by immunization of wild type mice with Ovalbumin and adjuvants.

Results:
We found profound alterations of bone marrow cellularity after sepsis, but only moderate effects on the number and function of memory immune cells.

Conclusion:
This is to our knowledge the first study to investigate the long term impact of sepsis on pre-existing immunological memory. Our results contribute important indications for the development of immune-monitoring and immune-modulation strategies to improve the long-term prognosis of sepsis survivors.
Influence of regulatory T cells during mCMV infections

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Cytomegaloviruses establish lifelong chronic infections in their hosts. Under immunosuppressive conditions the infection can lead to a life threatening disease. To test whether regulatory T cells (Tregs) contribute to the establishment of persistency we made use of the well established murine model for cytomegalovirus (mCMV) infection.

First, we infected DEREG mice which allow the depletion of Foxp3+ Tregs by Diphtheria toxin application. Upon mCMV infection we detected a higher activation status of T cells in the spleen and the salivary glands in Treg-depleted mice as compared to wildtype mice. In line with this we found a significant reduction of the viral load in the salivary glands, suggesting that Tregs downregulate effective T cell responses against mCMV.

However, monitoring the percentage of Tregs at different time points after infection revealed a significant decline of Treg frequencies within the spleen. Since Tregs might have an impact on the outcome of infection irrespectively of their expansion but due to changes in their phenotype we analyzed their phenotype in more detail.

Interestingly the immunosuppressive cytokine IL-10, which has already been reported to have a strong impact on mCMV infections, was up-regulated in CD4+Foxp3+ Tregs as well as CD4+Foxp3- cells from mCMV-infected mice in comparison to their non-infected counterparts.

Thus our data suggest that regulatory T cells have an impact on the outcome of mCMV infection and that this impact might be mediated by the expression of IL-10, which has to be analyzed in further experiments.
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MPLA and anti-DEC205 Combined targeting enhances uptake of Nanocapsules by murine liver NPCs

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Introduction:
The liver is target and host of various infectious diseases such as Hepatitis A, B and C, Malaria and human Cytomegalovirus infection. The development of chronic liver infections is in part attributed to the tolerogenic liver environment. Key cells in regulating liver immune responses are the non parenchymal liver cells, in particular, Kupffer cells, liver sinusoidal cells and Dendritic cells. Targeting these cells with functionalized nanocapsules is a promising approach to deliver antigen in conjunction with compounds that promote Th1-type immune responses.

Aim of the present study was to evaluate the properties of nanocapsules (NCs) functionalized with anti-CD40, anti-DEC205, and MPLA with respect to the overall uptake, the phenotypic characteristics of NC-positive cells and the cytokine profile secreted after ingestion of NCs.

Material & Methods:
NCs consisting of hydroxyethyl starch (HES) serve as a carrier for anti-CD40/DEC205 and MPLA. Xenogen IVIS Spectrum was used to detect IR labelled NCs in 6-week old female C57BL/6 mice. Therefore mice were inoculated i.v. via the tail vein with 300µg NCs in 300µl 0.9% NaCl. To determine cy5 labelled NCs in Kupffer cells NPC were isolated from the liver. NPCs were stained with fluorochrome-conjugated CD45, CD11c, CD40, CD205 and F4/80 antibodies for flow cytometric cell analysis (LSR II, BD). Cytokine secretion like IL-6 and TNFa was measured using enzyme-linked immunosorbent assay.

Results:
IR labelled HES NCs are primarily deposited in the liver. Additional coating of NCs with anti-CD40 or anti-DEC205 resulted in pronounced uptake in the liver rather than deposition in the lungs. NCs were detected primarily in Kupffer cells. NCs coated with anti-CD40/DEC205 and MPLA were significantly faster ingested by NPCs (12%). In vivo uptake was documented by the release of DXM. Ingestion of NC coated with anti-DEC205 and MPLA induced significant release of IL-6 and TNFa.

Conclusion:
HES NCs are a promising delivery system which can be exploited for targeting antigen and other therapeutic compounds to the liver. Functionlization with anti-CD40/DEC205 and MPLA promote faster uptake and cytokine release by murine liver NPCs.

Further studies will evaluate intrahepatic immune responses to relevant antitgens.
How much can phagocytes eat - Quantification of neutrophil phagocytic events by use of flow cytometry

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Aspergillus fumigatus is a ubiquitous mold which can be detected with a normal frequency of 5-10 fungal spores (conidia) per cubic meter environmental air. This prominent occurrence leads to an inhalation of several hundred conidia per day by a human being. In immune competent individuals the facultative pathogen gets eradicated by parts of the innate immune system very efficiently. One key function which is crucial for the immunological fight towards the fungus is the phagocytic uptake by professional phagocytes such as tissue resident macrophages or neutrophil granulocytes. For different immunological questions it is therefore of high importance to assess the phagocytosis rate of immune cells under in vitro and in vivo conditions. Standard quantification approaches usually employ light microscopy and manual counting as method of choice. This procedure unfortunately is very slow resulting in a low number of analyzed events and high statistical errors. In addition it is often hardly possible to distinguish between internalization and surface attachment of particles if the analyzed microscopic material is of 2D nature.

To circumvent these problems and to get hands on a fast and reliable screening tool we have established a flow cytometric approach for these quantitative measurements. The basic principle of this assay is to use genetically modified fungal strains which express fluorescent proteins in their spores. If these entities are used in phagocytosis experiments phagocytes which co-localize with glowing conidia are indicated by acquisition of the fluorescent signal. However, the gain of fluorescence can simply be an indication for just the surface attachment of the glowing conidia to the immune cell and not for real internalization. In order to distinguish between phagocytosis and attachment we are currently generating protocols for the use of pH-sensitive dyes in these experiments. We hope that as soon as spores loaded with these tracers are taken up by the acidic phago-lysosomal compartment of the immune cells the shift in pH can be visualized, indicating a real uptake. Such a tool would be the basis for larger screening attempts in which molecular phagocytosis-relevant factors can be nicely investigated. Additionally it would also provide us with a new option to clearly follow phagocytic events during in vitro and intravital microscopy.
The Alveolar Macrophage - Indispensable cell type in the fight against Aspergillus fumigatus?

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Alveolar macrophages together with neutrophil granulocytes are believed to mediate innate immune responses towards the opportunistic mold Aspergillus fumigatus and to be essential for host survival following inhalation of fungal spores. Preliminary experiments of our group had suggested that the relative contribution of macrophages to the clearance of the infection might be less important than postulated by others.

To characterize the contribution of alveolar macrophages in more detail, we depleted these cells in living mice and analyzed the effect on neutrophil recruitment. One strategy to eliminate alveolar macrophages in living mice was the intratracheal application of clodronate liposomes, a toxic agent for this cell type. After pulmonary infection with fungal spores the so treated animals showed neither an alteration in neutrophil recruitment nor in survival rates compared to untreated or control groups that just received PBS liposomes. In contrast, macrophage ablation in a transgenic mouse model was associated with a significantly increased mortality. In this system the avian diphtheria toxin receptor is expressed under control of the CD11c promotor. An intratracheal treatment of these animals with diphtheria toxin (DTX) led to local cell death of all CD11c positive cells, including alveolar macrophages. The increased susceptibility of these mice to a subsequent Aspergillus infection could neither be linked to an inhibited neutrophil recruitment nor to an increased fungal burden of the lung.

A set of recent data meanwhile leads to the speculation that the remarkable death of just the DTX treated CD11c-DTR mice upon fungal infection might be explainable by an overreaction of recruited neutrophils. One observation leading us to this conclusion was the elevated amount of myeloperoxidase (MPO), a protein which is released during the respiratory burst of neutrophils in lungs of DTX treated CD11c-DTR mice. Additionally we also found significantly increased levels of reactive oxygen species (ROS) in neutrophils obtained by bronchoalveolar lavage (BAL) of DTX treated and infected transgenic animals. Surprisingly, we could also show that just a chemotactic recruitment of neutrophils into the lung of macrophage depleted animals (DTX model) by intratracheal application of the chemokines CXCL1 and CXCL2 led to a clearly increased mortality compared to control animals.

In ongoing experiments we are currently evaluating possible reasons for this dramatic phenotype. We hypothesize that either the CD11c-DTR mouse line harbors neutrophils of a pre-activated status and that these cells induce the fatal outcome upon infection or that we deplete a cell subset of regulatory function by diphtheria toxin treatment which has the important function to dampen neutrophil responses under infection and/or resting conditions. To answer this question we will mainly employ complex flow cytometry approaches.
Functional characterization of the colocalization of GBP proteins with intracellular pathogens

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Interferon-γ (IFNy) is a major cytokine that mediates resistance against intracellular pathogens. IFN-γ stimulation leads to a strong upregulation of several GTPases, including the p65 guanylate-binding proteins (GBPs). In recent years, our group could show that several mGBPs quickly relocalize from an initial vesicular-like localization to the parasitophorous vacuole (PV) of intracellular Toxoplasma gondii after infection, indicating an anti-parasitic role for the mGBPs within the cell. Also, targeted disruption of mGBP1 or mGBP2 leads to increased susceptibility of mice to T. gondii infection.

We are interested in the cellular mechanisms which lead to the accumulation of the GBPs at the Toxoplasma PV membrane. The uptake of the parasite is host cell independent and the cellular signaling processes triggered by the invasion are not well described.

To understand the fundamental processes involved in mGBP relocalization to the parasite requires detailed study of the molecular events within host cells after pathogen entry. For explaining the events that lead to GBP localization around the PVM we used live-cell imaging techniques. We could observe pathogen entry and rapid GFP-mGBP2 accumulation around the invading parasite within the first minutes, so we assume an important role for mGBP2 in the early steps of initiating an immune response.

We are interested in the cellular mechanisms which lead to the accumulation of the GBPs at the Toxoplasma PV membrane. The uptake of the parasite is host cell independent and the cellular signaling processes triggered by the invasion are not well described.

Our results indicate a role for the autophagy related protein Atg5 in the recruitment of mGBP2 at the PV of T. gondii, since Atg5 deficient fibroblasts show less accumulation of mGBP2 around the T. gondii PV. However, inhibition of autophagy by chemical inhibitors had no effect on mGBP2 recruitment. Thus, we suggest an Atg5-dependent but autophagy independent mechanism involved in mGBP trafficking.

Some members of the mGBP family do not accumulate around the PVM of T. gondii. To identify protein domains and motifs which are important for the relocalization around the PV we utilized a recruiting mGBP (mGBP6) and a not-recruiting (mGBP10) protein to clone fusion constructs. Interestingly, these mGBP family members localize differently after pathogen entry even though they show a high sequence similarity, they differ only in 14 amino acids. Immunofluorescence staining with the fusion construct reveals that the C-terminal part is crucial for localization around the PVM. Single and multiple amino acid substitutions of mGBP6 to the mGBP10 counterparts revealed a motif in the N-terminal part of the protein which could be important for colocalization with the PV membrane directly or indirectly by interaction with other proteins.
Introduction:
The development of an effective vaccine against Tuberculosis (Tb) represents one of the biggest medical challenges of this century. *Mycobacterium bovis* BCG, the only vaccine available at present, is mostly effective in preventing disseminated Tb in children, but it shows variable protection against pulmonary Tb, the most common form in adults. The reasons for this poor efficacy are not completely known, but there is evidence that Tregs might play a role. Particularly, BCG efficacy is lower in developing countries, where people are continuously exposed to low levels of environmental mycobacteria. Strategies to deplete Tregs during BCG infection have shown an increased IFN-γ response, but only marginal effects on bacterial burden or protection against *Mycobacterium tuberculosis* (Mt). However, previous studies have mainly relied on anti-CD25 antibodies to deplete Tregs, a strategy that is known to also eliminate activated effector T cells. During mycobacterial infection, an expansion of Tregs parallel to the expansion of effector T cells has been observed both in mice and humans. Foxp3+ Tregs have been found not only in the lymphoid organs of Mt-infected mice, but also within lung granulomas. Despite this, it is not clear why absence of Tregs does not affect bacterial burden and whether Tregs in an infectious setting are also suppressive. Moreover it is not known whether mycobacteria can directly act on Tregs to modulate their activation status or suppressive capacity.

Objective:
Our aim was to investigate the dynamics of Treg expansion and activation during mycobacterial infection and to determine the effect of specific Treg depletion at different time points during BCG vaccination.

Materials and Methods:
Here we used DEREG BAC transgenic Foxp3 reporter mice, which express a fusion molecule of the diphtheria toxin receptor (DTR) and eGFP (enhanced green fluorescent protein) under the control of the Foxp3 locus. This reporter construct not only allows the detection, but also *in vivo* depletion of Foxp3+ T cells (Tregs) by injection of diphtheria toxin. Using DEREG mice infected i.v. with BCG, we determined the bacterial burden (CFU) upon DT treatment at different time points after infection. Moreover, we analysed the activation status, as well as the suppressive capacity of Tregs during the course of infection by using FACS analysis and suppression assays.

Results:
Our results show that depletion of GFP+Foxp3+Tregs using DEREG mice during the course of BCG infection lead to a rebound of Tregs in favour of GFP+Foxp3+Treg cells. This population may account for the poor ability of DT treatment to increase BCG clearance in DEREG mice. In addition, we could define the activation status of Tregs at different time points during BCG infection as characterized by the expression of markers such as Helios, Nrp-1, GITR, CD103, CTLA-4, CD25 and CD62L. We are currently investigating the *in vivo* relevance of these cells in great detail.

Conclusion:
Our present study using DEREG mice contributes to the understanding of Treg dynamics during mycobacterial infection and suggests that the Treg pool upon repetitive DT-mediated Treg depletion is repopulated by a previously underestimated population of GFP+Foxp3+ cells.
Fingolimod treatment does not aggravate mortality in a murine model of polymicrobial abdominal sepsis

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Introduction:
Immunosuppressive therapy is a key component in the treatment of autoimmune diseases. However, immunosuppression puts patients at increased risk for infections. The new immunosuppressive drug fingolimod attenuates the adaptive immune response by sequestering T cells within secondary lymphoid organs rendering them incapable of migrating to the sites of inflammation. Since April 2011, fingolimod is licensed for the treatment of multiple sclerosis (MS) in Germany. Thus, health care providers and surgeons will be confronted with a growing number of patients under fingolimod medication. To date it is unknown whether fingolimod increases the postoperative risk for infectious complications after abdominal surgery, i.e. peritonitis and polymicrobial sepsis.

Objectives:
In the present work we assessed the impact on fingolimod medication on the severity of postoperative intraabdominal infection in a murine model of polymicrobial sepsis.

Methods:
Colon ascendens stent peritonitis (CASP) was induced in fingolimod (0.5 mg/kg) pretreated or placebo treated C57 BL/6 mice. Briefly, fingolimod and placebo treated mice were subjected to median laparotomy and a 16 gauge plastic catheter was inserted into the colon ascendens. Fifty percent of each group were treated with antibiotics (Ceftriaxone (25 mg/kg KG) und Metronidazol (12.5 mg/kg KG)). Survival was measured in five experimental groups: placebo treated, fingolimod treated, placebo/antibiotic treated, fingolimod/antibiotic treated and sham operated mice.

Results:
Concurrent treatment with fingolimod did not impact on mortality in a murine model of polymicrobial abdominal sepsis. Under antibiotic treatment, survival was increased in untreated as well as fingolimod treated animals to a similar extent.

Discussion:
Immunosuppressive treatment is a risk factor for postoperative infectious complications. However, the risk for infection depends on the pharmacodynamic mechanism of the immunosuppressant. Here we show that fingolimod did not impact on mortality in a murine model of polymicrobial sepsis. A detailed analysis of fingolimod actions on cellular peritoneal infiltrates, cell populations in secondary lymphoid organs, local and systemic cytokine secretion in CASP is currently under way.
Cellular immunity in acute and persistent infection with *Orientia tsutsugamushi*

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*Orientia tsutsugamushi* is an obligate intracellular bacterium that replicates in the cytoplasm of its host cells. The pathogen is widely distributed in Southeast Asia and causes an acute febrile disease called scrub typhus. Today the disease can be effectively treated by antibiotics when diagnosed correctly and early enough. In the preantibiotic era infections could lead to death, however also untreated cases of scrub typhus eventually resolved in most cases (60 - 95%, dependent on the *Orientia tsutsugamushi* strain). In a mouse model the clinical signs of the infections disappear during the third week post infection. During this time the bacterial burden is drastically reduced. It was shown that cellular immunity is responsible for the recovery. However in humans as well as in mice a small number of bacteria can persist for a long time, probably lifelong.

We investigated the discrepancy between the effectiveness of the cellular immunity to overcome the acute phase of scrub typhus and the ineffectiveness to eradicate the bacteria in the persistent phase. Therefore we first asked which cells and effector mechanisms are responsible for the reduction of the bacterial burden in the acute phase. We then investigated the mechanisms that are responsible for the containment of the pathogen during the persistent phase.

To model these aspects we developed a subcutaneous infection model of *Orientia tsutsugamushi* in mice that mimics both, the acute and the persistent phase of scrub typhus. We could show that in this model CD8⁺ T cells, IFN-γ, and perforin dependent cytotoxicity were indispensible for the immunological control of *Orientia tsutsugamushi* during the acute phase of the infection. We could also demonstrate that CD8⁺ T cells and IFN-γ were responsible for the control of this pathogen in the persistent phase. However we also obtained hints that CD4⁺ T cells played a role for the long-term containment of *Orientia tsutsugamushi*. Therefore we currently investigate the effect of CD4⁺ T cells on the CD8⁺ T cell fate as well as their role in immune regulation.

We conclude that CD8⁺ T cells act not only to dramatically reduce the pathogen burden during the acute phase of an *Orientia tsutsugamushi* infection but also to contain persistent bacteria. However they are unable to fully eradicate *Orientia tsutsugamushi* from infected mice.
Acquired resistance of *Listeria monocytogenes* in and escaped from liver parenchymal cells to gentamicin is caused by being coated with their plasma membrane

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*Listeria monocytogenes* is a facultative intracellular bacterium, which can survive and propagate not only in professional phagocytes such as macrophages (Mφ), but also in nonprofessional phagocytes such as liver parenchymal cells (LPC). Whereas *L. monocytogenes* is susceptible to gentamicin (GM), it has been considered that this bacterium phagocytosed by Mφ shows resistance to this antibiotic, which is caused by its low plasma membrane permeability. Yet, evidence exists that GM penetrates into Mφ and kills *L. monocytogenes* phagocytosed by Mφ. Although LPC are a habitat of *L. monocytogenes*, it remains to be determined whether *L. monocytogenes* in and escaped from LPC acquire resistance to GM. In the present study, we compared the susceptibility of *L. monocytogenes* outside, within and escaped from LPC to GM using HepSV40 and AII. *L. monocytogenes* cultured without LPC lines was killed by 2 µg/mL of GM (minimal inhibitory concentration (MIC): 2 µg/mL). *L. monocytogenes* in LPC lines showed resistance to 250 µg/mL of GM (MIC: >250 µg/mL), and *L. monocytogenes* escaped from LPC lines showed, though lesser degree, resistance to GM (MIC: >5 µg/mL). *L. monocytogenes* escaped from infected LPC lines was coated with their plasma membrane and the acquired resistance of *L. monocytogenes* escaped from infected LPC lines to GM was abrogated by saponin. Our results not only indicate that *L. monocytogenes* in and escaped from LPC show resistance to GM, but also suggest that the acquired resistance of *L. monocytogenes* escaped from LPC to GM is caused by encapsulation of *L. monocytogenes* by their plasma membrane.
Influence of the components of the immune system on biofilm formation by *Pseudomonas aeruginosa*

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The formation of biofilms by *Pseudomonas aeruginosa* in the lungs of cystic fibrosis patients is the major complication of this inherited disease. It will eventually lead to the death of the patient. In this case, the conversion of planktonic *P. aeruginosa* to the mucoid form is favored by the chronic inflammatory state in the lung. To find the molecular mechanisms that induce the formation of such biofilms will help to design new intervention strategies.

Our recently established model employing solid murine tumors was used to investigate biofilm formation *in vivo*. BALB/c wild type or syngenic lymphopenic (Rag1−/−) mice were subcutaneously (s.c.) inoculated with CT26 colon carcinoma cells and intravenously (i.v.) infected with the *P. aeruginosa* wild type strain PA14. Bacteria colonized the tumors in the lymphopenic animals but in contrast to wild type mice no biofilm formation took place. After reconstitution of such lymphopenic mice with T cells, biofilm formation could be observed. How T cells are able to interfere with bacteria still needs to be determined.

During these experiments we noticed that the tumors shrank very quickly after systemic infection and tumor colonization by the bacteria. Some mice eventually even cleared the tumors. Reconstitution of lymphopenic animals with spleen cells from such mice revealed the presence of tumor specific T cells, while no such activity was detected with cells from mice that did not clear the tumors. Interestingly, when spleen cells of uninfected mice bearing CT26 tumors were transferred, a similar anti-tumor activity could be demonstrated. Obviously, the infection of tumor bearing mice activates T cells that are already present in such mice but are not able to reject the CT26 tumors.
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The GTPase activity of murine guanylate-binding protein 2 (mGBP2) controls the intracellular localization and recruitment to the parasitophorous vacuole of *Toxoplasma gondii*

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Introduction:
One of the most abundantly IFN-γ-induced protein families in different cell types are the 65-kDa guanylate-binding proteins (GBPs) which are recruited to the parasitophorous vacuole (PV) of the intracellular parasite *T. gondii*. Activity against pathogens has been shown for several members of the murine GBP family.

Objectives:
We wanted to elucidate the relationship between biochemistry and cellular host defense functions of mGBP2 in response to *T. gondii*.

Materials & methods and results:
The wild type (WT) protein exhibits in its monomeric form low affinities to guanine nucleotides, self-assembles upon guanosine 5’ triphosphate (GTP) binding, forming tetramers in the activated state and stimulates the GTPase activity in a cooperative manner. The products of the two consecutive hydrolysis reactions are both GDP and GMP. The biochemical characterization of point mutants in the GTP-binding motifs of mGBP2 revealed amino acid residues that decrease the GTPase activity by orders of magnitude and strongly impair nucleotide binding and multimerization ability. Live cell imaging employing Multiparameter Fluorescence Image Spectroscopy (MFIS) using a homo-FRET assay shows that the inducible multimerization of mGBP2 is dependent on a functional GTPase-domain. Furthermore, the role of single mGBP2 protein domains on the localization and function were investigated using truncated mGBP2 mutants. We show that the isoprenylated C-terminal region harbours the PV targeting motif. Additionally, we are analysing the GTPase activity of the respective peptides and are endeavouring to crystallize the full length protein. To illuminate the intracellular function of mGBP2 we are estimating the interaction partners of mGBP2.

Conclusion:
The consistent results indicate that GTP-binding, self-assembly and stimulated hydrolysis activity are required for physiological localization of the protein in infected and uninfected cells. Ultimately, we show that the GTPase-domain regulates efficient recruitment to *T. gondii* in response to IFN-γ. The mechanisms involved in the recruitment of mGBP2 proteins to the PV and their molecular and biochemical activity are the major focus of our current studies.
Role of LILRA3 in viral immune defence as a potential mediator of TLR-8 stimulation

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Question:
LILRA3 is a soluble protein which is a member of the leukocyte immunoglobulin like receptor family. It can exist as a deletion genotype, with the homozygous deletion present in 3% of caucasians, but overrepresented in patients with multiple sclerosis, Sjögren’s syndrome [1, 2], and HIV (own data). Our group has previously shown that LILRA3 could induce proliferation of CD8 T-cells and NK-cells in an allogeneic setting, induce various proinflammatory cytokines and upregulate the expression of the antigen presentation machinery in monocytes and B-cells, which also express an as yet unknown receptor for LILRA3 (paper under revision). Due to the selective proliferation of the cytotoxic cell compartments induced by LILRA3, we hypothesized that LILRA3 plays a role in viral immunity, in which case LILRA3 expression should be stimulated in the event of a viral infection.

Methods:
Real time-PCR primers against LILRA3 and various proinflammatory cytokines were designed. PBMCs were incubated with various TLR agonists and LILRA3 expression analyzed against unstimulated controls. For the time course experiment, PBMCs incubated with ssRNA40, ssRNA41 or without stimulation, were harvested at various time points and the expression of various cytokines and LILRA3 were measured and calibrated to a calibrator cDNA pooled from 10 PBMC donors using the $2^{-\Delta\Delta CT}$ method.

Results:
LILRA3 appeared to be selectively upregulated by ssRNA40 in 3 out of 4 donors, but not other TLR agonists (Figure 1A). ssRNA40 is a single stranded RNA whose sequence is derived from HIV-1 and is a known TLR-8 agonist. In order to confirm the preliminary result, we expanded the analysis of ssRNA40 induced LILRA3 expression to a larger cohort of healthy individuals (n=8), including ssRNA41 (ssRNA40 with uridine replaced as adenine) as control. ssRNA40 significantly upregulated the expression of LILRA3 whereas ssRNA41 did not (Figure 1B). This means that LILRA3 is potentially a mediator of TLR-8 induced immune mechanisms. In order to have a general idea where LILRA3 lies in the scheme of the TLR-8 mediated response, we compared the expression of LILRA3 to other prominent cytokines upregulated by TLR-8 stimulation. We performed a time course experiment to measure the expression of LILRA3, TNF, IFN-β, IFN-γ and IL-1β at 4, 8, 12, 24, 48 hours. Although TNF, IFN-β, IFN-γ and IL-1β were already prominently upregulated at 4 hours, LILRA3 expression peaked later at 24 hours (Figure 2). Whether this means that LILRA3 is induced by the upregulation of other proinflammatory cytokines, or that it is a later phase response to direct TLR8 stimulation remains to be answered.

Conclusions:
We show here that LILRA3 expression can be induced by TLR8. This in combination with our previous functional studies on LILRA3, strongly indicate a functional role of LILRA3 in viral immune defense.

Figure legend:
Figure 1. (A) PBMC from 4 different donors were used to analyze the expression of LILRA3 after 24-hour stimulation with various TLR agonists. (B) Expression of LILRA3 was measured after 24-hour stimulation with ssRNA40 and ssRNA41 (n=8). (**p
Figure 2. Expression levels of various cytokines and LILRA3 were measured from PBMCs harvested at various time points after stimulation with ssRNA40, ssRNA41 or without stimulation.

References:
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Figure 1: Fold change in LILRA3 expression for different donors under various stimuli.

Figure 2: Calibrated gene expression over time for TNF, IFN\(\gamma\), and IL1\(\beta\) under different stimulations.
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Sphingosine-1-Phosphate and its Role in Polymicrobial Sepsis

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Acting via its five receptors (S1P1-S5), sphingosine-1-phosphate (S1P) has been shown to be a versatile modulator of numerous physiological and pathophysiological pathways including, but not limited to: vascular permeability, inflammation, cell migration, and heart rate. Currently, however, its role in sepsis pathophysiology remains poorly understood and requires further research. In the current study, using both human and murine in vitro and in vivo models, the physiological and pathophysiological effects of S1P signaling on inflammatory responses, specifically immune system modulations and vascular dysfunction, are examined.

Part of these studies include the analysis of cytokine secretion in the murine macrophage cell lines Raw264 and J774.1 upon stimulation with the immune modulator and functional S1P receptor antagonist, FTY720, as well as, the S1P receptor agonist, FTY720-phosphate (FTY-P). Preliminary results demonstrate that exogenous S1P administration is capable of modulating cytokine, in this case TNF-α, and possibly chemokine secretion levels. This will provide a firm basis from which to develop broader cytokine expression profile. Previous studies have also shown that S1P signaling, via the S1P, receptor, is an important modulator of vascular stability. We, therefore, established an in vitro model, using human umbilical vein endothelial cells (HUVEC) to study the effect of S1P on endothelial cell integrity. As expected our results support these findings and using thrombin as a consistent inducer of vascular permeability, allow for further examination of inflammatory vascular dysfunction. These models will be used to shed light on the role of macrophage stimulation on cytokine secretion and on the contribution of S1P-regulated endothelial cell permeability under inflammatory conditions. In the near future, our experimental framework will be expanded to include in vivo studies utilizing mouse models expressing altered sphingolipid metabolism. In particular, these studies will be designed to further analyze the role of sphingolipids using Peritoneal Contamination and Infection (PCI), a model for induced polymicrobial sepsis.
Modifications of sphingosine-1-phosphate (S1P) receptors and S1P kinases during sepsis

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Various studies have elucidated the participation of the lipid mediator sphingosine-1-phosphate (S1P) signaling in regulating many processes during an immune response including antigen presentation, lymphocyte egress and their cytokine secretion as well as vascular development and permeability. However, the regulation of sphingosine kinase 1 (SPHK1) and SPHK2 as well as the incorporation of various G-protein coupled S1P receptors (S1PR1-5) in the early and late phase of infection, e. g. in case of sepsis, are not fully understood. Further, information about the intracellular targets that link S1P signaling to immune cell trafficking and possible alteration of T and B cell differentiation is scarce. Currently, we are investigating the modifications of S1P receptor and SPHKs expression with relation to local and systemic levels of S1P in murine models of sepsis as well as in LPS-treated cells. The initial approaches are real-time PCR studies using Taqman method to reveal S1P receptors profiles in various cells and tissues in order to continue with the analysis of related signaling pathways and cellular functions. Furthermore, we are testing some pharmacological compounds that were shown to alter S1P signaling for their influence on sepsis progression. We intend to present preliminary results on the way to help understand some of the S1P-related immune processes that will strengthen the link of lipid metabolism and immune regulation and reveal therapeutic potential of targeting S1P-signaling in sepsis.
Evasion strategies during echinococcosis: Involvement of Treg and alternatively activated macrophages

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Cytic echinococcosis is one of the world’s major zoonotic infections. It is caused by the parasite *Echinococcus granulosus* (*E.g.*). It usually manifests as unilocular cyst(s) mainly located in the liver. We have previously highlighted an evident role of laminated layer (LL, acellular layer of hydatic cyst) in parasite survival by impairment of Th1 protective response.

The purpose of this study was first, to investigate the effect of LL extract (LLs) on IL-10 production by human mononuclear cells (PBMC) *ex vivo*. Second, the effect of LLs on macrophages phenotype and expression of some M1/M2 markers was also investigated. In this way, NOS2 and Arginase activities were assessed in PBMC cultures induced by LLs. Moreover, implication of mannose receptor (MR) and TGF-β on Arginase activity were evaluated using mannose (MR antagonist) and Anti-TGFβ. Activity of NADPH oxidase was also evaluated by chemiluminescence. Finally, TLR2, CD14 and CD23 expression was measured by flow cytometric immunoassay.

Interestingly, we showed that LLs enhanced IL-10 production. Moreover, while NOS2 and NADPH oxidase activities are inhibited, Arginase activity is activated. MR and TGF-β are involved in the Arginase induction by LLs. Furthermore, LLs increases TLR2 and CD14 expression and decreases CD23 expression in monocytes.

Collectively, ours finding suggest that *E. g.* laminated layer induced M2 phenotype. These macrophages induce Treg cells and impair M1/Th1/Th17 protective responses allowing parasite survival. Inhibition of these mechanisms constitutes an important issue to address an anti-hydatic treatment design.
Chronic filarial infection improves *E. coli* induced sepsis through a TLR2 mediated cross-tolerance mechanism

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Helminths modulate the immune system of their hosts and induce a regulatory, anti-inflammatory milieu that enables long-term parasite survival within the host, but may also benefit the host. Thus, helminth infections have been shown to improve allergies and autoimmune diseases. In the current study we investigated whether chronic infection with the filarial nematode *Litomosoides sigmodontis* (*L.s.*) improves the outcome of an acute systemic inflammation caused by i.p. *E. coli* injection in BALB/c mice.

Chronic *L.s.* infection significantly improved *E. coli* induced hypothermia, bacterial clearance and sepsis survival compared to *E. coli* only injected controls. The *L.s.* mediated protective effect correlated with significantly increased levels of anti-inflammatory TGFβ and reduced concentrations of pro-inflammatory cytokines after *E. coli* challenge. Depletion of peritoneal macrophages prevented the filaria mediated protection against *E. coli* challenge. Improved bacterial clearance in *L.s.* infected animals was not due to an increased phagocytic capacity of macrophages, but correlated with a reduced loss of peritoneal macrophages and an alternatively activated phenotype after *E. coli* challenge. However, the *L.s.* mediated protective effect was still given in IL-4 and IL-4R/IL-5 deficient animals, suggesting that alternatively activated macrophages are not required. Endosymbiotic *Wolbachia* bacteria, that are present in most human pathogenic filaria and *L.s.*, signal via TLR2 and may induce cross-tolerance to TLR4 stimuli. Accordingly, in vitro experiments revealed that pretreatment of macrophages with crude *L.s.* antigen reduced LPS induced activation and lack of TLR2 signaling in *L.s.* infected mice prevented the protection against *E. coli* challenge.

Our study suggests that chronic *L.s.* infection can have a beneficial effect on acute bacterial infections. Current experiments are ongoing to test whether this is due to exposure to *Wolbachia* bacteria in chronically *L.s.* infected mice that prevents *E. coli* induced excessive macrophage activation by a TLR cross-tolerance mechanism.
Influenza infection of MHC-I transgenic mice reveals that ERAP is necessary and sufficient for generation of the B27-specific immunodominant epitope

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Background:
Although HLA-B27 and ERAP are known to confer susceptibility to spondyloarthritis (SpA), the role of these elements in modulating host response to infection is undefined. Despite co-dominant expression of class I MHC alleles, immune response to viral infections is characterized by immunodominance (ImDc). The exact mechanisms underlying ImDc are not clear. Defining factors contributing to ImDc has proved difficult due to multiple MHC-I allele co-expression in humans and normal mice.

Methods:
To overcome this limitation, we generated human MHC-I transgenic (Tg) mice deficient for endogenous mouse MHC-I molecules and expressing only one human MHC-I allele (e.g. HLA-B7, HLA-B27, HLA-A2). To assess whether co-expression of additional MHC-I alleles in the presence or absence of ERAP influences the pattern of anti-flu CTL epitope recognition and ImDc, novel Tg mice in the context of ERAP deficiency were established.

Results:
In flu-infected, double Tg A2/B7 or A2/B27 mice, IFN-γ ELISpot assays with the flu epitopes A2/M1.58-66 and B7/NP418-426 or B27/NP383-391 showed specific recognition of both peptides by both alleles respectively. In contrast, flu-infected B7/B27 Tg mice demonstrated a significantly reduced B27-restricted CTL response to NP383 while there was no change in the response of B7-restricted CTL response to NP418. Profiling the T cell response revealed that co-expression of B7 and B27 is associated with i) a partial deletion of Vβ8.1+ B27-restricted/NP383 CD8+ T cells and ii) a failure of Vβ12+CD8+ T cell expansion following flu infection in B7/B27 Tg mice. Studies in flu infection of ERAP-deficient Tg B27 and Tg B7/B27 mice revealed complete abrogation of the B27-restricted response to NP383-391, indicating the importance of ERAP in generation of this peptide.

Conclusions:
The HLA-B27 immunodominant response to infection is critically dependent on ERAP. This provides a possible mechanistic basis for the findings in genetic studies of the interdependence of B27 and ERAP in conferring susceptibility to SpA.
Influence of regulatory T cells on natural killer cells during murine cytomegalovirus infection

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Background:
Cytomegalovirus (CMV) infects 40-60% of the German population latently. Reactivation of the virus in patients due to immunosuppression, for example after an organ transplantation, can be life threatening. Regulatory T cells (T regs) are potent immune cells proposed to prevent transplant rejection by suppression of effector T cells. During acute CMV infection, Natural killer (NK) cells play a dominant role in controlling viral replication. In vitro studies by Ghiringhelli et al. (2005) revealed a functional control of NK cells by T regs in terms of cytotoxicity and IL-12 dependent Interferon-γ production. To this end, their interaction with NK cells in the context of CMV infection remains largely unclear.

Aims/Hypothesis:
In this study, we set out to elucidate the relationship between T regs and NK cells during acute MCMV infection and examine its influence on NK cell proliferation, activation and control of viral replication.

Methods:
We utilized Dereg mice generated in our laboratory allowing selective depletion of FoxP3+ T regs by Diphteria toxin administration and infected them with murine CMV (MCMV).

Results:
We observed an increase in NK cell number in depleted mice under homeostatic conditions as described previously. However, in contrast to this finding, NK cell numbers remained unchanged irrespective of T reg depletion in MCMV infection. Furthermore, only a notable difference in viral burden could be detected upon T reg depletion on day 3 post infection (p.i.). Results from later time points are not a definite consequence of T reg/NK cell interaction as a more pronounced T effector cell response in T reg depleted mice starting at day 4 p.i. was observed. Further functional analyses on NK cells at later time points p.i. will shed light on a possible boosted contribution of these cells in Treg deficient mice.

Conclusion:
Thereby our study suggests that during the acute phase of infection, NK cell proliferation remains unaltered in infected T reg depleted mice while functional suppression would need further elucidation. The impact of this study could provide valuable knowledge for dealing with virus reactivation in transplant medicine.
Transfection of melanoma cells by viral RNA mimetics enhances their T-cell sensitivity

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Question:
Recognition of tumor cells by cytotoxic CD8+ T cells (CTL) leads to specific elimination mediated by an interaction of T cell receptors and major histocompatibility complex (MHC) class I molecules. Efficiency of CTL against tumor cells is influenced by several factors such as density of specific MHC surface complexes. However, as tumor cells are genetically unstable, MHC class I negative variants can grow out (1). Such tumor cells cannot be detected by T cells but are potential targets of natural killer (NK) cells (2). NK cells are cytotoxic effectors of the innate immune system that can recognize and eliminate cells of an MHC class I-low/-negative phenotype, but only in case target cells additionally express surface ligands that bind to stimulating NK cell receptors. Thus, agents that enhance detection of melanoma cells by CTL or NK cells by modulating the tumor’s immunological phenotype might be of interest as an additive in melanoma immunotherapy. The aim of this study is to determine whether mimetics of viral dsRNA can be used for enhancement of immunogenicity of malignant melanoma.

Methods:
To answer this melanoma cells were transfected with 3pRNA, a mimic of viral dsRNA, and analysed for the expression of molecules involved in the recognition by T cells and NK cells. Alterations in the expression of MHC class I molecules and of components of the antigen processing machinery were measured by quantitative real-time PCR, Western blot and flow cytometry. Modified recognition of 3pRNA transfected melanoma cells by T cells and NK cells was determined by IFN-γ ELISPOT assay, ELISA and degranulation assay.

Results:
RIG-I signalling upregulated the expression of MHC class I molecules and of components of the antigen processing machinery, that in turn enhanced T cell recognition of 3pRNA transfected melanoma cells. Unexpectedly NK cell ligand expression was only slightly affected and NK cell activation was reduced after 3pRNA transfection. This is most likely due to increased MHC class I expression, which represents an inhibitory signal for NK cells.

Conclusion:
Taken together these data demonstrate that 3pRNA transfection of malignant melanoma cells enhances their T cell sensitivity, which suggests its potential use in melanoma therapy.

Reference:
Generating and characterizing WT1-specific T cells - research towards adoptive tumor therapy

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The Wilms tumor antigen 1 (WT1) is expressed at high levels in leukemic cells, but not in healthy tissue [1,2]. WT1, therefore, is a favorable target antigen for allogeneic T cell therapy to prevent or treat leukemic relapse after stem cell transplantation.

WT1-specific T cells have been detected in healthy individuals in low frequencies [3,4]. However, the efficient expansion of these T cells for clinical use has remained a major challenge.

To date, generation of WT1-specific T cells has mostly been restricted to priming and expansion of either cytotoxic T lymphocytes (CTL) or T helper cell clones [5,6,7]. A recent approach to obtain polyclonal cultures of CD4⁺ and CD8⁺ T cells resulted in only low frequencies of WT1-specific T cells. Moreover, a comprehensive analysis of the expanded T cells was omitted [8].

In this study we aim to develop an improved method for the generation of functionally potent, polyclonal WT1-specific T cells from human peripheral blood of healthy donors and an in-depth characterization of these T cells.

Collectively, our findings show that WT1-specific, reactive T cells can be efficiently enriched directly from peripheral blood mononuclear lymphocytes by magnetic separation of T cells based on the increase in CD137 expression after antigen-specific stimulation. A short 9-day expansion period yields a T cell product, containing both CD4⁺ and CD8⁺ T cells. The expanded T cells show specific reactivity against WT1-presenting autologous cells, as detected by cytokine production after antigen-specific restimulation, cytotoxic activity against antigen-loaded target cells and stain positive for WT1-MHC-I tetramers.

Comprehensive phenotypic and functional characterization revealed a polyclonal WT1-specific T cell culture with a non-exhausted phenotype, thereby suggesting good persistence and functionality of the obtained T cell product in vivo. Thus, our approach holds great potential for the GMP-compliant, automated generation of WT1-specific T cells for future clinical use.

References:
Differential Role of IKK on Cell Cycle in Primary and Cancer Cell Lines

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Introduction:
Duplication and division of cells involves sequentially ordered series of events known as cell cycle. Cell cycle is regulated by variety of molecular pathways which are initiated by various stimuli such as foreign antigen, immune reactions, stress and cytokines. One of the most crucial pathways is called NF-κB pathway, which is found to influence key cell cycle regulators. Signal induction of NF-κB pathway by some conventional oncogenes gives it full transforming potential. Activity and translocation of NF-κB transcription factor is regulated by a set of inhibitory proteins called inhibitor of NF-κB (IκB) which degrade after being phosphorylated by two subunits of kinase complex called IκB kinase alpha (IKKα) and IκB kinase beta (IKKβ). These kinases influence many regulatory molecules in the cell. We hypothesized involvement of IKKs in cell cycle.

Method:
We induced NF-κB pathway in primary and cancer cell lines and analyzed cell cycle progression by flow cytometry.

Results and Conclusion:
Different effect of IKK stimulation on different cell lines indicates their involvement.
Preclinical Evaluation of Ganglioside GD2-directed Trifunctional Bispecific Antibodies

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Introduction:
Trifunctional bispecific antibodies (trAb) are novel anticancer drugs recruiting and activating different types of immune effector cells at the targeted tumor where they cause effective tumor cell destruction. Ektomab, a new promising trAb, is directed against human CD3 on T cells and the tumor-associated ganglioside GD2, which is an attractive target for immunotherapy of small cell lung cancer, neuroblastoma, glioma, or melanoma in humans.

Objectives:
For studying this specificity in mice, we used a murine melanoma engineered to express GD2 and the surrogate trAb Surek (anti-GD2 x anti-murineCD3). In former studies, we already showed the therapeutic efficacy of intraperitoneally (i.p.) administered Surek and its capability to induce a long-lasting antitumor response in mice. For use in the clinics, the intravenous (i.v.) or alternatively subcutaneous (s.c.) route of antibody administration appears more feasible. In this study we investigate whether the s.c. administration of Surek is as effective as the i.p. administration.

Methods & Results:
To answer this question, we first studied the pharmacokinetics of the trifunctional bispecific antibody Surek by comparing three different routes of administration - i.v., i.p. and s.c. Additionally, the development of mouse anti-mouse antibodies (MAMAs) and mouse anti-rat antibodies (MARAs) was determined. To evaluate the tumor-protective potential of s.c. drug administration in vivo, mice were treated with melanoma cells and with the trifunctional bispecific antibody, administered intraperitoneally and subcutaneously, respectively. In the therapeutic trial s.c. administration of Surek showed the same survival, compared to i.p. administration. It is postulated that trAbs form a tri-cell complex, comprising T cell, tumor cell and accessory cell. Therefore, we conducted immunohistochemical analyses of the skin after s.c. administration of Surek. As expected, recruitment of dendritic cells as well as T cells was demonstrated. In addition, we examined the clinical applicability and the occurrence of local adverse effects by monitoring skin reaction after s.c. drug administration. Thereby, no inflammatory skin reaction was observed - neither macroscopically nor histopathologically.

Conclusion:
Taken together, these findings are relevant for the clinical application of trifunctional bispecific antibodies. We conclude that the s.c. administration of trAbs directed against GD2 can serve as an appropriate application scheme in clinics, providing therapeutic efficacy along with a good safety profile.
Tumor Immunology

Virus-induced hepatocellular carcinomas induce antigen-specific local tolerance

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T cell surveillance is often effective against virus-associated tumors because of their high immunogenicity. It is not clear why surveillance occasionally fails, particularly against hepatitis B or C virus-associated hepatocellular carcinoma (HCC). We established a transgenic murine model of virus-induced HCC by hepatocyte-specific adenovirus-induced activation of the oncogenic SV40 large T antigen (TAg). Adenovirus infection induced cytotoxic T lymphocytes (CTLs) targeted against the virus and TAg, leading to clearance of the infected cells. Despite the presence of functional, antigen-specific T cells, a few virus-infected cells escaped immune clearance and progressed to HCC. These cells expressed TAg at levels similar to HCC isolated from neonatal TAg tolerant mice, suggesting CTL clearance does not select for cells with low immunogenicity. Virus-infected mice revealed significantly greater T cell infiltration in early compared to late stage HCC, demonstrating progressive local immune suppression through inefficient T cell infiltration. Programmed cell death protein-1 (PD1) and its ligand PD-L1 were expressed in all TAg specific CD8+ T cells and HCC, respectively, which contributed to local tumor-antigen-specific tolerance. Thus, we have developed a model of virus-induced HCC that may allow for a better understanding of human HCC.
Tumor-associated neutrophils promote metastasis by enhancing the immune escape of the tumor cells

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Neutrophils are emerging as important regulators of tumor progression and have been associated with poor clinical outcome in several types of solid cancer. Recently, we demonstrated that high neutrophilic infiltration associated with advanced disease and poor survival in head and neck cancer (HNC) patients. Here, we characterized the molecular and functional interactions between neutrophils and HNC cells, and determined how these interactions might affect the progression of HNC.

We found that HNC cells promoted the recruitment, survival and proinflammatory functions of neutrophils. This modulation of neutrophil biology and functions was partly mediated by MIF (macrophage migration inhibitory factor), which was abundantly expressed and released by HNC cells. We further found that HNC cells induced neutrophils to release proinflammatory factors (such as CCL4, CXCL8 and MMP9) by activating p38-MAPK and, subsequently, p27 and CREB in neutrophils. Interestingly, the factors released by neutrophils upon HNC stimulation promoted the aggressiveness of HNC cells in a feedback manner. Specifically, we observed that HNC cells stimulated with neutrophil-derived factors metastasized significantly more than ‘wild-type’ HNC cells when injected into nude mice. We then tested which metastasis-associated properties of the HNC cells might be modulated by neutrophils. We found that neutrophils enhanced the migration, invasion and, interestingly, the resistance to NK -induced cell death of HNC cells in vitro. Our explorative studies in NSG mice (which lack NK cells) revealed, in contrast to nude mice, equal metastatic rates for unstimulated and neutrophil-stimulated HNC cells. These results suggest that neutrophils enhance HNC metastasis by increasing the resistance of cancer cells to NK cytotoxicity.

In summary, our study reveals novel mechanisms of tumor progression during cancer-related inflammation, via induction of a metastatic immune escape phenotype in tumor cells by tumor-associated neutrophils.
Superior and long-term tumor control by combining DC-SIGN targeting vaccines with curtailing regulatory T cell function


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Current tumor immunotherapies focus on in-vivo delivery of antigens to dendritic cells (DCs) to elicit the generation of tumor-specific CD8+ effector T cells. However, T cell priming, effector function and/or tumor infiltrating capacity are often compromised, limiting the efficacy of these therapeutic approaches. Particularly, tumor-associated regulatory T cells (Treg) and inefficient antigen cross-presentation may represent major obstacles for successful immunotherapy. To tackle this we designed a strategy that comprised targeting of tumorantigens to the endocytic C-type lectin DC-SIGN, highly expressed on immature DCs, in combination with controlled elimination of Tregs. Utilizing humanized mice expressing human DC-SIGN on DCs (hSIGN), we show that targeting of DC-SIGN in vivo using LewisB- and anti-DC-SIGN-modified Ovalbumin (OVA) increased cross-priming of endogenous CD8+ T cells as revealed from higher frequencies of TNF and IFNγ-secreting OVA-specific CD8+ T-cells compared to vaccination with native OVA. Combining DC-SIGN targeting formulations with Treg depletion in B16-OVA melanoma-bearing bacterial artificial chromosome-transgenic DEREG x hSIGN mice not only significantly delayed tumor growth but also caused long-term tumor regression in 50% of mice. Treg depletion alone or in combination with non-targeted OVA immunization did not induce tumor regression, albeit some delay in tumor growth was observed. Deletion of Treg permitted the systemic presence of activated CD8+ T cells with tumor-infiltrating capacity, yet no OVA-specific T cells emerged. Importantly, the combination of Treg depletion with a DC-SIGN-targeting vaccine resulted in the maximal generation of OVA-specific CD8+ T cells, which infiltrated B16-OVA tumors. Thus, inhibition of Treg-mediated immune suppression and DC-SIGN targeting strategy synergize to induce efficient tumor-specific immunity. These results may have tremendous implications for human anti-tumor therapy.
Novel RNA-lipoplexes with immunostimulatory and targeting properties induce potent antitumoral immunity

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Cancer immunotherapy with antigen-encoding RNA has been proved to be a potent tool for efficiently inducing T cell responses as well as anti-tumor immunity in preclinical models, and direct local administration of RNA is currently being tested in clinical trials with promising results. However, systemic administration of RNA is hindered through its degradation by RNases present in the serum as well as lack of proper cellular targeting. In order to circumvent this obstacle, we used liposomes to form RNA-lipoplexes providing protection against RNases. Optimizing their biodistribution, we were able to target pharmacologically optimized RNA-lipoplexes specifically to the spleen where they were selectively internalized by resident antigen-presenting cells (APCs) such as dendritic cells (DCs) and macrophages. Repetitive immunization with RNA-lipoplexes led to profound proliferation of functional antigen-specific CD8\textsuperscript{+} and CD4\textsuperscript{+} T cells to exogenous as well as endogenous antigens. Importantly, RNA-lipoplexes were capable of conferring complete tumor protection in the B16-OVA melanoma and the CT26 colon carcinoma models, and were able to eliminate progressing tumors in the therapeutic CT26 lung metastasis model. In addition to encoding antigenic information, RNA is known to exhibit immunostimulatory properties through TLR recognition. RNA-lipoplexes were also capable of inducing an inflammatory milieu in the spleen as strong but transient upregulation of activation markers was observed in all DCs, as well as in macrophages, T, B and NK cells. Upon RNA-lipoplex administration, high levels of IFNa originating from the spleen were detected in the serum, and the inflammatory environment in the spleen was shown to be IFNa-dependent. Moreover, T cells primed in the absence of IFNa signaling displayed impaired effector functions and were not able to prevent tumor growth. These results cumulatively demonstrate that systemic delivery of antigen-encoding RNA-lipoplexes with immunostimulatory properties represents a novel and potent class of anti-cancer immunotherapeutics.
Neuropilin-1 guides regulatory T cells into VEGF-producing tumors

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Introduction:
CD4⁺CD25⁺FoxP3⁺ regulatory T (Treg) cells are supposed to be an important factor in tumor immunity as they locally suppress an effective anti-tumor immune response and therefore promote tumor development. In former studies, we identified the neuronal surface receptor Neuropilin-1 (Nrp-1), which also acts as receptor for vascular endothelial growth factor (VEGF) to be highly expressed on murine Tregs. However, it is still unclear how Nrp-1 contributes to Treg cell function in vivo.

Objectives:
The aim of this study was to analyze the role of Nrp-1 on Treg cells, especially during tumorigenesis by using Nrp-1⁻/⁻ x CD4-cre (Nrp-1KO) mice to ablate Nrp-1 expression specifically in T cells.

Materials & Methods:
Lymphocyte distribution and frequencies as well as their Nrp-1 expression were analyzed in wildtype (Nrp-1WT) and Nrp-1KO mice by flow cytometry. In tumor transplantation models, 5x10⁵ tumor cells/100µl Matrigel were injected subcutaneously into the right flank of 6-8 week old mice. Tumorsize was quantified by caliper measurements and lymphocyte populations within the tumor and the tumor-draining lymph nodes were quantified and characterized by flow cytometry and immunohistochemistry. For in vitro proliferation assays, 10⁵ sorted CD4⁺CD25⁺ and CD4⁺CD25⁻ responder T cells were cultured alone or co-cultured with CD4⁺CD25⁺ or CD8⁺ responder T cells in the presence of 1 µg/ml anti-CD3 and 4x10⁵ irradiated splenocytes for 72 h. Proliferation was measured either by [³²P]-thymidine incorporation or by loss of CFSE dye by flow cytometry.

Results:
In the present study we show for the first time in several transplantation models, but also in a spontaneous, endogenously driven murine tumor model, that T cell-specific deletion of Nrp-1 leads to delayed tumor formation and growth, accompanied by an increased CD8⁺ T cell mediated anti-tumor immune response and, more importantly, a decreased infiltration of Foxp3⁺ Treg cells into the tumor tissue. Interestingly, adoptive transfer of Nrp-1 expressing Treg cells to Nrp-1KO mice outweighed this phenotype. Since we did not observe any differences in the suppressive capacity of Nrp-1 deficient and WT Treg cells, we suppose that Nrp-1 mediates infiltration of Treg cells into the tumor in dependency on tumor-derived VEGF. In vitro studies revealed that indeed, Nrp⁻¹, but not Nrp-1 deficient Treg cells migrate towards VEGF. Moreover, deletion of VEGF expression from tumor cells resembles a similar phenotype as Nrp-1 deletion from the tumor-bearing mouse.

Conclusion:
Consequently, our results suggest that Neuropilin-1 is not involved in the immunosuppressive activity of Treg cells, but rather controls infiltration of Foxp3⁺ Treg cells into tumor tissues, mediated by tumor-derived VEGF.
Inherent CD40L expression complements CD8\(^+\) T cell dependent anti-tumor immunity

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Introduction & Objective:
Adoptive therapy of cancer with tumor-specific CD8\(^+\) T cells is promising. However, which subset or phenotype of CD8\(^+\) T cells is more suitable for achieving effective and durable responses in the strong immunosuppressive context of cancer is still a matter of debate and investigation. We detected that a major subset of memory CD8\(^+\) T cells expresses CD40L, a strong immunostimulatory molecule of activated CD4\(^+\) T helper cells, and analyzed the role of this particular CD8\(^+\) T cell subset during immune responses against cancer.

Methods & Results:
We assessed the expression of CD40L on CD8\(^+\) T cells in an experimental model of tumor rejection where B6 mice were immunized with SV40 T antigen-expressing cancer cells. Here CD40L\(^+\) CD8\(^+\) T cells represented 50\% of the protective tumor-specific CD8\(^+\) T cell response. To analyze the impact of CD40L expression on CD8\(^+\) T cells \emph{in vivo} we challenged Rag1\(^-/-\) mice with cancer cells and injected wt or CD40L\(^-/-\) CD8\(^+\) T cells. Application of wt CD8\(^+\) T cells prevented the establishment of a solid tumor, whereas injection of CD40L\(^-/-\) CD8\(^+\) T cells alone or in addition with wt CD4\(^+\) T cells resulted in a non-controlled tumor progression similar to non-treated tumors. The necessity of CD40L on CD8\(^+\) T cells for tumor rejection was further demonstrated by injecting cancer cells in mice that lack CD40L expression only on mature CD8\(^+\) T cells. CD40L\(^{lox}\) x E8Icre mice were more susceptible to tumor formation than wt mice.

Conclusion:
These results disclose an essential functional relevance of CD40L expressed by CD8\(^+\) T cells in anti-tumor immunity. Especially, in tumor-driven tolerogenic conditions with limited danger signals CD40L-expressing CD8\(^+\) T cells may provide a crucial signal for anti-tumor immune responses and modulation of the tumor microenvironment and thus are potent T cells to execute or support effective anti-tumor immune therapies.
Remodelling of the lymphoma-stroma interface within secondary lymphoid organs involves fibroblastic reticular cells and dendritic cells


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Introduction:
Lymphoma cell survival and progression is critically dependent on nodal access and specific microanatomical localization within secondary lymphoid organs (SLO). The tumor stroma in SLOs consists of different cell types including cells of mesenchymal origin, cells of the innate and adaptive immune response, cells of the vasculature, but also of the extracellular matrix.

Objectives:
Here, we dissect the survival-promoting contribution of two major stroma cell populations, fibroblastic reticular cells (FRC) and dendritic cells (DC), respectively.

Materials and methods:
We applied an oncogene-driven transgenic mouse model, the Em-Myc strain, which mimics several aspects of Myc-induced aggressive human B-cell lymphoma. Lymphoma B-cells derived thereof were used for adoptive transfer experiments; applying immunohistochemistry and flow cytometry, progression and localization of lymphoma B cells were analyzed. The study involved MACS- and FACS-based isolation of FRCs and DCs derived from tumor-challenged or spontaneously diseased mice. Real time RT-PCR as well as cytometric bead arrays were used to assess the prevailing cytokine and growth factor milieu. Genetically-engineered mouse strains for a transcription factor, homeostatic cytokines and DCs were used to probe environmental factors upon adoptive lymphoma cell transfer.

Results:
Em-Myc lymphoma localized in the T cell zone in close proximity to a chemokine providing cell population, the gp38+ FRCs. Moreover, this myofibroblast-derived cell population provided prosurvival signals, foremost the hedgehog ligand Indian hedgehog. Other stroma cell populations, such as T cell zone-localized DCs, were also found to intermingle closely with infiltrating lymphoma B cells. Functionally, transfer of Em-Myc B cells in DC-depleted mice delayed lymphoma growth considerably. In vitro, co-culture of primary lymphoma cells with DCs counteracted the inherent apoptosis program of tumor cells. In gene expression analysis of tumor-derived DCs, we obtained a pattern of cytokines indicative of a transformation toward a putatively tolerogenic milieu. This observation could be corroborated in cytometric bead arrays. Furthermore, we identified the transcription factor C/EBPβ as a principal regulator of shaping a (pro-) inflammatory, tumor-permissive microenvironment within SLOs.

Conclusion:
Within nodal tumor cell niches, lymphoma cells initiate a reciprocal crosstalk with FRCs and DCs, thus effectuating synthesis and release of growth-promoting factors and inflammatory cytokines. This process is orchestrated by the transcription factor C/EBPβ.
CD4⁺ Foxp3⁺ regulatory T cells promote carcinogenesis in colitis-associated colon cancer

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Colorectal Cancer is the second most common carcinoma worldwide. Patients with inflammatory bowel diseases such as Crohn's disease (CD) or ulcerative colitis (UC) are at increased risk of developing colorectal cancer. Although the exact underlying mechanism of inflammation-associated tumor development still remains unknown, a role for the adaptive immune system has been implicated in colitis-associated cancer (CAC). The role of regulatory T cells (Tregs) is controversial. On the one hand they are able to dampen inflammation by inhibiting the activation of effector immune cells and thus can have protective effects in CAC. But on the other hand Tregs simultaneously can suppress antitumor immune responses. To investigate the role of CD4⁺Foxp3⁺ Tregs, CAC was induced in mice by a single i.p. injection of the procarcinogen Azoxymethan followed by the administration of dextran sulphate sodium (DSS) via the drinking water. After 12 weeks when tumors had developed CD4⁺Foxp3⁺ Tregs from CAC mice were compared to Tregs from healthy mice and mice which received only the DSS treatment. A strong increase of CD4⁺Foxp3⁺ Tregs in the colonic tumors but not in the mesenteric lymph nodes or spleens of mice suffering from CAC was found. Tumor-infiltrating CD4⁺Foxp3⁺ Tregs were highly activated, suggested by the increased expression of GARP, CD103, CTLA-4 and IL-10. Moreover, they exhibited an increased suppressive effect on CD4⁺CD25⁺ responder T cell proliferation and TH1-cytokine production ex vivo. Mice expressing a diphtheria toxin (DT) receptor-enhanced fluorescent fusion protein under the control of the foxp3 gene locus (DEREG mice) allow the depletion of Foxp3⁺ Tregs by DT injection. In vivo depletion of CD4⁺Foxp3⁺ Tregs during carcinogenesis in DEREG mice suppressed the tumor progression accompanied by an increase of CD8⁺ effector cells within the tumor. Furthermore, CD8⁺ T cells revealed an activated phenotype characterized by an increase of IFN-γ and Granzyme B expression. Our data suggest that selective, transient depletion of Tregs has the potential to evoke an efficient antitumor response and might have implications for therapeutic interventions in CAC patients.
Overcoming Treg mediated inhibition of tumor rejection

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Introduction:
Treg accumulation in human tumor is often associated with decreased survival.

Objectives:
1) assess the immune-suppressive role of Tregs in a quantitative way and 2) characterize the cellular and molecular mechanism involved in tumor rejection.

Materials & methods:
we have developed several strains of BAC transgenic Foxp3-Luci.DTR mice for depletion of increasing number of Tregs in tumor bearing mice. Immune population analyses were done by flow cytometry, cytokine profile was studied by Q-PCR and protein multiplex analysis.

Results:
We show that 90-95% Treg depletion resulted in complete regression of different large established tumors (e.g. MC38, RMA-Tag and M04) whereas 70% depletion, either using anti CD-25 or a different Foxp3-Luci.DTR strain, was ineffective. The observed tumor rejection is associated with a normalization of tumor vasculature and a strong immune cell infiltration, characterized by an increased number of CD4+ and CD8+ T cells and a shift from M2 macrophages towards M1-like cells. Importantly, specific CD8 T cells against tumor antigens reached 10% of all the CD8 cells. Treg depletion also induces a massive release of cytokines, changing the tumor microenvironment towards an acute inflammation profile (high levels of INFs, TNF, IL-6, IL-12 and chemokines) whereas angiogenesis related factors are downregulated. For tumor rejection not only CD8 T cells, but also macrophages are required, as shown by specific depletion experiments.

Discussion:
Treg depletion has two main effects: 1) It results in activation of endogenous anti-tumor T cells 2) it results in a modulation of tumor microenvironment, including normalization and activation of the vasculature, so that massive T cell infiltration and tumor rejection can occur. The Treg depletion model used here will allow the precise definition of mechanisms mediating tumor rejection, so that they can be used for the improvement of tumor immunotherapy.
In vitro activation of MUC1-specific T cells responses in healthy donors of different HLA type

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Objective:
Tumor-associated MUC1 is aberrantly glycosylated and overexpressed in carcinomas of the breast, ovary, colon, pancreas, prostate as well as in leukemias and multiple myelomas. The resultant epitopes are susceptible to recognition by cytotoxic T lymphocytes (CTL) facilitating treatment protocols with adoptive T cell transfer. In the present study we aimed at the in vitro activation of MUC1-specific T cells in peripheral blood mononuclear cell (PBMC) samples of healthy donors.

Methods:
CD8+ T cells were isolated from PBMCs of HLA-A2 positive and HLA-A1 positive healthy donors. The T cells were stimulated weekly with BP25 polypeptide loaded dendritic cells (DC) and interleukin-2. The BP25 polypeptide covers the MUC1 extracellular variable number of tandem repeats. The MUC1-specific T cell responses were assessed by tetramer staining and Cr51 cytotoxicity assay.

Results:
Using major histocompatibility complex-peptide tetramers, the frequency of MUC1-specific T-cells before stimulation ranged from 0.2-1.1% of CD8+ T-cells (mean 0.5%). Starting with 1*10^6 CD8+ cells the T-cell lines generated in 4 donors contained 3.6*10^7 to 2.0*10^8 (mean 1.0*10^8) CD8+ T cells after 3 stimulations with BP25 polypeptide loaded DC. However, in 1 donor the T cells did not expand but diminished within the 21 days of culture. In the other 4 donors expanded T cells contained MUC1-specific CD8+ T-cell responses. The number of MUC1-specific CD8+ T cells ranged from 0.1% to 1.6% (mean 0.5%). Functional analysis of these expanded MUC1-specific T-cells proved their specific cytotoxicity by lysing both BP25 loaded T2 cells and MUC1+ H1650 lung cancer cells.

Conclusions:
Using BP25 polypeptide loaded DC MUC1-specific CTL can be activated and expanded in vitro in healthy donors of different HLA type. Potentially in patients with malignant diseases these T cells can be used in allogeneic T cell transfer protocols.
Environmental cues determine localization and survival of leukemia B cells in a murine chronic lymphocytic leukemia model


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Introduction:
B cell chronic lymphocytic leukemia (B-CLL) is the most frequent leukemia subtype affecting adults in western countries. It develops as an expansion of clonal mature CD5⁺ B, and gene expression profiles of CLL cells show that these malignant B cells resemble antigen-experienced memory B cells. In B-CLL patients, tumor cell survival and progression are linked to interactions between leukemia cells and non-tumor cells in microenvironments of the spleen, peripheral blood (PB), and bone marrow (BM). Little is known about how CLL cells get access to a putative proliferation and survival niche within lymphoid organs, and a spatial and kinetic resolution of this process would be desirable.

Objectives:
Here, we aimed to link the mechanisms of leukemia cell activation and the microenvironment dependence during CLL pathogenesis in Eu-Tcl1 mice.

Materials and methods:
We applied an oncogene-driven transgenic mouse model, the Eu-Tcl1 mouse strain, which phenocopies several aspects of human B-CLL. Leukemia B-cells derived thereof were used for adoptive transfer experiments. Applying immunohistochemistry, flow cytometry, and sequential static imaging, progression and localization of leukemia B cells were analyzed.

Results:
Using the Eu-Tcl1 CLL model, we demonstrate that CXCR5-controlled access to follicular dendritic cells (FDC) is required to confer proliferative stimuli to leukemia B cells. Sequential static imaging revealed a marginal zone B-cell-like leukemia cell trafficking route and suggested extended interaction times with FDCs. Murine and human CLL cells reciprocally stimulated resident mesenchymal stromal cells through lymphotoxin-ß receptor activation, resulting in CXCL13 secretion and stromal compartment remodeling. Furthermore, therapeutic inhibition of lymphotoxin/lymphotoxin-ß receptor signaling or inhibition of the CXCL13/CXCR5 signaling axis could break this cycle, retarding leukemia progression.

Conclusion:
CXCR5 activity defines a mechanism that links tumor cell homing, shaping a survival niche, and antigenic stimulation.
Stabilin-1 is expressed on tumor-associated macrophages on early stages in breast cancer and supports tumor growth in animal model for breast carcinoma by clearance of SPARC

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Introduction:
Tumour-associated macrophages (TAM) are crucial participants in malignant progression. Acquisition of M2 (alternatively activated) phenotype by TAM during tumour progression enhances the immunosuppressive and tumour-supportive properties of TAM including support of tumour invasion, metastasis and angiogenesis. Scavenger/sorting receptor stabilin-1 is a marker of M2 macrophages and was found to be expressed by TAM in several murine tumour models. However, its role in tumour progression was not defined.

Results:
In order to identify the role of stabilin-1 in tumour progression, the model for mammary adenocarcinoma (TS/A) was established in BALB/c mice with stabilin-1 knockout. Growth of TS/A mammary adenocarcinoma in stabilin-1 knockout (ko) mice was suppressed by 36%. To identify the role of stabilin-1 in TAM biology and reveal functions of stabilin-1 that are important for tumour progression, isolation of high purity TAM from TS/A murine adenocarcinoma was established and optimized. Flow cytometry quantification revealed that adhesion/internalisation of extracellular SPARC was decreased in the isolated stabilin-1 ko TAM compared to wt TAM. Immunofluorescent/confocal microscopy analysis showed that transport of SPARC into the endocytic pathway was significantly impaired in the stabilin-1 ko TAM. SPARC has inhibiting effect on the development of breast cancer. Therefore knock-out of stabilin-1 in TAM leading to the increased concentrations of SPARC results in suppression of tumor growth. Analysis of two cohorts of female patients with breast carcinoma of different stages demonstrated that stabilin-1 is expressed on significant part of tumor associated macrophages. Three types of TAM were identify by co-staining with a-stabilin-1 RS1 antibody and a-CD68 antibody: CD68+stabilin-1–, CD68+stabilin-1+ and CD68+stabilin-1+. Highest levels of stabilin-1 expression and highest amount of stabilin-1+ TAM were found on stages I and IIa, suggesting that stabilin-1 is required for support of tumor growth at early stages of tumor progression.

Conclusions:
Out data indicate that stabilin-1 expression on TAM is needed on the early stages to tumor growth in human cancer, genetic knockout of satbilin1 results in decrease in tumor growth in mouse adenocarcinoma model, and tumor associated macrophages deficient in stabilin-1 have significantly decreased ability for the endocytic clearance of SPARC.
A mass spectrometry approach for low abundant epitope identification - applied for detection of HPV epitopes for therapeutic vaccine design

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Introduction:
Human papillomavirus (HPV)16 has been identified as the causative agent in 50% of all cervical cancer cases and in virtually all extra-cervical mucosal HPV-induced tumors. The development of prophylactic vaccines to prevent HPV infection in previously unexposed individuals was a great achievement in combating HPV. Therapeutic vaccination options for already infected patients are under active investigation. As the induction and maintenance of the malignant phenotype depends on two consistently expressed viral oncoproteins, E6 and E7, they are ideal targets for immunotherapy.

Objectives:
As HPV affects the cellular antigen processing machinery, not every epitope derived from viral proteins is presented by MHC molecules on the cancer cell surface. Even the naturally processed and presented epitopes are present in very low abundance. To directly identify these low-abundance epitopes, we here apply a highly sensitive MRM (multiple reaction monitoring) nano-UPLC-ESI-MS² and -MS³ mass spectrometry approach for direct epitope identification on the cancer cell surface.

Materials and Methods:
Prospective epitopes from the HPV16 E6 and E7 proteins for the five major HLA supertypes, allowing >95% population coverage, were predicted in silico and ranked according to their binding affinity. The best predicted binding peptides were synthesized and tested in competition-based cellular binding assays. The presentation of epitopes on HPV16-transformed cancer cells was analyzed by the above mentioned MS methodology.

Results:
In silico predictions resulted in over 360 possible HLA binding peptides derived from the HPV17 E6 and E7 proteins. So far, over 150 novel binders were identified for the investigated HLA supertypes in the cellular binding assays. MS analysis was successfully established and validated for HLA-A2, and is currently ongoing for HLA-A24.

Conclusions:
We here show that ascertaining the actual cellular presentation of low abundance T cell epitopes is feasible from a limited amount of cells. As the included HLA supertypes have a cumulative population prevalence of >95%, the resulting set of verified epitopes may be used for the formulation of a widely applicable therapeutic HPV vaccine, or for immunomonitoring purposes in any HPV immunotherapy studies.
Exploring the mTOR inhibitor Rapamycin to generate EBV-reactive CD8\(^+\) T cells with stem-cell-like and central memory properties for adoptive immunotherapy

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Introduction:
Cellular immunotherapy using in vitro generated tumor- and virus-reactive T cells for adoptive transfer has evolved as a promising strategy to treat cancer and virus-associated malignancies such as Epstein-Barr Virus (EBV)-mediated post-transplant lymphoproliferative disease observed after allogeneic hematopoietic stem cell transplantation. However, high avidity effector T cells (T\(\text{EFF}\)) obtained from extensive culture conditions to elicit highly effective antitumor immunity have been reported to exhibit limited homing and self-renewal capacity to establish sustained immunity and memory.

Objective:
Based on previously established cytokine optimized culture conditions we thus explored the modulation of mTOR-mediated signaling pathways reported to promote T cell memory formation to generate HLA-A*0201 restricted EBV-reactive T cells with stem-cell-like (T\(\text{SCM}\)) and central memory-type (T\(\text{EM}\)) properties.

Methods:
Naïve CD8\(^+\) T cells isolated from peripheral blood mononuclear cells (PBMCs) of healthy donors using the Naive CD8\(^+\) T-Cell Isolation Kit (Miltenyi Biotec) were repetitively stimulated with EBV-peptides presented first by autologous dendritic cells (DCs) followed by HLA-A2\(^+\) T2 cells or autologous PBMCs in the presence of the interleukins (IL)-7, -15, and -21 for 4 weeks. mTOR activity was inhibited in the presence of different concentrations of Rapamycin added to cultures. Phenotypic and functional analysis were performed by flow cytometry and IFN-g ELISpot assays, respectively, at different time points of culture. The biological activity of these in vitro generated EBV-reactive CTL in vivo is currently being tested by adoptive transfer into NOD/scidIL2Rgc-null (NSG) mice previously engrafted with autologous EBV-transformed B cells (B-LCL).

Results:
Upon repetitive stimulation of naïve CD8\(^+\) T cells for 28-33 days we obtained good enrichment of EBV-reactive T\(\text{SCM}\) and T\(\text{EM}\) CTL characterized by a CD8\(^-\)CD45RA\(^+\)CD45RO\(^-\)CD95\(^+\)CD27\(^+\)CD28\(^+\)CD62L\(^+\)CCR7\(^+\) phenotype when compared to CTL stimulated without inhibitor. Molecular and flow cytometric studies revealed reduced cell size and reduced expression of pro-effector transcription factor T-bet confirming our phenotypic analyses on T cell differentiation. Rapamycin treated and control CTL populations elicited largely comparable reactivity against B-LCL in vitro as shown by IFN-g secretion. Adoptive transfer studies to evaluate the biological activity of Rapamycin treated EBV-reactive CTL as compared to controls are currently in progress in NSG mice engrafted with autologous B-LCL, and the results will be discussed.

Conclusion:
The results of this study strongly suggest that EBV-reactive CTLs with stem-cell-like and central-memory properties can be successfully generated upon inhibition of mTOR activity in the presence of optimized culture conditions for improved cellular therapy.
Macrophages in T cell/histiocyte rich large B cell lymphoma strongly express metal-binding proteins and show a bi-activated phenotype


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Abundant macrophage infiltration in tumors often correlates with a poor prognosis. T cell/histiocyte rich large B cell lymphoma (THRLBCL) is a distinct aggressive B cell lymphoma entity showing a high macrophage content.

To further elucidate the role of tumor-associated macrophages in THRLBCL, we performed gene expression profiling of microdissected histiocyte subsets of THRLBCL, nodular lymphocyte predominant Hodgkin lymphoma (NLPHL), Piringer lymphadenitis, sarcoidosis, nonspecific lymphadenitis, and monocytes from peripheral blood.

In a supervised principal component analysis, histiocytes from THRLBCL were most closely related to epithelioid cells from NLPHL, with both types of cells expressing genes related to proinflammatory and regulatory macrophage activity. Moreover, histiocytes from THRLBCL strongly expressed metal-binding proteins like MT2A, by which histiocytes of THRLBCL can be distinguished from the other histiocyte subsets investigated. Interestingly, the validation at the protein level showed a strong expression of TXN, CXCL9, MT2A and SOD2 not only in macrophages of THRLBCL but also in the tumor cells of NLPHL and classical Hodgkin lymphoma (cHL).

Overall, the present findings indicate that macrophages in the microenvironment of THRLBCL have acquired a distinct gene expression pattern, that is characterized by a mixed M1/M2 phenotype and a strong expression of several metal binding proteins. The microenvironments in NLPHL and THRLBCL appear to have a similar influence on the macrophage phenotype. The high expression of metal binding proteins in histiocytes of THRLBCL may be diagnostically useful, but a potential pathophysiological role remains to be identified.
Induction of regulatory T cells by AML bone marrow stroma

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Question:
In leukemia, and acute myeloid leukemia (AML) in particular, cross-talk between the malignant cells and stromal cells in the bone marrow (BM) microenvironment is considered to play a role in blocking of cell death, stimulation of proliferation, drug resistance induction, and immune suppression. In the work presented here, we studied the influence of soluble factors released by BM stroma cells on the induction of regulatory T cells.

Methods:
To study such stromal effects in more detail and with an unlimited source of cells we had established three BM stroma cell lines from samples of two AML patients (K-AML and CZ-AML) and a sample from a normal donor (N-KM). The technique used for immortalization is considered to maintain the phenotype of the primary cells as much as possible. The cells reveal a common mesenchymal stem cell surface marker profile and have been in continuous culture for >1 year. Here, we studied the immunomodulatory properties of soluble factors derived from these cell lines in comparison to four AML derived primary stroma cells on the induction of regulatory T cells.

Results:
After the incubation of PBMCs in presence and absence of stromal cell line’s supernatant during 7 days, we observed that the percentage of CD4+CD25+FOXP3+ cells did not change in the flow cytometric analysis. In contrast, an 1.5-fold increase of FOXP3 mean fluorescence intensity (MFI) was observed for CD4 T cells cultured with supernatant of the K-AML cell line compared to the CZ-AML BM stroma cell line and the normal donor. Culturing PBMCs with supernatant of AML derived primary stroma cells from four different AML patients revealed for two supernatants an induction of FOXP3 comparable to the K-AML supernatant, whereas the other two primary AML stroma supernatants showed no effect on the FOXP3 expression level. These results were verified by quantitative FOXP3 specific Real-Time PCR. Moreover, the same experimental setting has been analyzed by the Amnis Image Stream instrument. The biophysical characterization showed that the CD4+CD25high+FOXP3+ cells seem to be smaller than the CD4+CD25low+FOXP3+ cells.

Conclusions:
The supernatant of the two novel BM stroma cell lines derived from AML patients showed a converse behavior in case of the up-regulation of FOXP3 in CD4+CD25+ T cells, which was confirmed by comparison of AML-derived primary stroma cell supernatants. Thus, it seems that these cell lines are qualified to investigate immune modulatory properties of soluble factors being part of a leukemic BM microenvironment, e.g. modulation of immune cells.
Free but not exosome-derived soluble HLA-G molecules are of prognostic relevance in lung cancer patients

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Question:
HLA-G exerts multiple immune suppressive regulatory functions and can be released into circulation as a free molecule or via microvesicles (MV) such as exosomes. Here we analyzed the clinical relevance of free and exosomal sHLA-G molecules in plasma samples of 23 small cell lung cancer (SCLC) patients and of 114 non-small cell lung cancer patients.

Methods:
In each sample the total amount of sHLA-G (sHLA-Gtotal) and of exosomal HLA-G (sHLA-Gexo) after ExoQuick precipitation were determined by specific ELISA. The corresponding increment values were defined as the amount of free sHLA-G (sHLA-Gfree) molecules. MV were characterized for their sizes by Nanoparticle Tracking Analysis (NTA) and for the presence of exosomal markers by SDS-PAGE and specific Western Blotting.

Results:
The majority of MV revealed an average size of 100 nm. MV preparations of patients and healthy controls were found to be positive for the typical exosomal markers CD9, CD63, CD81, TSG101, and rab5b. Compared to 20 controls sHLA-Gtotal (p=0.03) and sHLA-Gfree (p<0.001) were significantly increased in patients, but sHLA-Gexo was only strikingly increased in controls (p<0.001). Although in the small group of SCLC patients sHLA-Gtotal did not differ from controls, higher sHLA-Gfree and lower sHLA-Gexo levels were found compared to controls. Among all patients an association of sHLA-Gexo with the absolute number of monocytes, with IL-10 and with TGF-beta levels (p<0.05) were observed, whereas sHLA-Gfree was related to tumor burden (p=0.002). High sHLA-Gfree levels but not the levels of sHLA-Gtotal or sHLA-Gexo were associated with a worse five-year overall survival (OS). Importantly, multivariate analysis revealed disease stage (p<0.0001, Hazard Ratio: 11.2) and sHLA-Gfree (p=0.007, Hazard Ratio 2.1) but not sHLA-Gtotal and sHLA-Gexo as independent prognostic factors for OS.

Conclusions:
This study clearly demonstrates for the first time that free but not exosomal sHLA-G molecules are of prognostic relevance in lung cancer. Thus, it seems necessary to differentiate between free and exosomal sHLA-G molecules to evaluate the diagnostic relevance of sHLA-G in future studies.
High but not low affinity TCR prevent relapse of cancer in TCR gene therapy

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Introduction:
Engineering of T cells with tumor-reactive T cell receptors (TCR) - TCR gene therapy - provides a powerful approach for adoptive T cell therapy of cancer. The anti-tumor effect of TCR-engineered T cells is largely determined by the transgenic TCR that recognizes tumor cell-derived peptides presented on the patient's tumor tissue. However, current therapeutic approaches are impeded because although the function of selected TCR can be analyzed in vitro, parameters that predict in vivo efficacy are not established. We developed a mouse model system for the evaluation of the therapeutic efficiency of TCR gene therapy on established cancer.

Material and Methods:
We generate human MHC-transgenic mice (HHDxRag2/2) and a syngeneic tumor cell line that can be modified to express human tumor antigens of interest. T cells were engineered with murinized human tyrosinase-specific TCR of high affinity (isolated from a non-tolerant repertoire) or lower affinity TCR (isolated from a tolerant repertoire) (Wilde et al., Blood, 2009). The anti-tumor effect of the T cells was analyzed in vitro and by adoptive transfer into mice bearing established, tyrosinase-positive cancer. Therapeutic efficacy was assessed by monitoring tumor growth. In parallel, cross-presentation of antigen by tumor stroma was analyzed by functional assays and by in situ confocal microscopy.

Results:
Adoptive transfer of T cells engineered with high affinity tyrosinase-specific TCR resulted in complete rejection of cancer, whereas lower TCR affinity selected for escape variants and relapse. The tumor-derived human tyrosinase was cross-presented by tumor stroma cells (CD11b-positive cells). Although T cells engineered with both the high or lower affinity TCR showed identical in vitro tumor cell killing, only T cells expressing the high affinity TCR recognized cross-presented antigen on tumor stroma.

Conclusions:
This in vivo model provides a versatile, pre-clinical test system of adoptive T cell therapy of cancer and allows the prediction whether or not TCR-engineered T cells eradicate large tumors or select escape variants. Our results indicate that only TCR gene therapy with high affinity TCR prevents relapse, as opposed to use of lower affinity TCR which are typically isolated from the tolerant human repertoire against tumor-associated antigens. We suggest that the high affinity of the TCR enables the T cells to recognize and perhaps eliminate tumor stroma cells cross-presenting the melanoma antigen tyrosinase, resulting in bystander elimination of escape variants.
HLA-independent recognition of human melanoma cells by autologous CD8+ α/β-TCR+ T cells

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Introduction:
T cell-tumor interactions are constrained by immune barriers and escape mechanisms such as HLA loss. In a human melanoma model (MA-MEL-86/INTH) we observed T-cell receptor (TCR)-mediated recognition of autologous, HLA class I-negative tumor cells by CD8+ T cells. So far, only few examples of HLA-independent, but T-cell receptor (TCR)-mediated recognition by CD8+ and CD4+ T cells have been reported.

Objectives:
We aimed at the identification of the antigens targeted by HLA-independent T-cell clones derived in the MA-MEL-86 melanoma model and at the cloning of their T-cell receptors.

Materials & methods:
Four permanent tumor cell lines had been generated from independent lymph node metastases of melanoma patient MA-MEL-86. They carried putative signs of immune escape, such as total loss of melanosomal antigens, total loss of HLA class I molecule expression due to inactivation of the beta-2-microglobulin genes and HLA class I haplotype loss, respectively. Peripheral blood mononuclear cells (PBMCs) separated from the patient's blood at different time points were stimulated with different melanoma lines in mixed lymphocyte-tumor cell cultures (MLTC). MLTC responders and T-cell clones derived thereof were applied to cDNA library expression screening for target identification as described (Lennerz et al., PNAS 102:16013, 2005). Antigen-specific (TCRs) were identified and cloned according to Birkholz et al. (J. Immunol. Methods 346:45, 2009).

Results:
Typing with autologous and allogeneic melanoma cells indicated that the HLA-independent, CD8+ T cells recognized at least four distinct antigens. Lysis by these T cells was blocked with anti-CD3 antibodies, but not with antibodies against HLA molecules. Two antigens were identified so far. These were CSF2RA [GM-CSF receptor alpha chain; syn.: colony stimulating factor 2 receptor, alpha, low affinity (granulocyte-macrophage)] and TRP-2 (tyrosinase-related protein 2; syn.: L-dopachrome tautomerase). Recognition of both molecules required their presence on the cell surface and occurred across species barriers upon transfection of antigen-encoding plasmid DNA only. TRP-2-reactive T cells cross-reacted with almost all allogeneic TRP-2+ melanoma lines and primary melanocytes, irrespective of their HLA phenotype. T cells against CSF2RA reacted with a significant proportion of allogeneic melanoma lines, acute myeloid leukemia (AML) blasts as well as with pancreatic, cholangiocellular, colon, lung and ovarian cancer cell lines, but not with melanocytes and blood-derived mononuclear cell. α/β-TCRs against both antigens were cloned and were shown to mediate HLA-independent tumor cell lysis in vitro after transfer into allogeneic T cells.

Conclusion:
Due to their ability to redirect T cells against naturally occurring HLA-loss variants, TRP-2 and CSF2RA appear to be promising targets to safeguard all efforts in generating efficient and sustained anti-tumor immune responses. Although recognition by HLA-independent T cells is confined to cell surface molecules, their therapeutic use would counteract numerous escape mechanisms affecting HLA/peptide-specific T cells and also allow to circumvent the dictates of individual HLA phenotypes.
Dedifferentiation of malignant melanoma: a potential barrier to antigen-specific immunotherapy

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Melanoma differentiation proteins (Melan-A/MART-1, gp100, tyrosinase, tyrosinase-related proteins 1 and 2 (TRP-1 and TRP-2)) are well known as target antigens of cytotoxic CD8⁺ T lymphocytes. Accordingly, among the peripheral blood lymphocytes from melanoma patient Ma-Mel-86 we detected tyrosinase-specific CD8⁺ T cells. These T cells recognized only one of two autologous melanoma cell lines (Ma-Mel-86a, Ma-Mel-86c) that were established from sequential lymph node metastases. Precisely, tyrosinase-specific CD8⁺ T cell clones recognized Ma-Mel-86c cells in an HLA-dependent manner, but did not respond to Ma-Mel-86a cells. This led us to ask for the molecular mechanisms of MDA loss in Ma-Mel-86a.

All MDA and their transcriptional regulators MITF, Sox10 and LEF1 were expressed in Ma-Mel-86c cells according to Western blot and qRT-PCR. In contrast, none of these molecules was detectable in Ma-Mel-86a cells. The dedifferentiated Ma-Mel-86a phenotype was stable during culture and could not be reverted by transfection of the cells with MITF and/or Sox10. Treatment of the tumor cells with Vemurafenib could not induce de novo MDA expression in Ma-Mel-86a cells but enhanced MDA expression in Ma-Mel-86c. Even in the presence of the histone deacetylase inhibitor trichostatin A and the DNA demethylating agent 5-azacytidine differentiation of Ma-Mel-86a cells could not be induced, pointing to a definitive reprogramming of the cells. Importantly, mutations shared by both cell lines, including alterations in BRAF and p53, allowed us to confirm the melanoma origin of Ma-Mel-86a cells.

Since the anti-melanoma T-cell repertoire of patient Ma-Mel-86 involved besides tyrosinase-specific T cells also T cells against gp100 and TRP-2 we hypothesize that MDA-specific immune responses selected for the outgrowth of dedifferentiated Ma-Mel-86a cells emphasizing the limitations of MDA-specific mono-immunotherapy.
Antigen-armed antibodies targeting B lymphoma cells effectively activate antigen-specific CD4+ T cells

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Introduction:
The treatment of non-Hodgkin lymphomas has benefited enormously from the introduction of monoclonal antibodies directed against surface antigens. The inclusion of anti-CD20 antibody in treatment protocols against B cell lymphomas epitomizes this approach and the list of potential targets is steadily growing. However, the efficacy of antibody-based therapy varies with the lymphoma subtypes and typically decreases with the occurrence of subsequent relapses. Thus, efforts have been made to optimize their antitumor activity by conjugating them with cytotoxic drugs or with radionuclides, these antibodies are highly effective but have had mitigated translational success to date due to their higher toxicity. We have generated a new type of armed antibody, known as antigen-armed antibodies (AgAbs), in which we couple an antibody targeting B cell surface receptors (CD-19, -20, -21 and -22) to the peptide of an immunodominant T cell epitope of a common pathogen. Treatment with these antibodies leads to presentation of the peptide to specific T cells, which become activated and can kill the targeted B cells.

Objective:
To determine the efficacy of AgAb treatment of various B lymphoma cell lines in vitro with regards to activation and cytolytic effect of antigen-specific T cells. AgAb treatment will be evaluated as a potential therapeutic strategy against B lymphoma.

Materials & methods:
Recombinant antibodies directed against human CD19, CD20, CD21 and CD22 were designed to include different immunodominant EBV T cell antigens from proteins such as EBNA2, EBNA3B and EBNA3C. The antibodies were produced by transient expression of HEK293 cells. T cell response assays were performed in lymphoblastoid cell lines (LCLs) and Burkitt’s lymphoma (BL) cell lines treated with AgAbs. T cell activation in vitro was determined by interferon γ secretion detected by Enzyme-linked immunosorbent assay. Target cell killing was also determined in vitro by standard 4 hour Chromium 51 release assay.

Results:
Treatment of LCLs and BL cell lines with AgAbs led to antigen presentation, T cell recognition and target cell killing, and was superior to peptide treatment alone. The efficiency of action of the AgAbs paralleled the abundance of the targeted molecules on the lymphoma cells as well as their HLA class II expression levels.

Conclusion:
AgAb treatment demonstrates potential as an effective therapeutic strategy against B lymphomas and this strategy warrants further investigation in additional B lymphoma subtypes, especially for those that retain a high expression of MHC class II.
Cancer/testis antigen 96 (dual specificity protein kinase TTK) is expressed in Hodgkin’s lymphoma cells and involved in chemoresistance

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Introduction:
A significant number of patients with a Hodgkin’s lymphoma (HL) cannot be cured with current therapy regimes. In addition, this therapy is associated with severe late side effects. Therefore it is important to identify new therapeutic targets to improve the current therapy and create new therapy options.

Objectives:
The main goal of the present study was the identification of new genes which might be associated with the resistance of HL cells against cytostatic drugs. In addition, we tested whether one of the identified genes can be used as antigen for the development of HL-specific immunotherapy.

Materials & methods:
We established a cDNA library from the chemoresistant HL cell line KM-H2. This library was used for the identification of new potential genes which are involved in the resistance against cytostatic drugs. For this end, we transfected sensitive cells with the library and treated them with cisplatin. From surviving cells the transfected vectors were re-isolated and sequenced. Expression of identified resistance-associated genes in tumour cells and normal tissues was analyzed by conventional and quantitative reverse transcription-polymerase chain reaction (RT-PCR). For immunospot analysis we transfected lymphoblastoid cell lines with in vitro synthesized TTK RNA. Thereafter, these cells were used as antigen presenting cells for priming of CD8+ T-cells from healthy donors. After priming we analyzed the reactivity of stimulated CD8+ T-cells against HL cells by using interferon gamma enzyme-linked immunospot (ELISPOT).

Results:
One gene which was identified by the transfection experiments was the dual specificity protein kinase TTK (TTK, also known as cancer/testis antigen 96, CT96). RT-PCR revealed that TTK is expressed in HL cell lines and other cancer cell lines (e.g. Ewing sarcoma cell lines, neuroblastoma and leukemia cell lines) but not in normal blood cells. In normal tissues, TTK was highest expressed in testis. Cancer/testis antigens like TTK are interesting candidates for novel immunotherapeutic approaches. We used in vitro synthesized TTK RNA for transfection of antigen presenting cells and primed CD8+ T cells in vitro. Interferon gamma ELISPOT indicated that TTK RNA-primed T cells were able to react against TTK expressing HL cells. Reactivity against TTK-negative HL cell lines was significantly lower.

Conclusion:
The tumour antigen TTK is an interesting target for the development of new therapy strategies for patients with Hodgkin’s lymphoma.

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Cancer is driven by multiple genetic events followed by further clonal evolution, rendering disease elimination with single-targeted drugs a difficult task. The multiplicity of gene mutations derived from sub-clone heterogeneity may represent an ideal setting for multi-epitope tumor vaccination. Vaccines are particularly suited for the expansion of antigen-specific CD4+ and CD8+ T cells and allow precise targeting of tumor-associated molecular alterations. In the last years, multiple tumor-associated antigens (TAA) have been identified and categorized based on tumor-specific deregulation of gene expression. In addition, neo-epitopes can be created by non-synonymous, somatic mutations. As human cancers carry 100-300 non-synonymous mutations on average which are not subject to central immune tolerance, these mutations can be ideal candidates for vaccine development. We propose that cancer can be targeted by T cells induced by poly-epitopic vaccines based on non-synonymous individual tumor-specific mutations. In order to test this hypothesis, we resorted to B16-F10 murine melanoma of which we have identified more than 500 non-synonymous mutations by whole exome sequencing. After selection of expressed genes and good potential MHC binders of the respective mutated epitopes, 50 mutations were chosen and validated by Sanger sequencing. In order to define the immunogenicity of the mutation-coding sequences, we designed 27-mer peptides incorporating either the mutated or the wild-type amino acid to immunize C57BL/6 mice. Using IFNg ELISpot, we found one third (16/50) to be immunogenic, and out of these, 60 % elicited immune responses preferentially directed against the mutated sequence as compared to the wild-type sequence. Anti-tumor potency of all immunogenic epitopes was confirmed in a transplantable B16-F10 melanoma model where mice immunized with mutation-encoding in vitro transcribed RNA revealing tumor control in the protective and the therapeutic setting for a substantial number of mutations sequence encoded epitopes. Surprisingly we identified one MHC class II restricted epitope (Mut-30) that confers tumor control beyond the efficacy of known immunodominant tumor associated antigens like Trp2 or gp100. In summary our data prove that non-synonymous mutations are vaccine targets that code for naturally processed epitopes inducing efficiently potent anti-tumor T-cell responses. Future studies will characterize the mechanistic basis for the observed effects and pave the way to clinical translation of the concept.
The IVAC approach: non-synonymous mutations of the CT26 colon carcinoma cell line are a source of tumor targets

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Recently, understanding of the dynamic interplay between the tumor and immune system has led to the development of novel immunotherapies, including cancer vaccines. In the last years, multiple tumor associated antigens (TAA) have been identified and categorized based on tumor-specific deregulation of gene expression. In addition, neo-epitopes can be created by non-synonymous, somatic point mutations. As human cancers carry 100-300 non-synonymous mutations on average which are not subject to central immune tolerance, these mutations can be ideal candidates for individual vaccine development.

We have previously shown that by next generation sequencing (NGS) with subsequent bioinformatic analysis we are able to identify immunogenic mutations. To this end, the highly tumorigenic B16F10 murine melanoma was used. As proof-of-concept 50 highly expressed mutations were selected according to their potential MHC binding affinities and validated by Sanger sequencing. We could show that a fraction of these mutations are able to induce mutation specific T-cells with anti-tumoral potency.

To confirm the potential of this approach, we extended the proof-of-concept studies to test the CT26 colon cancer model in the Balb/c background. After bioinformatic analysis more than 600 non-synonymous expressed mutations were identified in the tumor cell line compared to the WT background. In this study we defined 96 mutations to test for in vivo immunogenicity and confirmed them by Sanger-sequencing. The mutations were chosen from different subgroups, e.g. for high and low affinity binding to MHC molecules. To test the mutations for immunogenicity 96 IVT-RNAs were produced encoding the mutated amino acid with the flanking 12 amino acids up- and downstream of the mutation. Those sequences were introduced in our MITD-cassette allowing maximal MHC class-I and class-II presentation. We immunized Balb/c mice four-times with 2 mutation-encoding RNAs in each group and analyzed the induced T cell reactivity via IFN-γ ELISPOT analysis. For the ELISPOT splenocytes of the mice were restimulated with mutation and WT-coding long peptides and bone marrow-derived dendritic cells transfected with the respective mutated RNA.

Twenty-two of the 96 mutations induced mutation-specific reactivity. In three cases the mutations did also induce WT-reactive cells. The mutation-specific T cells were further analyzed for their MHC-restriction and were found to be CD8+ and CD4+ T cells. In addition, more immunogenic mutations were found in the subgroup with the predicted better MHC-binding. The mutations are currently being evaluated for anti-tumoral activity.

In summary the data proof and extend the validity of NGS based mutation identification in conjunction with bioinformatic prioritisation and the potency of RNA based anti tumoral vaccination. This study paves the way for clinical translation of the RNA based individualized vaccine against cancer (IVAC).
Cancer is a disease caused by DNA aberrations: tumors contain 10s to 100s of mutations that are unique to the tumor cells and thus can serve as the basis for highly individualized, patient-specific mutation-targeting therapeutic cancer vaccines. Recently we provided the pre-clinical proof of concept that non-synonymous point mutations identified via next generation sequencing (NGS) are immunogenic, targetable by individualized vaccines, and provided tumor control. In the B16F10 murine melanoma model, we found a total of 563 non-synonymous expressed somatic single nucleotide variants. One third of the mutations tested were immunogenic and immunization conferred in vivo tumor control, qualifying mutated epitopes as source for effective vaccines. We now investigated other types of potentially immunogenic genomic alterations to increase the number of potential targets for individualized therapeutic cancer vaccines. To this end, we examined insertions and deletions (indels) in the B16 cells: translated indel-containing mRNAs would create long, novel and possibly immunogenic peptides.

However, methods to identify, validate and determine expression of indels are not well established. Indel identification from NGS data has low accuracy due to read alignment difficulties; validation is complicated by the frequent occurrence of both indel-containing and WT sequences, causing difficult to interpret Sanger traces; and mRNA expression confirmation, which may be biologically impacted by nonsense-mediated decay, is complicated by the presence of mutated and WT alleles. Addressing these difficulties, we developed a lab and computational workflow for identification of potentially immunogenic indels. We identified nine frameshifting indels in the NGS data. Eight were confirmed by re-sequencing of the genomic loci. Seven of those indels are expressed as mutation-containing mRNAs, as determined by RNA-Seq and Sanger sequencing. The eight indels cause frameshifts, leading to predicted novel amino acid sequences of between one and 185 residues.

Using our RNA vaccine platform, we designed RNA constructs encoding for the entire novel frameshift amino acids. Those sequences were introduced in our MITD-cassette allowing maximal MHC class-I and class-II presentation. To ensure endogenous expression and presentation, we vaccinated naive C57BL/6 mice with the indel encoding RNA five times in two weeks. Five days after the last vaccination we tested for the induction of target specific T cells via IFN-γ ELISpot utilizing overlapping peptides for readout. In total we discovered specific T cell reactivity against four epitopes in two different indels. Further analysis by flow cytometry confirmed that the mutation-specific T cells are CD4+ and CD8+ T cells. Anti-tumoral potency of vaccinations with the RNA constructs encoding the immunogenic indels are being evaluated.

In summary, our data show that indels can be identified by a NGS based mutation discovery pipeline and demonstrate that they are a prolific source of targets for individualized vaccines against cancer (IVAC). Further studies in multiple models to characterize the therapeutic potential of indels for cancer vaccination are underway.
Determination of HLA type and expression of cancer cell lines from RNA-Seq data

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Cancer cell lines are a tremendous resource for cancer biology and therapy development. Given the advent of next-generation sequencing (NGS) technology, they are an ideal tool to examine the genetic origin of cancers; to identify potential novel tumor targets, such as tumor antigens for vaccine development; and to use in preclinical studies. For this purpose, we need highly characterized cell lines. Mutations, gene expression, and drug sensitivity have been determined for many cell lines. However, the HLA type and HLA expression of cell lines, characterizations necessary for the development of cancer vaccines, are largely incomplete and, when available, distributed in many publications.

Here, we used a previously described in silico method to determine HLA type and expression of publically available RNA-Seq samples from cancer cell lines. We use standard NGS RNA-Seq short reads from “whole transcriptome” sequencing, map reads to known HLA types, and statically determine HLA type, heterozygosity, and expression. First, to our knowledge, we report previously unreported HLA class I and II genotypes and HLA expression of cancer cell lines. Second, these results provide a fundamental cell line “barcode” to track samples and prevent sample annotation swaps and contamination. Third, this provides insights into HLA downregulation and loss in cancer. Finally, these results are a fundamental resource for vaccine drug development. We show how to integrate the cancer cell-line specific HLA types and HLA expression determined here, with public available cell-line specific mutation information and existing MHC binding prediction algorithm toto make a catalog of likely immunogenic mutations in each tumor cell line.
Molecular and functional characterization of the highly specific cancer target T-066

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Introduction:
Ideal antigens for cancer immunotherapies should be expressed specifically in tumor cells, sparing normal cells from being targeted by immunotherapeutic treatments. A confounding factor of targeted cancer therapies, immunotherapy or otherwise, is the possibility of non-expressing tumor cells, either through target down-regulation or tumor heterogeneity. Understanding of the regulation and function of tumor specific antigens might reveal possible mechanisms for their re-expression in tumors and thus result in more efficient tumor eradication. A group of promising targets for cancer immunotherapy are tight junction molecules. Expression of these molecules is frequently deregulated in cancer resulting in disruption of tight junctions, a characteristic often associated with a higher metastatic potential.

Objectives:
In this study, we present the tight junction molecule T-066 as a novel target candidate for cancer immunotherapies. T-066 is expressed during early stages of embryogenesis only and is absent in normal adult somatic tissues; however, the function and regulation of T-066 in cancer is largely unknown. Here, we summarize our efforts to elucidate the function and regulation of T-066 in cancer.

Methods:
mRNA expression of T-066 was analyzed by quantitative RT-PCR in human primary tumor and normal tissues; bisulfite sequencing was used to analyze the T-066 promoter methylation in human primary ovarian and lung tumor and normal tissues as well as human tumor cell lines. RNAi mediated silencing was used for functional analysis of T-066 in different cellular in vitro assays.

Results:
We found high mRNA expression of T-066 in multiple cancer tissues, especially ovarian, testicular and lung cancer, whereas it is not expressed in several human adult normal tissues analyzed in our study. Differences in promoter methylation between T-066 expressing and T-066 non-expressing tumor cell lines and between primary ovarian and lung tumor tissues were detected. Additionally, we determined that T-066 mRNA levels correlate significantly with the cancer-testis antigen BORIS in ovarian and lung cancer. BORIS is able to exert regulation of gene expression in dependence on DNA-methylation. siRNA mediated silencing of T-066 in cancer cells did not result in any detectable significant effects in several functional cellular in vitro assays.

Conclusions:
In conclusion, we introduce T-066 as a novel candidate target for cancer immunotherapy. Our data imply that promoter methylation is involved in the regulation of T-066. However, presumably tumor-specific transcription factors like BORIS complete the up-regulation of T-066 in cancer. Further experiments have to clarify which transcription factors are involved and if other epigenetic mechanisms like histone modifications are responsible for deregulation of T-066 in cancer. The function of T-066 in cancer cells remains elusive, ongoing studies will complete the characterization of T-066.
Regulatory T cells and coinhibitory molecules in a mouse model of spontaneously arising B-cell lymphoma

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Introduction & Objectives:

Tumour transplantation models do not reflect the immunologic situation of endogenously developing tumours. Therefore, we examined the induction of adaptive immune responses in a c-myc-transgenic mouse model of spontaneously arising B-cell lymphoma. NK cells in these tumours show an activated phenotype but their effector functions are considerably suppressed. As IFN-γ that is required for effective antitumour T-cell responses is mainly derived from NK cells, it was anticipated that a missing NK-cell help may hamper the T-cell response.

Methods & Results:

In addition to CD4⁺ and CD8⁺ effector T cells (Teff) an augmented fraction of CD4⁺ Foxp3⁺ regulatory T cells (Treg) was detected in spleen and lymph nodes of tumour-bearing c-myc animals. Compared with wildtype mice, in c-myc spleens, all three T-cell subpopulations (CD4⁺ and CD8⁺ Teff and Treg) showed an activated phenotype as evidenced by up-regulated CD69 and down-regulated CD62L. Additionally, T cells seem not to be functionally suppressed as expected because they express IFN-γ, proliferate in vivo and show degranulation (CD8⁺ Teff) following in vitro stimulation.

On the other hand, CD4⁺ T cells producing IL-10 were detected which indicates an immunosuppressive tumour microenvironment. This was confirmed by the presence of several coinhibitory receptors expressed on intratumoral T cells. Thus, the receptor PD-1 was strongly up-regulated. CTLA-4 expression could only be detected on the Foxp3⁺ Treg population.

To interfere with the PD-1/PD-L1 and the CTLA-4/B7 inhibitory signalling pathways as a therapeutic option, we injected healthy c-myc mice intraperitoneally with anti-PD-1 and anti-CTLA-4 mAb together or alone. The combination therapy yielded a significant survival benefit compared with the untreated control, whereas the group treated with anti-PD-1 or anti-CTLA-4 mAb alone did not.

The role of Treg in the c-myc model was examined by Treg ablation in vivo. Indeed, depletion of Treg provided a significant longer survival.

Conclusion:

Part of the T cells in tumour-bearing mice seems to be exhausted. Together with the apparently important Treg population, these T cells reflect an immunosuppressive tumour microenvironment which favours tumour growth.
Improving adoptive T cell therapies of cancer by ectopic expression of miR181a to repress inhibitory phosphatases

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Adoptive T cell therapy using antigen-specific T lymphocytes is a powerful immunotherapeutic approach against cancer. Nevertheless, many T cells against tumor-antigens exhibit only weak anti-tumoral response. To overcome this barrier it is necessary to improve the potency and anti-tumoral efficacy of these T cells. Activation and activity of T cells are tightly controlled to inhibit unwanted T cell responses and to reduce the risk of autoimmunity. Both are regulated by extrinsic signals and intrinsic mechanisms which suppress T cell activation. The intrinsic mechanisms include the expression of phosphatases that counteract the activation-inducing kinases. Modifying the expression of these phosphatases allows the targeted modulation of T cell reactivity that could be used to enhance the potency of T cells for adoptive cell therapy of cancer. MicroRNAs (miRNAs) are small noncoding RNAs identified as important regulators of gene expression. By repressing specific target genes at the post-transcriptional level they play a key role in diverse biological processes, such as development, differentiation, and functionality. miR181a has been shown to be highly expressed in immature T cells that recognize low-affinity antigens. Here we show that overexpression of miR181a in primary T cells improves their reactivity as indicated by up-regulated expression of activation-induced molecules and effector cytokines. These effects are achieved by the down-regulation of multiple phosphatases involved in the T cell receptor signaling pathway. To test miR181a overexpressing T cells \textit{in vivo}, we established a mouse tumor model using a B cell lymphoma cell line (A20-HA) expressing the Influenza hemagglutinin (Infl.-HA) antigen. The expression of model antigens in tumor cell lines enables targeted elimination of tumors using TCR\textsuperscript{Tg} T cells and provides the opportunity to study the influence of intrinsic modulations of T cells. The transfer of miR181a overexpressing Infl.-HA TCR\textsuperscript{Tg} T cells leads to improved tumor control and prolongs survival of A20-HA tumor-bearing mice. This effect is characterized by higher amounts of effector T cells (T\textsubscript{eff}) and the expansion of Infl.-HA TCR\textsuperscript{Tg} CD8\textsuperscript{T} T cells. Our results demonstrate that miR181a is able to enhance effective tumor control and is, therefore, an interesting candidate for improving adoptive cell therapy.
The need for improving current cancer therapies as well as developing novel strategies is of utmost importance in biomedical research. Bacterial-mediated tumor therapy represents an old concept, revoked in recent times by technological advances (i.e. genetic engineering). As it has become possible to tailor bacteria according to the requirements of efficacy and safety, many research efforts have been placed at selecting the best candidate strain on basis of the better intrinsic tumor therapeutic properties, and at tailoring it subsequently. Thus far, efforts have been focused on the employment of Salmonella for tumor therapy. Recently however, clinical trials with attenuated Salmonella have failed to demonstrate significant clinical efficacy at tolerated dose, suggesting that it might be difficult to reconcile the therapeutic benefit with the safety of the treatment. Consequently this has prompted us to reevaluate the use of pathogenic bacteria for tumor therapy. That is, in terms of how vital the intrinsic anti-tumor properties are for the ultimate therapeutic success of the candidate bacterium when at the same time considering to endow the bacterium with desired qualities like tumor specific toxicity. Rather than focusing on the attenuation of pathogenic strains like Salmonella, probiotics E.coli offer a nonpathogenic bacterial alternative. Using a murine transplantable-tumor model system the relative tumor therapeutic potential of wild type and recombinantly strengthened E.coli probiotics Mutaflor and Symbioflor®2 were investigated in terms of specificity of colonization and anti-tumor effects. Relative to Salmonella, their intrinsic properties provide a safer and more specific tumor targeting delivery system. The relative possibility of specifically delivering natural tumor toxic compounds like glidobactin and Luminmide or inflammatory mediators like IL10 provide only a few of many options of recombinantly strengthening the probiotics to maximize the effects against tumors either directly or through the enhancement of tumor immune surveillance, respectively.
Diverse effects of rituximab in two disseminated, bioluminescent tumor models of human B-cell Non-Hodgkins lymphoma in immunodeficient mice

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Bioluminescence imaging is an important tool for testing therapeutic options in animal models. Especially mice are well suited for this imaging tool. They are small enough to find nearly every transfected tumor cell subcutaneous, orthotopic or metastatic. Imaging of mice can be performed after injection of a specific substrate and an evaluated incubation time. Our aim was to establish a non-invasive method for monitoring tumor dynamics during and after therapy and perform different therapy regimens with cyclophosphamide and rituximab and combinations of both.

For establishing stably transfected lymphoma cell lines, we used two different electroporation systems. The transfected cell lines were selected by hygromycin in culture medium for three months before animal experiments started. For establishing bioluminescence imaging, five animals per group were used. For cell line OCI-Ly3 (luci), we had three groups, which were sacrificed after bioluminescence imaging at day 7, 14 and 21 after intravenous injection of cells. For cell line Jeko1 mice were sacrificed at day 7, 14, 21, 28 and 35. Tumor burden was calculated by FACS analysis of anti-human CD19 positive cells in combination with total cell count with Turk’s solution in a Neubauer counting chamber. Correlation with bioluminescence imaging and Lactate dehydrogenase was tested with Spearman rank correlation test. Then chemo- and immunotherapeutic experiments started with different time and dose regimens for testing influence of dose, combination and time line on efficiency of treatment. Rituximab treatment was done with two dosages once and with one dosage in combination with two cyclophosphamide doses. Dosage was dependend on cell line because of different sensitivities.

We compared the results of bioluminescence imaging, FACS analysis and cell count of spleen, bone marrow and brain and lactate dehydrogenase (LDH) levels in plasma. Results were promising as increase of tumor burden determined by FACS analysis and cell count correlated significantly with increase of photon emission in bioluminescence imaging and LDH. Also different treatment regimens showed interesting outcomes - especially with rituximab which had a cell line depending effect alone and improved treatment outcome in combination with cyclophosphamide.

Figure 1: Bioluminescence intensity correlated very well with cell count in vitro. Correlation coefficient OCI-Ly3(luci): R=0,991; p=0,000119; Jeko1(luci): R=0,944; p = 0,0159; [Sigma Plot 11.0]

Figure 2: Dorsal overlay image of mice injected with 1×10^7 cells of OCI-Ly3(luci) 20 days after treatment with 50, 100 or 150 mg / kg cyclophosphamide (from right to left). Highest tumor burden in mouse treated only with 50 mg / kg.
Adoptive T-cell receptor gene transfer with a high-affinity single chain p53(264-272)-specific TCR combined with depletion of tumor-infiltrating suppressive cells improves anti-tumor response

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Introduction:
Several studies have demonstrated the clinical efficacy of adoptive T cell therapy (ACT) for targeting cancer. Our group has demonstrated the feasibility of T-cell receptor (TCR) gene transfer into T cells to circumvent self-tolerance to the widely expressed human p53(264-272) tumor-associated antigen and developed approaches to generate high-affinity CD8-independent TCR. A safety concern of TCR gene transfer is the pairing of endogenous and introduced TCR chains resulting in the potential generation of self-reactive T cells (OFF-target autoimmunity). Therefore, an optimized single chain (sc.) p53-specific TCR was engineered to avoid the formation of mismatched TCR heterodimers.

Objectives:
The study aims to evaluate the anti-tumor response and the safety issues raised by the risk of p53TCR gene transfer-associated ON/OFF-target toxicities in vivo.

Methods:
The safety and therapeutic efficiency were evaluated in pre-clinical mouse models of ACT. Tumor eradication experiments were performed in A2.1K⁶ Human p53 knock-in (Hupki) mice using immortalized A2.1K⁶ mouse embryonic fibroblasts (MEFs) expressing mutant p53. To assess the impact of tumor-infiltrating suppressive cells on the anti-tumor response, myeloid-derived suppressor cells (MDSC) were depleted using an anti-Gr-1 antibody.

Results:
We could successfully demonstrate that optimized sc.p53-specific TCR-redirected T cells prevent TCR mispairing-mediated lethal OFF-target autoimmunity in contrast to the parental optimized double chain TCR. Moreover, by performing ACT experiments in Hupki mice our study demonstrated that high-avidity scTCR-engineered T cells were able to target p53⁺A2.1⁺ tumor cells without inducing self-directed toxicities. Based on previous experiments we observed that an insufficient anti-tumor response in vivo might be due to the potent immunosuppressive tumor microenvironment (TME), most prominently infiltrated by MDSCs. By additional application of anti-Gr-1 antibody, which induces a depletion of CD11b⁺/Ly6C⁺Ly6G⁺ MDSCs, we could observe further delay in tumor outgrowth.

Conclusion:
These mouse studies suggest that the optimized sc.p53(264-272)-specific TCR may represent a safe and efficient approach for TCR-based gene therapy. However, combinatory therapies are needed to enhance the efficacy of ACT-mediated anti-tumor responses.
Virus-like particles (VLPs) are formed by structural virus proteins which have the intrinsic property to self-assemble into multisubunit, highly repetitive protein structures. VLPs mimic the morphology of their corresponding native virus, but in contrast to authentic virus, VLPs lack any viral genetic information. Thus, VLPs are non-infectious, non-replicating and considered as a safe vaccine format. Among others, the strong humoral immunogenicity of VLPs is based on inherent structural features like epitope spacing and more importantly, highly repetitive and dense epitope display on the VLP surface. The cloning of heterologous epitopes, including self-antigen-derived ones, into specific sites of VLP subunit proteins confers the high VLP immunogenicity to the inserted sequence. This qualifies VLPs as an efficient antigen carrier system for vaccine development.

Recently, we developed a versatile antigen display platform based on modified hepatitis B virus core antigen (HBcAg) derived VLPs. In this study, we explored the effectiveness to elicit a strong target specific humoral immune response in relevant animal models by chimeric HBcAg-VLPs displaying a surface epitope of a novel, highly selective cancer specific claudin (CSC).

Bioinformatically selected, interspecies conserved CSC epitopes were inserted into surface exposed regions of the HBcAg backbone. After expression of the constructs in E.coli, chimeric VLPs were purified by a multistage disassembly/reassembly process, quality controlled and subsequently used for immunization studies in rabbits. Serum analysis by ELISA showed the induction of high titer polyclonal IgG responses against the immunogen as well as the inserted linear epitope. More importantly, we could demonstrate by immunofluorescence and FACS analysis that the induced auto-antibodies were capable to recognize the targeted CSC in its native conformation on the surface of living, CSC transfected HEK293 cells. This recognition is highly specific and no cross-reactivity to other claudin orthologs was detectable, which is a prerequisite for a successful vaccine development. A further key objective was to induce CSC specific IgGs capable of lysing target positive cells by Fc-mediated immune effector mechanisms such as complement-dependent cytotoxicity (CDC). Therefore, rabbit sera were subjected to an in vitro CDC assay. Sera from rabbits immunized with the developed HBcAg-VLPs lysed CSC bearing tumor cells in a complement dependent manner with high efficiency. The observed killing activity was depending on the displayed CSC epitope, since sera from rabbits immunized with chimeric HBcAg-VLPs displaying an unrelated epitope, failed to exert any cytolytic effect.

In this study, we could demonstrate that the vaccination with chimeric HBcAg-based VLPs is capable to break the pre-existing humoral tolerance against self-antigens and to elicit highly specific auto-antibodies against a novel CSC. Furthermore, the induced auto-antibodies recognize the target antigen in its native conformation and mediate cytotoxicity against CSC positive tumor cells. These results are encouraging for the further development of these chimeric HBcAg-VLPs into potential anti-cancer vaccines.
Therapeutic treatment with zoledronic acid liposomes reduces tumor growth, differentiating macrophages and myeloid-derived suppressor cells


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Zoledronic acid (ZA) is a third generation bisphosphonate (BP), which has the most potent inhibitory effect on osteoclast-mediated bone re-adsorption among currently available BPs. It is currently used in clinical treatments of osteoporosis and bone metastasis in breast cancer patients. The limited in vivo antitumor activity of ZA is probably due to the rapid clearance of ZA from the circulation and its preferential accumulation in bone. Thus, liposomal formulations carrying ZA (ZAL) are currently under intensive evaluation to improve its delivery and circulation in cancer patients.

An excellent model of metastatic breast cancer is the BALB/c-derived 4T1 tumor. Although a transplantable model, 4T1 is poorly immunogenic and shares many characteristics with human breast cancer. After orthotopic inoculation to the abdominal mammary fatpad, the primary tumor grows into a nodule with the histology of a high-grade breast cancer and sheds spontaneous systemic metastases to the lungs, liver, lymph nodes, bone marrow, and central nervous system. While prevention of metastasis and tumor growth by ZA and ZAL is reported in some transplantable tumor models, the role of immune cell infiltration and the characterization of tumor environment in primary breast tumors under ZAL therapy have not been examined yet.

In our study, we are using a liposomal system for ZA delivery in 4T1 tumor bearing mice to characterize its therapeutic effect in primary tumors and on circulating immune cells. Systemic and repetitive low dose ZAL injections in the therapeutic setting reduce the tumor growth of the orthotopically injected tumor cells. At late time point when the ZAL treatment shows the stronger effect on tumor reduction, we performed extensive FACS analysis of immune cells such as CD4+ CD8+ T cells, regulatory T cells, NK, macrophages (Mph), myeloid-derived suppressor cells (MDSC) and dendritic cells to evaluate their frequencies on lymphoid and non-lymphoid organs of tumor bearing mice. Frequencies of tested immune cells in spleen, blood as well as in tumor do not differ among the ZAL treated mice compared to control groups. It is known that Mph can polarize in ‘alternatively activated’ or M2-like cells, differing from the M1-cells in receptor expression, antigen presentation, function and cytokine production. These M2-like Mph are accepted to have pro-tumor and immunosuppressive effect. Interestingly, we found that ZAL differentiates Mph as well as MDSC isolated from spleens and tumors in M1-like cells. In particular, the level of mannose receptor type 1 (MRC1, also known as CD206) recognized as the most common marker of M2-like cells is dramatically reduced in Mph (F4-80+CD11b+) and MDSC (Gr1+Ly6G+CD11b+CD11c+ known as PMN-MDSC), recovered from ZAL treated mice. We detected in all treated and control tumors a very high infiltration of PMN-MDSC, low frequency of Gr1+Ly6G+CD11b+CD11c+ cells (known as M-MDSC) while Mph (F4-80+CD11b+CD11c cells) and DC (CD11c+F4-80 cells) very modestly infiltrate into tumors. Localization and frequency of Gr1+ cells, Ki67+ proliferating cells as well as CD31+ endothelial cells in tumor samples are similar in all groups as monitored by immunofluorescence and immunohistochemistry. Gene expression profile study by NGS from bulk tumors and sorted tumor infiltrated cells (Mph, PMN-MDSC and M-MDSC) is in progress to evaluate the therapeutic effect of ZAL treatment in the tumor environment.

From our results so far, we can conclude that repetitive injections of low dose ZAL show a therapeutic effect on tumor reduction and polarize pro-tumor Mph and PMN-MDSC into cells with an anti-tumor phenotype.
Combinatory Approaches in Immunotherapy against Cancer: Transcutaneous Immunization and low-dose Cyclophosphamide in a murine model

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Transcutaneous immunization (TCI) is a promising candidate for fighting tumors and infectious diseases. Based on a T cell epitope and the Toll-like Receptor 7 agonist imiquimod, we have established a protocol that induces a potent cytotoxic T lymphocyte (CTL) response in a non-invasive way. However, TCI alone shows only limited effectiveness in terms of durable tumor protection, in part due to suppression by regulatory T cells (T<sub>reg</sub>). In another instance, cytotoxic drugs like cyclophosphamide (Cy) are currently the basis for the treatment of various tumors including malignant lymphomas. Beyond this, low-dose Cy therapy can mediate inactivation of T<sub>reg</sub> cells. Hence, we conducted our present work to evaluate the efficacy and mechanisms of combined cytotoxic treatment with TCI for the treatment of malignancies.

To study the impact of Cy on TCI effectiveness, we performed TCI by applying imiquimod creme together with the CTL epitope SIINFEKL for two days on the shaved back skin of C57BL/6 mice after i.p. injection of various doses Cy (25, 50, 100 mg/kg) one day before. Induction of CTL responses was assessed on day 6 post treatment by analysis of the frequency of SIINFEKL specific CD8<sup>+</sup> T cells in the blood and in vivo cytolysis of target cells. Here, we observed no significant decrease in the induced CTL responses comparing TCI in the presence or absence of Cy indicating that Cy does not impair TCI-induced T cell responses at the chosen dose levels. Thus, the combinatory treatment is feasible for further evaluation.

To understand the mechanisms of Cy in this context, we investigated its influence on DC as crucial antigen-presenting cells for TCI-induced immune responses. Thus, we analyzed lymph nodes and spleen for the absolute number of DC, subpopulations and activation phenotype. In Cy/TCI treated mice, we found an increased expression of CD80 and CD86 in DC compared to untreated or TCI alone and a shift to CD4<sup>+</CD8<sup>–</sup></sup> DCs, indicating stimulatory effects of Cy on DC by this immunization protocol. To address the impact of T<sub>reg</sub> cells after vaccination with Cy/TCI, we depleted CD25<sup>+</sup> cells using a monoclonal antibody and detected only few differences in DC numbers and activation phenotype in the lymph nodes. Since antibody mediated depletion may exert secondary effects, we also depleted T<sub>reg</sub> cells by diphtheria toxin treatment of FoxP3-diphteria toxin receptor transgenic (DEREG) mice that were immunized by Cy/TCI or TCI alone. Specific lysis was greatly enhanced in both treatment groups suggesting that the effects of Cy in the context of TCI are not only mediated by the inactivation of T<sub>reg</sub> cells, but also by contributing to DC activation.

To evaluate durable tumor protection, we applied our treatment to a therapeutic tumor setting, using the EG.7 lymphoma model. When tumors were palpable (13 days after the s.c. injection of EG.7 cells), mice received Cy/TCI, TCI or Cy. Cy/TCI and TCI treatment both delayed tumor growth significantly and prolonged survival.

Taken together, we show that the combination of low-dose Cy with TCI does not diminish vaccination-induced immune responses and is therefore feasible. For the underlying mechanisms, we suggest that not only T<sub>reg</sub> cell inactivation, but also other mechanisms such as effects on DC activation may be important. However, further studies are needed to improve the efficacy of Cy in the tumor setting. Overall, our studies may contribute to the development of new therapeutic options against tumors by combining chemotherapy with immunomodulatory treatments.
The way to the embedded treasure: a protocol for RNA-sequencing of formalin-fixed paraffin-embedded (FFPE) tumors using Next-Generation Sequencing

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The ability to understand and elucidate the mechanisms of diseases and their corresponding therapeutic responses has been revolutionized by advances in gene expression profiling. The majority of gene expression studies profile high quality RNA from fresh frozen (FF) samples. In contrast to the smaller number of FF sample cohorts with clinical annotation, there are many cohorts comprising formalin-fixed paraffin-embedded (FFPE) tissue specimens worldwide in hospitals and biobanks. The ability to profile FFPE samples and analyze the results with the clinical annotation would enable, for example, large retrospective studies correlating molecular features with therapeutic response and clinical outcome and help reveal the molecular basis for many diseases. FFPE samples represent a highly stable, cheap and easily storable form of tissue and this embedding has therefore been the method of choice for decades, long before molecular biologists were concerned about the preservation of RNA. However, FFPE samples have not been considered a reliable source of RNA due to the FFPE-associated degradation and chemical modifications of RNA.

Modern mRNA profiling techniques such as quantitative real-time PCR (qRT-PCR) and microarray analysis have advanced our molecular understanding of disease. One of the most significant technological advances, however, is next-generation sequencing (NGS). The application of RNA sequencing (NGS RNA-seq) is fast superseding microarray technology, providing a superior digital readout, larger dynamic range, discovery of novel genes and splice forms, allele specific expression, and RNA-editing.

Here, we present mRNA gene expression profiling from FFPE-extracted and highly degraded RNA by Next Generation Sequencing using an Illumina HiSeq 2000. We tested multiple RNA extraction kits for performance with FFPE samples and evaluated many RNA-Seq library preparation methods: a total of 141 different RNA extractions and 208 RNA-Seq libraries were performed and analyzed. Using the best method, we benchmarked (i) the reproducibility by sequencing several intra- and inter-day replicates of the same samples and (ii) the sensitivity of methods by profiling matching FFPE and FF breast cancer tumor samples. Furthermore, we modified and optimized TRON’s established “Gene Expression Pipeline” for FFPE profiling. We were also able to determine new sample exclusion criteria. The selected, optimized and validated platform is able to effectively profile FFPE samples up to 20 years old.
High-throughput identification of functional T cell receptors (TCRs) from single antigen-specific T cells

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Introduction:
Adoptive transfer of TCR-engineered T cells has evolved as an effective treatment approach in the field of cancer and infectious diseases. But for many antigens the lack of available TCRs and known T cell epitopes may constrain a broad applicability of such new therapy approaches.

Objectives:
In order to establish a library of defined TCRs as “off-the-shelf” reagents for ACT, we developed a technology platform for systemic retrieval and functional characterization of TCRs from single antigen-reactive T cells in a high-throughput manner including the identification of epitope specificity.

Materials & Methods:
To obtain functional TCRs, a three-step procedure was established comprising isolation of single antigen-reactive T cells, cloning of TCR-α/β chains from sorted cells, followed by functional immunological validation of complete TCRs.

Sources of antigen-specific T cells are PBMCs of healthy donors or cancer patients with primary or vaccine-induced T cell responses as well as spleen cells from immunized HLA-A*0201-transgenic mice. Single antigen-reactive T cells are isolated by flowcytometry sorting based on activation-induced expression of cytokines or activation markers. TCR gene isolation from single T cells is realized by 5’RACE RT-PCR following amplification of TCR-α/β variable regions by multiplex PCRs. TCRα/β variable regions are cloned into vectors containing TCR-α/β constant region cassettes providing full-length templates for immediate in vitro transcription. Immunological validation of identified TCRs is subsequently conducted using IVT RNA transfer for rapid expression of TCRs in lymphocytes and HLA molecules in antigen-presenting cells.

To achieve a high sample throughput, the TCR cloning part was converted into an automated high-throughput procedure. The working steps including multiplex PCR-based gene amplification, purification of PCR fragments and their cloning into plasmid vectors were adapted to 96-well systems and automated on three liquid handling robots in combination with automated capillary electrophoresis systems. For continuous process monitoring, a barcode-assisted sample identification combined with a SQL-server-based sample tracking system was implemented.

Results:
Automated processing of multiple samples in parallel together with the process monitoring system reduced the labor effort and complexity of the TCR gene isolation procedure substantially leading to high sample throughput and minimized error risk. Multiple TCR genes can now be isolated and cloned into plasmid vectors in parallel within 9 work days. Up to 700 T cells are currently processed by two technicians within one month.

By implementing automated TCR gene isolation and cloning, we established a TCR platform with a unique sample throughput and an outstanding collection of validated TCR as output. So far we discovered more than 100 murine or human antigen-specific TCRs from CD8+ as well as CD4+ T lymphocytes directed against 10 different tumor antigens.

Conclusions:
The rapid retrieval of antigen-specific TCRs together with the identification of corresponding epitopes allows the generation of a well characterized TCR library supporting personalized immunotherapy concepts based on TCR warehousing and adoptive transfer of T cells reconstituted with autologous TCRs.
MiR-mediated regulation of antigen processing components: a novel mechanism for immune evasion of tumor cells

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In recent years, it could be shown that tumor escape is often associated with abnormalities in the surface expression and/or function of the major histocompatibility complex (MHC) class I antigens thereby limiting the CD8⁺ cytotoxic T cell response. Abnormalities of MHC class I surface expression are often mediated by defects within the antigen processing machinery (APM). While structural alterations of the APM components rarely occur, dysregulation of MHC class I APM components is the major mechanism, which could be mediated by epigenetic, transcriptional and/or posttranscriptional processes. One possibility of posttranscriptional regulation is the presence of microRNAs (miR), which are small, non-coding RNAs that bind to the 3′-UTR of the target mRNA and thus prevent their translation or induce degradation of the bound mRNA. We hypothesize that miRs exist, which bind to the 3′-UTR of selected MHC class I APM components thereby impairing their function leading to a reduced surface expression of MHC class I molecules. A discordant expression pattern of TAP1 has been described in many tumors including renal cell carcinoma and melanoma. Using an in silico approach, putative miRs were identified, which are able to bind to the 3′-UTR of TAP1 due to their sequence and structure. The expression profile of selected TAP1 expression regulating candidate miRs was determined in different tumor cell lines demonstrating a heterogeneous expression pattern. Furthermore, enrichment of miR-200a* with the 3′-UTR of TAP1 was demonstrated. Overexpressing miR-200a* into HEK293T cells caused a reduced TAP1 expression at both mRNA and protein level by qPCR and western blot. In addition, the overexpression of miR-200a* was associated with a downregulation of MHC class I surface expression. Currently, the role of the miR-200a*-mediated downregulation of TAP1 and MHC class I surface expression on the immune response is investigated. These data show for the first time that miRs might be involved in the regulation of MHC class I APM component expression in human tumors thereby leading to their evasion of immune surveillance.
A chimeric antigen receptor coexpressed with Ca is able to recruit endogenous CD3ζ and leads to specific killing of human tumor cell lines

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Introduction:
Chimeric antigen receptors (CARs) are recombinant receptors that provide both HLA independent scFv-mediated antigen-binding and T cell-activating functions. A multitude of CARs has been reported over the past decade targeting a panel of different cell surface tumor antigens. Their biologic functions were dramatically improved by incorporation of a costimulatory domain resulting in tripartite receptors (scFv, CD28, CD3ζ), termed 2nd generation CARs. CARs of the 3rd generation encompass additional domains of costimulatory molecules such as OX40 and 4-1BB to enhance the proliferative capacity and persistence of modified T cells.

CARs are a new class of drugs with great potential for cancer immunotherapy. Upon their expression in T lymphocytes, CARs direct potent, targeted immune responses that have recently shown dramatic clinical results after targeting the CD19 molecule in leukemia patients.

Objectives:
However, in a few cases the treatment of patients with CARs led to morbidity and death with GvHD-like symptoms due to off-target-reactivity against self-antigens expressed on normal tissues. This adverse reaction may originate from the artificial CAR design overexpressing the signaling and costimulatory moieties of CD3ζ, CD28 and 4-1BB leading to reactivity in the presence of even minimal amounts of antigen. This might foster the inappropriate shut-down of T-cell signaling which normally terminates any T cell-based immune reaction. In order to provide a more native mechanism of T cell activation, we used a novel CAR format relying on a previously published single chain (sc) TCR framework of the domain order Vα-Linker-Vβ-Cβ and coexpression of the Cα domain.

Furthermore, we compared antigen-specific reactivity of this novel format with a classical 2nd generation CAR and a native murine double chain (dc) TCR of the same antigen-specificity.

Materials & Methods:
Here, we assessed this novel CAR format to target a promising oncofetal antigen expressed on tumor entities from visceral organs such as ovary. For this, we hooked up a proprietary antibody VH/VL-linked scFv-fragment on a murine TCR-Cβ-domain and coexpressed the murine TCR Cα-domain in human T-cells. CAR-mediated effector functions were analyzed after RNA transfer in T cells of healthy donors by IFNγ-ELSPOT, luciferase-based cytotoxicity and CFSE-based proliferation assays.

Results:
CAR-transfected T cells recognized target cells either transfected with antigen encoding mRNA or endogenously expressing the relevant antigen resulting in specific IFNγ secretion, proliferation and cytotoxicity. Furthermore, we developed a 2A peptide-based bicistronic vector for simultaneous expression of scCAR and Cα genes. Initial analysis demonstrated that linkage of scCAR to Cα results in decreased surface expression and reactivity compared to coexpression of the two components.

Conclusions:
The novel scCAR/Ca construct specific for visceral tumors is highly reactive against antigen expressing target cells as analyzed by cytotoxicity and proliferation assays. Notably, the novel CAR format mediated efficient antigen-specific lysis of tumor cell lines comparable to a classical 2nd generation CAR or dcTCR format. The linkage of scCAR and Cα by a self-processing 2A-element decreased surface expression and reactivity requiring further improvements of the vector design.

These experiments represent the basis for preclinical analyses in depth regarding reactivity and toxicity in vivo.
Efficient Treg Depletion Induces Complete Rejection of Large Established Melanomas Expressing Natural Antigens

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Introduction:
In human tumors frequently high numbers of Foxp3(+) regulatory T cells (Treg) are present, which correlate with poor clinical prognosis. Several emerging therapies for cancer involve Treg depletion or modulation in an effort to enhance anti-tumor immune responses. Our group has shown that high-level Treg depletion induced rejection of tumors expressing ovalbumin or SV40 Large T antigen as tumor antigens. However, clinical tumors usually express self-antigens. Therefore, self/tumor specific CD8+ T cells are usually subjected to negative selection in the thymus, leading to relatively low numbers or low T cell receptor affinity. Although this mechanism is important for avoidance of autoimmunity, it will also negatively affect self/tumor specific T cells.

Materials and methods:
To study the effect of Treg depletion for tumors expressing natural self-antigens, we use an HCmel melanoma cell line recently derived from HGF-CDK4 mice that spontaneously develop melanoma. These tumors express natural melanoma antigens and recapitulate growth kinetics and histomorphology of parental primary melanoma when transplanted into mice. We utilized two different mouse models, namely Foxp3-DTR knock-in mice that allow 99% Treg depletion after DT application, and Foxp3-LuciDTR4 BAC transgenic mice that yield 90% Treg depletion.

Results:
We observed that 99% Treg depletion resulted in complete regression of large established HCmel melanoma whereas 90% depletion induced only partial rejection with subsequent outgrowth of the tumors. Both, 99% and 90% Treg-depleted tumors exhibit similar total levels of leukocyte infiltration, but 99% depletion induced stronger activation and infiltration of tumor-specific CD8+ T cells. Interestingly, infiltration of CD8+ dendritic cell (DC), the DC subset responsible for cross-presentation of antigens, into tumors was strongly enhanced after 99% Treg depletion. These observations suggest that the high number of CD8+ DCs induces efficient priming of tumor-specific CD8+ T cells. Intratumoral gene-expression profiling data showed that 99% depletion induced additional drastic changes in the tumor microenvironment, including macrophage polarization towards M1 and significantly stronger upregulation of intratumoral expression of Th1 cytokines.

Conclusion:
As only 99% Treg depletion results in complete tumor rejection and cure of the mice, but not 90% depletion, it follows that a relatively small percentage of remaining Tregs is sufficient to prevent effective tumor immunity. Since it is not possible to achieve a very high degree of Treg depletion in the clinic, we presently unravel the precise immunological parameters leading to complete tumor rejection, so that knowledge of respective pathways and factors can be employed for improvement of therapeutic strategies.
User vs. software-dependent variability of ELISPOT counts obtained from ten different laboratories

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Introduction:
There are a defined number of T cells specific for any given antigen within a PBMC pool. However, an accurate and reproducible method to measure this response, between different laboratories has been controversial. In ELISPOT assays, antigen-induced spots show a broad spectrum of sizes and densities over variable background. Therefore, even for experienced investigators using ELISPOT analysis software, it is difficult to judge the minimal spot size/density to be counted, and the gates for upper threshold to distinguish single cell-derived spots vs. clusters.

Methods:
We studied PBMC plated in serial dilutions, with 24 replicates per dilution, to establish the distributional properties of IFN-γ ELISPOTS elicited by antigens with defined HLA-Class I or Class II restriction. We also sent ELISPOT plates, and image files of such, to 10 different laboratories for independent counting. The plates were machine counted by each laboratory relying on the subjective counting parameters assessment by different investigators, and by the statistics-based auto-gating method in conjunction with auto-threshold algorithm.

Results:
Both these CD8- or CD4 cell-derived spots were found to closely follow log-normal distribution. These log-normal distributional properties of ELISPOTS permit to set the lower and upper gates for counting by means of statistics, automatically, with a 95% confidence interval.

Conclusions:
While counts based on judgment call of the investigator showed considerable variability, the counts obtained in the laboratories by the statistic gating method were comparable to each other and thus establishes the prospect of harmonizing ELISPOT counting.

Keywords: ELISPOT, Immunomonitoring, Standardization, Th1 response
Cytokine Status in Ukrainian Children with Irritable Bowel Syndrome Residing in a Radioactive Contaminated Area

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Background:
The effect of low dose radiation on immune system is shown. Ionizing radiation can affect cytokine production and polarization of T helper cells.

Objective:
The current study focused on ionizing radiation in Ukrainian children residing in a contaminated area with clinical irritable bowel syndrome.

Method:
Our study included 75 rural children population aged 4-18 yrs, who lived in a contaminated area exposed to natural environmental radiation with clinical irritable bowel syndrome (categorized in three groups) and 20 rural children participants aged 5-15 yrs who were living in areas with similar levels of radioactive contamination without clinical irritable bowel syndrome as control group. Internal radiation activity was measured by gamma-ray spectrometry. Serum levels of IL-4 and IFN-γ were measured by enzyme linked immunosorbent assay.

Results:
A trend towards increased levels of IL-4 was observed in children with clinical irritable bowel syndrome. In these children, IFN-γ levels were lower than that of the control group.

Conclusion:
The IBS symptoms in Ukrainian children residing in a contaminated area may have stemmed from Th1 to Th2 immune deviation and differential expression of IL-4 and IFN-γ.
Anti IgE (Omalizumab) is effective in treating severe asthma in patients with severe cardiovascular complications

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The human studies of omalizumab for treating allergic asthma and other allergic diseases have helped our understand the roles of IgE in the pathogenesis of various allergic diseases. Recent studies of omalizumab also reveal the effects of omalizumab in reducing T cell related cytokines and soluble TNF-related apoptosis inducing ligand (sTRAIL) and the systemic levels of oxidative stress markers. It is conceivable that mast cells residing in the nasal lining, lower airway, other areas of the mucosal tracts, and in the skin, differ in tryptase and chymase content, sensitivity, receptor regulation, and life span. Most asthmatic individuals respond satisfactorily to inhaled corticosteroids and β-adrenergic agonists; however, 5-10\% of them have severe, persistent symptoms that respond poorly to such treatment. Those patients, who cannot be adequately controlled with even high doses of corticosteroids and often require repeated emergency visits and hospitalization, are approved to use omalizumab by the health insurance systems in many countries. Herein, we report 4 cases, wherein severe, persistent allergic asthma patients with co-morbid severe cardiovascular complications were effectively treated with omalizumab, with improvements both in pulmonary and cardiovascular functions.

The asthma control test scores and quality of life measurements improved generally in the three patients. While there was a small decrease in thrombocyte count, thromboembolism was not observed.

Case I:
The male patient was a heterozygous carrier of factor V Leiden and prothrombin G20210A mutation, which is related to high risk of thrombosis \cite{7,8}. Bilateral pulmonary thromboembolism was observed in multiple segments by Tc99m DTPA/MAA radioaerosol ventilation-perfusion scintigraphy. The severity indicated by those tests suggested the need of a lung transplantation. The relevance of the heterozygosity for hereditary Protein C deficiency as a risk factor for venous thrombosis has been disputed, because heterozygotes without symptoms have been identified among blood donors and relatives of homozygotes. After anti-IgE therapy, he did not require lung transplantation and serum Protein C/S levels increase to normal ranges.

Case II:
The female patient was diagnosed of congestive heart failure (CHF) and hypertension (HT). A year ago she suffered an intracranial embolus with resultant facial palsy. After initiated anti-IgE treatment, cranial emboli event or any neurologic complication did not occur.

Case III:
Because of MVP, she had required mitral valve replacement 5 years ago. She currently used CCB and CP. Patient did not report any cardiac arrhythmias after initiated anti-IgE therapy. Besides, exercise ECG testing was normal while the patient was treated with anti-IgE.

Case IV:
He was diagnosed with an aortic aneurysm 8 years ago. Patient’s autologous serum skin prick test was positive. His past history revealed angioedema and urticaria exacerbations for 9 years which sometimes associated with larynx edema. After the third round of omalizumab, frequency of exacerbations decreased and after eleventh round it was completely disappeared. Aneurysm enlargement or complications related this aneurysm were not detected during the treatment with anti-IgE. The development of anti-IgE therapy over the past 20 years provides an interesting example of the emergence of a conceptually new, biotechnology-produced pharmaceutical. Our clinical experience together with international coordination on performing the omalizumab treatment in large number of patients with different clinical status helps us to relate this knowledge to clinical application in thromboembolism.
### Table

<table>
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<th>Age (y) and Gender</th>
<th>CASE I</th>
<th>CASE III</th>
<th>CASE IV</th>
<th>CASE V</th>
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<td></td>
<td>40 male</td>
<td>63 female</td>
<td>64 female</td>
<td>63 male</td>
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</table>

**Asthma Diagnosis**
- 31
- 12
- 34
- 15

**Co-morbidities**
<table>
<thead>
<tr>
<th>CASE I</th>
<th>CASE III</th>
<th>CASE IV</th>
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<td>Protein-Cystatin C</td>
<td>Neutropenia, abdominal gynecomastia, multiple pulmonary emboli</td>
<td>N00A, tumor replacement, CHF</td>
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<tr>
<td>Anemia, Anemia</td>
<td>Anemia, Anemia</td>
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</tbody>
</table>

**Protein C Activity**
- CASE I: 90%
- CASE III: 90%

**Protein S Activity**
- CASE I: 90%
- CASE III: 90%

**Protein C Activity**
- CASE I: 90%

**Protein S Activity**
- CASE I: 90%

**Protein C Activity**
- CASE I: 90%

### Figure 1

![Figure 1](image1)

### Figure 2

![Figure 2](image2)
TGN1412-induced human cytokine release and lymphopenia in a humanized mouse model

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Question:
Monoclonal antibodies (mAb) are widely used in therapeutic applications. However, mAbs can cause a variety of severe adverse effects including cytokine release syndrome (CRS). One mAb which induced a CRS during a first-in-human clinical trial is the superagonistic, CD28-specific antibody TGN1412. Nevertheless, also the first mAb approved for therapeutic use in humans, OKT3, an immunosuppressant anti-CD3 mAb, is well known to induce CRS. In this study, we established a humanized mouse model to evaluate its predictive value in preclinical testing of mAbs for the propensity to cause CRS.

Method:
For this purpose, we reconstituted immunodeficient mice (Nod-RAG1−/− Aβ−/− HLA DQ1−/− or IL-2Rγc−/−) with human peripheral blood mononuclear cells (hPBMCs).

Results:
Interestingly, human T cells are the predominant cell population within all populated immune organs as well as the peripheral blood whereas only very few human B cells and CD11b+ myeloid cells are detectable. Reconstituted mice (≥15% hCD45+ mononuclear cells in peripheral blood) were injected intravenously with OKT3 or TGN1412. After both OKT3 and TGN1412 application, all mice showed severe signs of illness, rapid drop of body temperature, and finally succumbed to antibody application 2-6 hours later. Interestingly, all mice showed loss of hCD45+ cells from the peripheral blood after TGN1412 application and loss of only human T cells after OKT3 injection as it similarly has been reported for TGN1412- or OKT3-treated humans, respectively. Moreover, upon OKT3 injection we detected selective CD3 down-modulation on human T cells in blood, spleen, lymph node, and on peritoneal exudate cells, one typical mode of action of OKT3. Importantly, we could measure release of human IFN-γ, TNF-α, and IL-10 in humanized mice upon OKT3 as well as upon TGN1412 application.

Conclusion:
Hence, the humanized mouse model provided here reflects several of the effects and adverse events observed in humans upon application of the mAbs OKT3 or TGN1412.
The heterogeneity in the healthy human immune system, and the immunological changes that occur in various diseases, have only been partially described. The accurate measurement of variations in the human immune system requires precise and standardized assays to distinguish true biological changes from technical artefacts. Besides standardization projects that rely on isolated cells in the blood and use cytometry as a standardization tool, the characterization of immune cells in different tissue types from different organs still needs to be elucidated. Thus, the tissue bank for inflammatory diseases (GEZEH) in Heidelberg, Germany, was founded in 2011 by the Institute of Pathology and the Department of Dermatology. It is a nonprofit organization with a completely evaluated legal and ethical framework and is embedded in the Biomaterial Bank Heidelberg (BMBH) concept. Its main aim is the acquisition and characterization of fresh-frozen and paraffin-embedded non-neoplastic human tissues according to the standards of good scientific practice and the promotion of interdisciplinary translational immunology research. It also offers expert project assistance: a project leader can submit a short proposal, and the tissue collecting/preparing process will be performed in cooperation with a specialised pathologist and, if applicable, an experienced clinical researcher. The tissue bank is also a central platform for the further development of innovative technologies for tissue handling, e.g., multi-tissue-array and virtual microscopy, with links to digital image analysis and bioinformatics. Hence, the GEZEH tissue bank represents a model for innovative biobanking and for institutions with active interdisciplinary translational immunology research.
The effects of omalizumab on soluble CD200, IL-1β and 25-hydroxyvitamin D in moderate to severe allergic asthma

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Background:
The mechanism of action of omalizumab in the treatment of asthma is believed to be multifactorial, and includes effects mediated through altered production of redox metabolites, and regulation of production of known inflammatory proteins. We examined the levels of serum soluble CD200 (sCD200), IL-1β, eosinophil cationic peptid (ECP) and 25-hydroxyvitamin D (25(OH)D) in allergic asthma patients treated with omalizumab therapy, to explore their relationship with disease activity.

Methods:
In the first group there were 17 patients of 7 male and 10 female, suffering from moderate to severe allergic asthma—allergic rhinitis, all of whom underwent anti-IgE therapy for 12 months within the product label (omalizumab; xolair) every 2 weeks. Blood samples were taken at all follow up visits from the time of first diagnosis (pre-omalizumab period, Group IA), and after 12 months of treatment during disease remission (post-omalizumab period, Group IB). Blood sampling was performed in May because this was known to represent their time of highest air pollination. Healthy volunteers (group II, n = 25) had no history of allergy/atopy, family atopy, cardiac liver, renal and pulmonary diseases. Concentrations of sCD200 in the serum samples were quantified using ELISA kit. IL-1β levels were measured using ELISA kit. Total and specific IgE levels were enumerated by fluoroenzyme immunoassay using an ImmunoCAP kit. Serum levels of 25(OH)D were quantified by a radioimmunoassay and categorized into sufficient (≥ 30 ng/ml), Blood samples for 25(OH)D, IL-1β, sCD200, total and specific IgE measurement were always taken in the morning between 8 and 10 am. Participants abstained from caffeinated drinks and food for 12 h before testing. Medical history, lung function tests, measurement of exhaled nitric oxide concentrations (FE NO) were performed on the same day.

Results:
ACT scores (p<0.0001), FEV₁ (p<0.0001), FVC (p=0.001), and serum 25(OH)D (ng/ml) (p<0.05) levels were significantly increased after omalizumab treatment. In contrast, total IgE (p<0.05), ECP (p<0.05), FeNO (p<0.0001) and sCD200 (p<0.0001) were all significantly decreased after omalizumab treatment. The difference of the IL-1β concentrations between control and patient groups were found non-significant. Regression analysis showed positive correlations between ACT and FEV₁ (r=0.548, p<0.05), FVC (r=0.637, p<0.05); between FeNO and age (r=0.572, p<0.05), ECP (r=0.491, p<0.05), SOA (r=0.615, p<0.05), and IL-1β and SOA (r=0.515, p<0.05), for group IA patients. Negative correlations were detected between ACT and IgE (r = - 0.555, p<0.05); between age and FEV₁ (r = - 0.499, p<0.05), , between FeNO and FEV₁ (r = - 0.555, p<0.05), FVC (r = - 0.498, p<0.05) for group IA patients. A positive correlation between age and FeNO (r=0.572, p<0.05), and negative correlations between BMI and IgE (r = - 0.496, p<0.05), IL-1β and FEV₁ (r = - 0.549, p<0.05) were also detected for Group IB patients.
Liver and renal function tests, serum IgG, IgA, IgM, complement (C)3, C4 levels were within normal ranges.

Conclusions:
These data suggests that routine clinical measurements of ECP, 25(OH)D and sCD200 may provide useful additional information to conventional clinical measures, which can help us understand the current clinical status of moderate to severe asthmatic patients, and guide an optimal therapeutic approach.
Clinical Immunology, Immunodeficiencies, Reproduction Immunology

Figure 1

Figure 2
Decreased expression of miR-146a and miR-155 contributes to an abnormal Treg phenotype in patients with rheumatoid arthritis

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Introduction:
Rheumatoid arthritis (RA) is a chronic inflammatory autoimmune disease characterized by development of bone erosions and severe joint destructions. Regulatory T cells (Tregs) have been implicated in the maintenance of peripheral tolerance and control of the disease development. deregulated expression of two miRNAs, miR-146a and miR-155 has been reported in RA. Both miRNAs are induced by the NF-κB signaling pathway and function as a negative regulator of NF-κB activity. Here, we investigated expression of these miRNAs and NF-κB activity in patients with RA.

Materials and Methods:
Total RNA were isolated from CD25+ CD4 T cells under stimulation with anti-CD3/CD28 for 0, 24h and 48h from patients with early, treatment naïve disease (n=10), patients with established RA(n=20) and healthy individuals (HC) (n=27). miR-146a and miR-155 expression was assessed using TaqMan miRNA expression assays. mRNA expression of TRAF6, IRAK1, IKKe, IL-17, TNF, IL-2 and IL-10 was assessed using TaqMan gene expression assays. To analyze NF-κB activity, CD25+ CD4 T cells were isolated from the peripheral blood of RA patients and healthy individuals and transfected with an NF-κB reporter vector. The endogenous NF-κB activity was measured by luciferase assay.

Results:
Patients with active untreated RA showed lower miR-146a and miR-155 expression levels as compared to HC. The expression level of miR-146a was however restored in response to treatment and negatively correlated with disease activity. In contrast miR-155 expression level remained decreased in RA patients independent of treatment and diseases activity. Their target genes, TRAF6, IRAK1 and IKKe, which are components of NF-κB signaling pathway, showed higher expression in RA patients as compared to HC after TCR stimulation. This increase in the expression level was not affected by treatment. NF-κB activity in response to TCR stimulation was lower in Tregs from RA patients as compared to HC. Interestingly, Tregs from treatment naive RA patients expressed elevated levels of proinflammatory cytokines IL-17, TNF and IL-2. The expressions of these cytokines were restored in response to treatment, however there was no correlation with treatment efficacy.

Conclusions:
Our data suggest that the expression of miR-146a and miR-155 which may function as negative regulators of NF-κB activation in CD25+ CD4 T cells are deregulated in patients with RA, resulting to an altered cytokine production may contribute to the disease pathogenesis.
A real-time cytotoxicity assay to analyze killer cell function and for personalized immunotherapy

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²Saarland University, Physiology, Homburg, Germany

To kill pathogen-infected or tumorigenic cells is a key immune function. However, the current standard cytotoxicity assays (lactate dehydrogenase (LDH) or chromium (⁵¹Cr) release assays) are not well suited for clinical applications because they are extremely labor-intensive endpoint assays, lacking kinetic information and may even require handling of radioactive material. Therefore, assays to analyze kinetics of cytotoxicity are promptly required. Here we present a sensitive, time-resolved assay with a broad dynamic range for quantification of killing kinetics and high-throughput use. By determining fluorescence loss as a function of the fraction of lysed cells, this assay measures the kinetics of cytotoxicity over a broad dynamic range. In comparison with the gold-standard LDH or chromium release assays, the real-time cytotoxicity assay has a broader dynamic range, reports reliable target cell killing values under all tested conditions and is much better time-resolved. The high time resolution of the assay also provides kinetic information with the potential to reveal neglected/immeasurable properties in the process of cytotoxicity of both purified human cytotoxic T lymphocytes and natural killer cells. To test its clinical potential in routine high throughput clinical applications, we have applied this assay to analyze NK cell activity of 30 human blood donors. The assay identified three killing phenotypes in healthy human donors, illustrating its importance in characterization of basic mechanisms, diagnosis of immune disorders and personalized cancer immunotherapy. We believe that combined with other parameters, this assay can provide a powerful tool to improve diagnosis and prognosis as well as optimization of treatment strategies in a variety of clinical applications, from immune disorder diagnosis to personalized immunotherapy of cancer.
Major visceral surgery inhibits the function of circulating CD56hi Natural killer cells in response to Staphylococcus aureus and is associated with disturbed IL-12/STAT4 signaling

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After major visceral surgery, patients frequently suffer from infectious complications due to the development of immunosuppression of unknown origin. Immune defense against bacteria requires the appropriate function of Natural killer (NK) cells. IL-12 is released from accessory cells like monocytes and dendritic cells upon stimulation with bacteria and activates NK cells, especially the CD56hi subset, for the release of IFN-γ that in turn increases the bactericidal activity of phagocytes. IL-12 signaling occurs via binding to the IL-12 receptor and subsequent phosphorylation of the transcription factor STAT4. IL-10 inhibits the formation of IFN-γ by NK cells. Moreover, binding of activating (e.g. NKG2D) or inhibitory receptors (e.g. NKG2A) on NK cells by ligands expressed on accessory cells modulate the degree of NK cell activation. In the present study, we investigated the impact of major visceral surgery on the activity of circulating CD56hi NK cells.

Inclusion criteria of the patients were age >18 years and elective laparotomy with planned partial or total organ resection and the absence of immunosuppression due to disease or drugs. Peripheral blood mononuclear cells (PBMC) were isolated from patients 24 h before and 24 h to 7 d after surgery. NK cells were analyzed in terms of the expression of surface molecules and the capacity to release IFN-γ in response to stimulation of leukocytes with inactivated S. aureus by means of flow cytometry. Purified CD56hi NK cells were stimulated with IL-12 and IL-18 to determine the release of IFN-γ. The expression of the IL-12 receptor and the phosphorylation of STAT4 were analyzed. NK-92 cells were cultured in the presence of patients’ sera obtained before and after surgery and the IL-12/IL-18-induced secretion of IFN-γ and the expression of NKG2A were determined 24 h later.

In total 58 patients were included in the study. Surgery induced a significant decrease of the absolute number of NK cells in the blood for at least 7 d. Upon stimulation of PBMC with S. aureus, the percentage of IFN-γ-producing NK cells significantly declined from 60% before surgery to 20% and 25% 24 h and 5 d, respectively, after surgery. The expression of CD69, CD62L, NKp46, and NKG2D did not change after surgery. The expression of NKG2A increased by 60% within 24 h after surgery but declined thereafter. The surgery-mediated suppression of the NK cell-derived IFN-γ synthesis was not reversed by neutralization of IL-10 or by blocking NKG2A. Supplementation with IL-12 but not with IL-18 during stimulation with S. aureus partly restored the production of IFN-γ. After surgery, purified CD56hi NK cells were inhibited in the secretion of IFN-γ upon direct stimulation with IL-12/IL-18. Surgery caused a decrease in the expression of the IL-12 receptor β1 chain on NK cells and was associated with an altered extent of STAT4 phosphorylation. Serum drawn after surgery had no effect on NK-92 cells in terms of NKG2A expression and IFN-γ secretion.

Major visceral surgery causes a loss of circulating NK cells possibly through emigration into the damaged tissue. Moreover, the capacity of CD56hi NK cells to secrete IFN-γ either in the context of S.aureus-stimulated accessory cells or upon direct stimulation with IL-12/IL-18 is suppressed. The unresponsiveness of NK cells after surgery is not mediated by IL-10 or by the enhanced expression of NKG2A but might be caused by a reduced expression of the IL-12 receptor and a disturbed STAT4 signaling. The impaired function of NK cells might explain the enhanced susceptibility to opportunistic infections after surgery.
HLA-Universal platelets infusions prevent platelet refractoriness in a mouse model

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Refractoriness to platelet (PLT) transfusion caused by alloimmunization against HLA class I antigens constitutes a significant clinical problem. Thus, it would be desirable to have PLT units devoid of HLA antigens. Previously, we showed that the generation of HLA class I-silenced (HLA-universal) PLTs from CD34+ cells using an shRNA targeting β2-microglobulin transcripts is feasible. Here, we assessed the functionality of HLA-silenced PLTs and their ability to escape HLA antibody-mediated cytotoxicity in vitro and in vivo. Platelet activation in response to ADP and thrombin were assessed in vitro. The immune-evasion capability of HLA-universal megakaryocytes (MKs) and PLTs was tested in lymphocytotoxicity assays using anti-HLA antibodies. To assess the functionality of HLA-universal PLTs in vivo, 1x10⁶ HLA-silenced MKs were infused into NOD/SCID/IL-2Rγc−/− mice with or without anti-HLA antibodies. PLT generation was evaluated by flow cytometry using anti-CD42a and CD61 antibodies. HLA-universal PLTs demonstrated to be functionally similar to blood-derived PLTs. Lymphocytotoxicity assays showed that HLA-silencing efficiently protects MKs against HLA antibody-mediated complement-dependent cytotoxicity. 80-90% of HLA-expressing MKs, but only 3% of HLA-silenced MKs were lysed. In vivo, both HLA-expressing and HLA-silenced MKs showed human PLT production (up to 0.5% within the PLT population) when anti-HLA antibodies were absent. However, in presence of anti-HLA antibodies HLA-expressing MKs were rapidly cleared from the circulation of mice, while HLA-silenced MKs escaped HLA antibody-mediated cytotoxicity and human PLT production was detectable up to 11 days. Our data show that HLA-silenced PLTs are functional and efficiently protected against HLA antibody-mediated cytotoxicity. Provision of HLA-universal PLT units may become an important component in the management of patients with PLT transfusion refractoriness.
Innate effector molecules as promising prognostic biomarkers in non-invasive molecular imaging

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Introduction:
In the onset of an inflammation, the activation and immigration of phagocytes leads to a vast release of the proinflammatory alarmins S100A8 and S100A9. Due to their favorable kinetics S100A8/S100A9 (calprotectin) could be characterized as a very early and sensitive biomarker in a broad spectrum of acute and chronic inflammatory disorders like rheumatoid arthritis, allergies, inflammatory bowel or lung diseases.

Objectives:
The purpose of our study was to avail these innate effector molecules for in vivo imaging of inflammation with the perspective of monitoring disease activity in clinically relevant disorders.

Materials & methods
S100A9-antibody was coupled to the fluorescence dye Cy5.5 for optical imaging studies by Fluorescence Reflectance Imaging (FRI). We investigated various mouse models of inflammatory and infectious diseases using anti-S100A9-Cy5.5 antibodies. Rab-IgG antibody without relevant specificity coupled with Cy5.5 and S100A9 knock-out-mice served as controls.

Results:
In the contact dermatitis model injection of anti-S100A9-Cy5.5 resulted in more than ten-fold higher contrast to noise ratios (CNR) compared to those of knock-out-mice and significantly higher CNRs compared to injection of rab-IgG-Cy5.5. With this specificity-proven marker we monitored inflammatory models of different immunological etiology, e.g. collagen-induced arthritis (CIA) and experimental leishmaniasis. In both models molecular imaging of S100A9 provides a sensitive and specific method of non-invasive monitoring even in subclinical disease stages.

Conclusion:
In conclusion S100A9 has the potential to monitor disease activities in vivo for various inflammatory diseases associated with a high phagocyte activity.
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Tertiary lymphoid organs in oral squamous cell carcinoma

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Introduction:
Tertiary lymphoid organs (TLOs) are accumulations of immune cells with considerable morphological similarity to follicles in secondary lymphoid organs (SLOs), especially to lymph nodes. Unlike SLOs, they arise in random locations under terms of chronic inflammation. As these highly organized structures contain all cells needed to generate a functioning immune response, they probably serve as supporters for SLOs by promoting a local anti-tumor effect. These specialized tissues have been reported in various types of tumors such as breast and ovarian cancer. In oral squamous cell carcinoma (OSCC), however, TLOs have not been described yet.

Objectives:
The aim of this study is to evaluate the presence of TLOs in OSCC and to characterize the inflammatory infiltrate. In addition, we want to correlate the disease-specific and overall survival rates of patients with OSCC to the presence of TLOs.

Patients & methods:
A total of 87 patients with OSCC were included in the study. TLOs were detected based on immunohistochemical staining for CD20, CD21, and CD3. High-endothelial venules (HEVs) were recognized by positive staining for PNAd, and their relevance in early detection of TLOs was assessed. To further characterize the inflammatory infiltrate, sections from all tumors were immunohistochemically stained for CD68 and CD163, and Giemsa staining was performed.

Results:
Up till now, TLOs have been noticed in 10 of 87 tumors (about 11%). They typically appeared as clusters of CD20+ B-cells and CD21+ follicular dendritic cells, surrounded by CD3+ T-cells. HEVs occurred both in sections with and without TLOs and were highly associated with lymphocyte infiltration. The correlation between the presence of TLOs and the prognosis of patients with OSCC remains to be elucidated.

Conclusion:
Whereas TLO formation was found in OSCC, the PNAd antibody probably does not serve as a marker for early detection of this phenomenon. It is known that the presence of these cell accumulations is associated with a favorable clinical outcome in other types of cancer, e.g. malignant melanoma and non-small-cell lung cancer. At present there are few good prognostic markers for patients with oral cancer, especially in patients with early stage cancer. A better understanding of TLOs in OSCC might lead to the development of new opportunities in the treatment of this cancer entity which is considered to be very aggressive and correlated with a high rate of metastasis.
Immunmodulatory Properties of Mesenchymal Stem Cells and Ghrelin in Crohn’s Disease


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Introduction:
With the discovery in the late 1980s that T helper (Th) cells differentiate into Th1 and Th2 cells, producing different sets of cytokines, it was quickly established that Crohn’s Disease seemed to be a Th1 cytokine-mediated disease characterized by increased production of interferon-γ. Mesenchymal stem cells are a heterogeneous population of self-renewing and multipotent cells isolated from the bone marrow and other tissues. MSCs raised hopes for their clinical exploitation for tissue-repair strategies and increasing experimental evidence supports their use also for immune-mediated diseases. Ghrelin, an endogenous ligand for the growth hormone secretagogue receptor, is a gastric hormone that has been found to have a wide variety of biologic functions including anti-inflammatory activities. The aim of the study was to investigate the synergistic therapeutic effect of MSC and Ghrelin on CD4+ Th-cells isolated from infested gut.

Methods and Results:
Therefore MSC and heterogeneous population of CD4+ Th cells were isolated from human gut and cytokine expression was analyzed using different methods including ELISA, PCR, and flow cytometry. Interestingly, no elevated number of MSC was isolated from infested gut as compared to healthy parts. After generation of MSC monolayer in vitro, MSC (minimal density 3x10^4 cells/ml) have significant effects on the cytokine production of isolated Th-cells population, as pro-inflammatory cytokines (interferon-γ, interleukin (IL)-1β, IL-6, IL-12 and IL-17) were downregulated and anti-inflammatory IL-10 was upregulated (p<0.05). This effect was most pronounced in cultures with cell-cell interaction, but also detectable when cells were separated by trans-well membranes. Also, the portion of CD4+CD25+FoxP3+ Th1 helper cells was significantly increased. Adding Ghrelin to co-cultures with MSC and CD4+ Th-cells, the anti-inflammatory were again significantly enhanced. It could be evaluated, that the anti-inflammatory properties of ghrelin were partially mediated by direct effects on CD4+ Th-population. But also MSC were integrated in the action of ghrelin. In MSC, the expression of transforming growth factor β, prostaglandin E2 and activity of indoleamine-2,3-dioxygenase (IDO; kyurenin-pathway) were increased as well as growth hormone secretagogue receptor (GHSR) 1a and 1b were detectable on MSC. Blocking GHSR 1a, GHSR 1b, and IDO lead to a decrease of anti-inflammatory effects mediated by MSC, especially in a statistically significant manner by blocking GHSR 1b. At least, the pre-incubation of MSC with ghrelin and subsequent co-cultivation with CD4+ TH1 cells (without ghrelin) lead to significant changes in cytokine expression of the last population.

Conclusion:
As a result, MSC and ghrelin have synergistic effects on the cytokine production of CD4+Th-cell population, so they might be a valuable tool in regenerative strategies for the treatment of Crohn’s Disease.
Physical exercise affects homeostasis of human regulatory T cells


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Question:
Physical activity has significant health benefits. The cellular and molecular mechanisms by which physical activity mediates its beneficial effects are poorly understood. Since the biological effects of physical activity are in part mediated through the amelioration of chronic low-grade inflammation we studied the impact of physical activity on the frequency of regulatory T cells, which are central regulators of adaptive immunity and inflammation.

Methods:
To study the effect of physical activity on the anti-inflammatory arm of the immune system we first assessed both the relative VO$_2$peak (relVO$_2$peak) values and T$_{reg}$ frequency in a test cohort of 245 elite athletes from different Olympic sport disciplines. The relVO$_2$peak served as a measure of the athletes’ cardiovascular fitness and aerobic endurance as well as indirectly the intensity of the training program. The frequency of T$_{reg}$ was determined by flow cytometric analysis of the CD3$^+$CD4$^+$CD25$^{high}$CD127$^{low}$ T$_{reg}$ subset in the peripheral blood.

Results:
Athletes had significantly higher frequencies of T$_{reg}$ than age-matched healthy controls, which engaged only in recreational physical activity (7.04% vs. 6.11%, P=0.0068). Furthermore, we observed a significant gender difference. Female athletes had a 10% lower frequency of T$_{reg}$ than VO$_2$peak-matched male athletes (6.57% vs. 7.29%, P=0.0021). This provides further evidence for a hormonal influence on T$_{reg}$ homeostasis. In order to assess whether a higher training-load was associated with higher proportions of T$_{reg}$ cells the athletes were divided into three groups based on their relVO$_2$peak values (Low (n=82), Intermediate (n=81), High (n=82)). The intermediate group had a significantly higher mean frequency of circulating T$_{reg}$ than the low group (6.92 vs. 6.39%, P=0.0378). With a mean T$_{reg}$ frequency of 7.83% the high group had a significantly higher mean frequency of T$_{reg}$ than both the intermediate and low group. Importantly, an exercise-dependent increase in T$_{reg}$ was observed in both sexes, indicating that the anti-inflammatory effects of exercise are gender-independent. To validate these findings in an independent second cohort and to prove causality we performed a longitudinal study of athletes undergoing time-dense, high-intensity training. 19 members of the German Olympic Hockey team preparing for the 2012 Olympic Summer Games agreed to serve as the validation cohort. We obtained blood samples immediately before and after a one-week training program and found that at the end of the observational period, just prior to participation in the Olympic Summer Games and winning the Olympic gold medal, the average frequency of T$_{reg}$ had significantly increased from 7.21% to 8.14% (p=0.0210). This 12.9% average increase of T$_{reg}$ following the one-week intense training phase proves that physical exercise can affect T$_{reg}$ homeostasis.

Conclusion:
Our data provide the first direct evidence for a causal link between endurance exercise and an increase in circulating T$_{reg}$ in humans. Furthermore, the results suggest a dose-dependent effect of training load on the T$_{reg}$ frequency and illustrate how lifestyle factors such as physical activity can influence immune system homeostasis. The beneficial effects of exercise on disorders, which are associated with chronic inflammation such as atherosclerosis, autoimmunity or cancer, may therefore be partially attributable to the exercise-induced increase in T$_{reg}$ which shifts the balance of the immune system towards a more anti-inflammatory state.
Fighting chronic inflammation - CD64 specific elimination of M1 macrophages

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Macrophages are key players of the innate immunity and versatile cells that can adapt to microenvironmental signals resulting in distinct polarization states with altered phenotype and function¹. Although they have been studied for many years, only recently growing awareness has been raised that these cells are composed of distinct subpopulations, called M1 and M2². The dynamic ratio of M1 and M2 polarized macrophages is critical for the appropriate development and resolution of inflammation. M1 dominate in the early inflammatory phase, as opposed to M2 in the late and repair phase³,⁴. In chronic disease, this normal progression seems arrested where M1 macrophages remain dominant, leading to chronicity of the response and tissue damage⁵,⁶.

In our study, we induced M1 and M2 macrophages and characterized these by a set of membrane and soluble markers, both in human and in mice. CD64 was found to be one marker with highly increased expression by M1. We show the exclusive elimination of M1 type macrophages by a CD64 targeted immunotoxin, H22(scFv)-ETA', both in vitro and in vivo in a transgenic mouse model for chronic cutaneous inflammation. Above this, we could selectively eliminate M1 macrophages, thus inducing an overall switch from a Th1 to a Th2 response, using a human skin biopsy specimen from a patient with atopic dermatitis. In addition, we show that both populations (M1 and M2) show plasticity and that after conversion into either type only M1 polarization results in sensitivity for the CD64 immunotoxin. This implies that CD64 is a good target to specifically influence M1 macrophages and thereby alter their function or role in chronic inflammation. Specific targeting of M1 macrophages enables intervention in processes in which this subtype plays a pathological role. This concerns the majority of chronic inflammatory diseases, e.g. rheumatoid arthritis and chronic wounds. Our findings open the way for the development of completely novel, M1 macrophage targeted treatment strategies for chronic diseases, which is presently in great demand.

References:
T cell activation status determines the cytokine pattern induced by zymosan and bacterial DNA both in thymocytes and splenocytes

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Question:
Microbial factors have the ability to induce (over) production of pro-inflammatory cytokines which can cause in SIRS, sepsis or septic shock [1,2]. We here investigated the influence of two microbial stimulants, zymosan and bacterial DNA from E. coli K12, on mature and immature immune cell activation by analysing pro- and anti-inflammatory cytokine production.

Methods:
To study the role of T cell activation status, we investigated ex-vivo cultures of female C57.BL/6 and SJL/J mice (after PLPp 139-151/CFA/PTX-induced EAE) with and without αCD3 or PLPp stimulation. Alterations in their cytokine secretion pattern were measured by cytokine ELISPOT and FACS analysis.

Results:
In naive mice, we found that zymosan and E. coli DNA strongly co-stimulate αCD3-induced IFN-γ and IL-6 secretion in the thymus and in the spleen, but stimulate IL-17 production only moderately. Zymosan also increases PLPp-specific IFN-γ and IL-6 production in EAE.

Conclusion:
Zymosan and E. coli DNA affect mainly cells of the innate immune system in mature and immature immune cell populations. Moreover, T cell activation status is crucial for the cytokines secreted by an immune cell population encountering a microbial pathogen.

References:
1 Giamarellos-Bourboulis EJ. What is the pathophysiology of the septic host upon admission? Int J Antimicrob Agents 2010; 36 (Suppl. 2):S2-S.

Figure legends:
Fig. 1. Cytokine secretion pattern of thymocytes and splenocytes from naive C57.BL/6 mice in response to zymosan and αCD3 stimulation for the cytokines indicated as measured by ELISPOT assays and FACS analysis. (a) exemplars for ELISPOT wells (IL-17-producing cells) and (b) frequencies of cytokine-producing cells. Each bar represents the mean of two independent experiments of eight mice ± standard error of the mean. (c) flow cytometry for IFN-γ and IL-6 in thymocytes and splenocytes. Cell populations were gated on lymphocytes and living cells. Numbers in quadrants indicate percentage of positive cells. Data represent one of two independent experiments. * P<0.05; ** P<0.01; *** P<0.001.

Fig. 2. Cytokine secretion pattern of thymocytes and splenocytes from naive C57.BL/6 mice in response to E. coli DNA and αCD3 stimulation for the cytokines indicated as measured by ELISPOT assays and FACS analysis. (a) exemplar for ELISPOT wells (IL-17-producing cells) and (b) frequencies of cytokine-producing cells. Each bar represents the mean of two independent experiments of eight mice ± standard error of the mean. (c) flow cytometry for IFN-γ and IL-6 in thymocytes and splenocytes. Cell populations were gated on lymphocytes and living cells. Numbers in quadrants indicate percentage of positive cells. Data represent one of two independent experiments. * P<0.05; ** P<0.01; *** P<0.001.

Fig. 3. (a) Clinical disease course of PLPp 139-151/CFA/PTX-induced EAE in female SJL/J mice. Symbols represent the mean clinical EAE score of 4 mice in one experiment ± standard error of the mean. (b) Frequencies of cytokine-producing cells for the cytokines indicated as measured by ELISPOT. Cytokine production was assessed in the spleen 14 days after immunization. Splenocytes were stimulated with PLPp, zymosan or both agents in combination. Data of one experiment with four mice are displayed. Each symbol represents one animal and bars the mean. * P<0.05.
The EFECT Study: Inhalation of ectoine has a beneficial long-term effect on neutrophilic cell numbers

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Neutrophilic lung inflammation is one feature of chronic obstructive lung disease (COPD). Unfortunately, neutrophils do not respond well to the typical anti-inflammatory therapies. In earlier studies we were able to demonstrate that the compatible solute ectoine has effects on neutrophilic lung inflammation by preventing membrane-coupled signaling steps necessary for the induction and perpetuation of inflammation. The current study aimed to investigate these effects in humans which were recruited from an epidemiological cohort suffering from mild COPD correlated to the enhanced exposure to traffic-related air pollution.

The study was designed as a double blinded cross-over trial. 33 volunteers (age 76.32 ± 2.42 years) were randomly chosen to inhale the ectoine or placebo (0.9% saline) once a day over a period of 28 days. Subjects were examined for respiratory symptoms, lung function and inflammatory parameters, prior to inhalation, directly after the inhalation period and 28 days later to investigate a long term effect. Beside the survey on changes in life quality, lung function investigation assigned as forced expiration, forced vital capacity and the resulting Tiffenau Index were determined. Furthermore, inflammatory markers such as IL-8, neutrophilic cell numbers, TNF-α, C reactive protein (CRP), total nitrogen and protein content in the sputum as well as leukotriene B4 (LTB4), fractional exhaled nitric oxide and total nitrogen in the breath condensate were assessed.

Inflammatory markers such as IL-8 and the number of inflammatory cells showed no significant reduction after inhalation of Ectoin® Inhalation Solution compared to placebo. However, total nitrogen in the sputum was significantly reduced (by 35%) after ectoine inhalation regimen. Analysis of quality of life and lung function data did not show significance to support superiority of one of the treatment arms. Upon monitoring possible adverse effects, no indication for increased health risks could be detected. A long term reduction of neutrophils by Ectoin® Inhalation Solution lasting over the wash-out period was observed.

Inhalation of ectoine can reduce inflammatory markers and the number of neutrophils. The results of the EFECT study encourages to test Ectoin® Inhalation Solution in COPD patients.
Natalizumab affects the T cell receptor repertoire in patients with multiple sclerosis

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Question:
To assess changes in the T cell receptor (TCR) repertoire in peripheral venous blood and cerebrospinal fluid (CSF) of patients with multiple sclerosis (MS) treated with natalizumab and the potential implication for developing progressive multifocal leukoencephalopathy (PML), and PML-immune reconstitution syndrome (IRIS).

Methods:
The TCR repertoire in blood and CSF was assessed by complementarity determining region 3 spectratyping in 59 patients with MS treated with natalizumab for at least 18 months, 5 cases of natalizumab associated PML, 17 age- and gender-matched MS patients not treated with natalizumab, and 12 healthy controls.

Results:
MS patients presented with peripheral TCR repertoire expansions in blood, which appeared less prominent during therapy with natalizumab. TCR repertoire restrictions observed in CSF were most pronounced in MS patients treated with natalizumab. In patients who developed PML with longitudinal samples available, new identical TCR receptor lengths expansions in blood and CSF were observed following plasma exchange, and preceded the development of IRIS.

Conclusions:
Profound TCR repertoire restrictions in CSF of patients treated with natalizumab reflect an altered immune surveillance of the central nervous system, which may contribute to an increased risk of developing PML. Natalizumab seems to prompt an impaired or delayed peripheral expansion of antigen-specific T cells, whereas increased reconstitution of peripheral T cell expansion following plasma exchange may trigger PML-IRIS. Our data corroborate that treatment with natalizumab results in broader changes in the T cell immune repertoire beyond lymphocyte migration.
Liver Sinusoidal Endothelial Cells Contribute to CD8 T Cell Tolerance Toward Circulating Carcinoembryonic Antigen in Mice

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Immunity against cancer is impeded by local mechanisms promoting development of tumor-specific T cell tolerance, such as regulatory T cells, myeloid-derived suppressor cells, or immunosuppressive factors in the tumor microenvironment. The release of soluble antigens, such as carcinoembryonic antigen (CEA) from colorectal carcinoma (CRC) cells, has been investigated for diagnostic purposes, but not for its immunological consequences. Here, we address the question of whether soluble CEA influences tumor-specific immunity. Mice were injected with soluble CEA protein, and CEA-specific CD8 T cells were analyzed for their phenotype and functionality by means of restimulation ex vivo or antitumor efficacy in vivo. We furthermore characterized the CD8 T cell population in peripheral blood mononuclear cell (PBMCs) from healthy donors and colorectal carcinoma patients. In mice, circulating CEA was preferentially taken up in a mannose receptor-dependent manner and cross-presented by liver sinusoidal endothelial cells, but not dendritic cells, to CD8 T cells. Such systemically circulating CEA promoted tolerization of CEA-specific CD8 T cells in the endogenous T cell repertoire through the coinhibitory molecule B7H1. These CD8 T cells were not deleted but were rendered nonresponsive to antigen-specific stimulation and failed to control growth of CEA-expressing tumor cells. These nonresponsive CD8 T cells were phenotypically similar to central memory T cells being CD44highCD62LhighCD25neg. We found T cells with a similar phenotype in PBMCs of healthy donors and at increased frequency also in patients with colorectal carcinoma. Conclusion: Our results provide evidence for the existence of an unrecognized tumor immune escape involving cross-presentation of systemically circulating tumor antigens that may influence immunotherapy of cancer.
Antibody dose and corticosteroid sensitivity separate TGN1412 induced inflammatory cytokine release from activation of regulatory T-cells

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Introduction:
CD28 superagonists (CD28SA) are CD28-specific mAb able to activate T-cells without overt ligation of the TCR. In rodents, CD28SA preferentially activate regulatory T-cells (Treg cells), allowing effective treatment of a broad spectrum of immunopathologies including models for multiple sclerosis and rheumatoid arthritis (RA). In contrast, application of the CD28SA TGN1412, to healthy volunteers during a clinical study in 2006 led to an immediate cytokine release syndrome (CRS), which resulted from the activation of CD4 effector memory T-cells. Recent studies have shown that the dose applied during this trial was in saturation with regard to the triggering of pro-inflammatory cytokine release.

Objectives:
The aims of this study were: 1) to investigate the ex-vivo bioactivity of the humanized, CD28 superagonistic mAb TGN1412 to expand regulatory T-cells in peripheral blood mononuclear cells (PBMC) from healthy donors and from patients with rheumatoid arthritis, 2) to determine if the inclusion of the corticosteroid methylprednisolone (MP) is compatible with the expansion of Treg cells by CD28SA and has an effect on the cytokine release, and 3) to evaluate the suppressive capacity of the TGN1412 induced regulatory T cells.

Materials and methods:
High density cultured PBMC from healthy and RA donors were stimulated with TGN1412 in the presence and the absence of methylprednisolone (MP), 24 hours after stimulation the cytokine concentrations were measured in culture supernatants. Five days after stimulation Treg expansion and proliferation were measured. A CFSE based suppression assay was used to determine the suppressive activity of TGN1412 induced Treg cells.

Results:
We report robust expansion of activated Treg cells with TGN1412 under restove conditions. Moreover, activation of conventional T cells (Tconv) as demonstrated by induction of cell proliferation and cytokine release can be separated from activation of Treg cells by both TGN1412 dose and sensitivity to methylprednisolone. At low TGN1412 doses, or in the presence of MP, secretion of pro-inflammatory cytokines is completely lost, whereas considerable activation of Treg cells can be observed. Moreover, TGN1412 induced Treg cells are highly efficient in suppressing the activation of Tconv.

Conclusion:
Our results strongly suggest that by applying low doses of TGN1412 and/or corticosteroid co-medication, the human CD28SA may effectively activate Treg cells in humans to target autoimmune-inflammatory diseases such as RA.

References:
Characterization of Th17 cells in Multiple Sclerosis patients and controls

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Objective:
Multiple Sclerosis (MS) is a relapsing-remitting, disseminated CNS inflammation with focal plaque-like CNS lesions, which is driven by autoimmune T cells. The mechanisms underlying relapses, remission and irreversible tissue destruction in MS are not clear. Our recent findings in the animal model experimental autoimmune encephalomyelitis (EAE) revealed that long-lasting contacts of Th17 cells led to severe, localized and partially reversible fluctuation in neuronal intracellular Ca2+ concentrations in living animals and that TH17 cells were capable of inducing cell death in cultured neurons in an antigen unspecific manner. We were now interested to translate these findings into the human system. Therefore, we analyzed human Th17 cells in MS patients and healthy persons. Furthermore, in order to check the pathogenicity of potentially encephalitogenic cell types, we established Th17 cultures from human Peripheral Blood Mononuclear Cells (PBMCs).

Methods:
PBMCs were isolated from blood of either Natalizumab- or Fingolimod treated patients and age- and sex-matched healthy donors via gradient density centrifugation. Cells were stimulated and stained for IL-17, IFN-γ, IL-22, IL-4 and GM-CSF. For Th17 cultures, cells were stimulated for 4 h and a magnetic bead based IL-17 secretion assay was performed on the cells. The cells were cultured for 6 days and characterized at the end of the culture.

Results:
The cytokine profiles of 40 patients treated with either Natalizumab (Tysabri ®) or Fingolimod (Gilenya ®) as well as healthy donors show highly significant differences in lymphocyte numbers and cytokines. While Natalizumab treated patients show very high lymphocyte numbers and high amounts of IL-17 producing cells per ml blood, only very few lymphocytes in Fingolimod treated patients are detectable. In Natalizumab treated patients IL-4 (a Th2 cytokine) has been detected in a raised level.
Th17 cultures with cells isolated from patients compared to healthy controls showed stable IL-17 expression concomitant with the cytokines IFN-γ, IL-22 and IL-4. First experiments suggest higher amounts of IFN-γ and IL-22 on day 6 in Natalizumab-treated patient derived Th17 than detected in cultures from cells of healthy donors. In contrast, IL-17 levels were similar in both cultures.

Conclusion:
Differences in cytokine profiles and numbers in both patient groups versus controls could be shown. High IL-17 producer numbers in Natalizumab treated patients suggest, that Th17 cells are sequestered in the blood. In comparison, Fingolimod treated patients largely lack these cells in the blood. This might be related with previously reported rebound phenomena of disease activity in MS patients after cessation of Natalizumab therapy.
Survival and differentiation defects contribute to neutropenia in glucose-6-phosphatase-beta (G6PC3) deficiency in a model of mouse neutrophil granulocyte differentiation

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Differentiation of neutrophil granulocytes (neutrophils) occurs through several steps in the bone marrow and requires a coordinate regulation of factors determining survival and lineage-specific development. A number of genes are known whose deficiency disrupts neutrophil generation in humans and in mice. One of the proteins encoded by these genes, glucose-6-phosphatase-beta (G6PC3), is involved in glucose metabolism. G6PC3-deficiency causes neutropenia in humans and in mice, linked to enhanced apoptosis and ER-stress. We used a model of conditional Hoxb8-expression to test molecular and functional differentiation as well as survival defects in neutrophils from G6PC3-/- mice. Progenitor lines were established and differentiated into neutrophils when Hoxb8 was turned off. G6PC3-/- progenitor cells underwent substantial apoptosis when differentiation was started. Transgenic expression of Bcl-XL rescued survival; however, Bcl-XL-protected differentiated cells showed reduced proliferation, immaturity and functional deficiency such as altered MAP kinase signaling and reduced cytokine secretion. Impaired glucose utilization was found and was associated with ER-stress and apoptosis, associated with the up-regulation of Bim and Bax; down-regulation of Bim protected against apoptosis during differentiation. ER-stress further caused a profound loss of expression and secretion of the main neutrophil product neutrophil elastase during differentiation. Transplantation of wild type Hoxb8-dependent cells into irradiated mice allowed differentiation into neutrophils in the bone marrow in vivo. Transplantation of G6PC3-/- cells yielded few mature neutrophils in bone marrow and peripheral blood. Transgenic Bcl-XL permitted differentiation of G6PC3-/- cells in vivo. However, functional deficiencies and differentiation abnormalities remained. Differentiation of macrophages from Hoxb8-dependent progenitors was only slightly disturbed. A combination of defects in differentiation and survival thus underlies neutropenia in G6PC3-/- deficiency, both originating from a reduced ability to utilize glucose. Hoxb8-dependent cells are a model to study differentiation and survival of the neutrophil lineage.
Mutation Screening In STAT1, CARD9 And Protein Kinase C-delta In Patients With Chronic Mucocutaneous Candidiasis

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Introduction:
Chronic mucocutaneous candidiasis (CMC) is characterised by recurrent or persistent infections of skin, nails or mucosa with Candida species. Sporadic as well as autosomal dominant and recessive inheritance has been reported. Recent research suggests that autosomal dominant CMC may be due to heterozygous gain-of-function mutations in STAT1 or in the loss of CARD9 signalling. Moreover, more recently, protein kinase C-δ (PRKCD) was identified as an essential part of the Syk-CARD9 pathway.

Objective:
In order to find the genetic cause of their disease, we analysed thirteen unrelated families with autosomal dominant CMC inheritance and fifteen sporadic patients with diagnosed CMC.

Methods:
The three candidate genes STAT1, CARD9 and PRKCD, were analysed in 43 patients from thirteen families with autosomal dominant CMC and fifteen sporadic cases with clinically diagnosed CMC. We performed our analysis by sequencing genomic DNA of all patients with Sanger’s method.

Results:
Among the individuals studied, 28 patients from ten families had a heterozygous STAT1 mutation. In three families, no mutation was found. In addition, sequencing confirmed STAT1 mutations in seven out of fifteen sporadic CMC patients. Overall, we identified thirteen different heterozygous missense mutations in STAT1, one located in the N-terminal region, eight located in the coiled-coil domain and four located in DNA-binding domain. At the time of abstract writing we have not identified any mutation in CARD9 or PRKCD, but sequencing efforts continue.

Conclusions:
Missense mutations in the coiled-coil and DNA-binding domain of STAT1 are the cause of approximately 50% of sporadic and 75% of familial CMC in our cohort. Hence, STAT1 mutations have to be taken into account when diagnosing this condition. CARD9 mutations are much rarer in CMC patients and PRKCD mutations have yet to be identified.

References:
Hypomorphic, homozygous mutations in \textit{Phosphoglucomutase 3} impair immunity and increase serum IgE levels

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Recurrent bacterial and fungal infections, eczema and elevated serum IgE levels characterize patients with the hyper-IgE syndrome (HIES). However, glycosylation defects have not been described in HIES. Here we identified three hypomorphic, homozygous mutations (E340del, L83S, and D502Y) in \textit{Phosphoglucomutase 3} (\textit{PGM3}) that segregated perfectly with affection status and recessive inheritance of nine patients with HIES. Functional studies showed that these hypomorphic mutations have impact on the biosynthetic reactions involving UDP-GlcNAc, as glycomics analysis revealed an aberrant glycosylation pattern in leukocytes exemplified by a reduced level of tri-/tetra-antennary N-glycans. We concluded that the impairment of PGM3 function leads to a novel primary (inborn) error of development and immunity, as biallelic hypomorphic mutations are associated with impaired glycosylation and a hyper-IgE-like syndrome.
The extended clinical phenotype of 58 patients with DOCK8 deficiency


Background:
Hyper-IgE syndromes (HIES) are rare primary immunodeficiencies. STAT3 deficiency causes the majority of autosomal-dominant HIES (AD-HIES), whereas DOCK8 deficiency causes the majority of autosomal-recessive HIES (AR-HIES). These two syndromes share some, but not all clinical features. Distinguishing AD- from AR-HIES is of clinical importance due to a difference in prognosis and management. DOCK8 deficiency, being an AR trait, is characterized by affected children having healthy parents, but many patients have a de novo STAT3 mutation, so the absence of an affected parent does not effectively exclude AD-HIES.

Objectives:
To characterize the clinical phenotype of AR-HIES due to DOCK8 mutations. To determine if AR-HIES patients with DOCK8 mutations can be distinguished from those without based on clinical features. To establish diagnostic guidelines to distinguish between DOCK8- and STAT3-deficiencies. To identify new mutations in DOCK8.
Methods:
DOCK8 was analyzed in 76 patients from 54 families with the phenotype of AR-HIES. Regression analysis was performed to compare clinical data from 35 index patients with and 10 index patients without a DOCK8 mutation. A second analysis was performed to compare the same 35 patients with a DOCK8 mutation to 64 AD-HIES index patients with STAT3 mutation. A machine-learning approach was used to identify features that better predict a DOCK8 or STAT3 mutation, respectively.

Results:
Among the individuals studied, 58 from 44 families had DOCK8 deficiency. For 54 persons from 40 families, a DOCK8 mutation was confirmed by sequencing or PCR analysis. Four persons in four families had less direct evidence of a DOCK8 deficiency. We identified 27 deletions/insertions, 12 splice site or nonsense mutations, and one gene transcription failure. The clinical phenotype of AR-HIES is too heterogeneous to distinguish patients with or without DOCK8 deficiency. A combination of five clinical features can predict DOCK8 mutations vs. STAT3 mutations in individuals with a diagnosis of HIES, although misclassifications can occur. Some features such as eosinophilia and recurrent viral infections are more severe in DOCK8 deficiency than in STAT3 deficiency.

Conclusions:
We propose the following diagnostic guidelines for DOCK8 deficiency:
Possible: Diagnosis of HIES plus a weighted score of clinical features >12 based on hypereosinophilia and upper respiratory tract infections (weighing positively) and parenchymal lung abnormalities, retained primary teeth, and minimal trauma fractures (weighing negatively). Probable: Above plus consanguineous parents, severe viral infections, allergies, and/or low IgM levels. Definitive: Homozygous or compound heterozygous mutation in DOCK8 and/or lack of full-length protein expression.
TNF-alpha mediates a delayed anti-inflammatory autocrine feedback mechanism in the response of monocytes to LPS

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Introduction:
Activation of monocytes by microbial products is an essential mechanism for host response against invading pathogens. This process has to be tightly controlled, because overwhelming inflammatory reaction can cause excessive host tissue damage as seen for instance during hyper inflammatory phase of sepsis. However, the underlying mechanisms in regulating inflammation during prolonged monocyte activation by bacterial endotoxin are not completely understood.

Objective:
We analysed long term Lipopolysaccharide (LPS)-stimulated human monocytes to identify mechanisms allowing resolution of inflammatory response.

Materials & Methods:
Human monocytes were challenged for 16 hours with LPS. Using oligonucleotide microarrays we carry out a sophisticated genome-wide analysis to define the delayed LPS-triggered expression profile. Functional assays were performed to characterize the phenotype of monocytes in more detail.

Results:
Remarkably, statistical analysis demonstrated that LPS induced an up-regulation of anti-inflammatory rather than pro-inflammatory molecules 16 hours after stimulation. Thus, prolonged LPS treatment resulted in the generation of an anti-inflammatory monocyte phenotype. To determine whether monocytes challenged for 16h with LPS can actively suppress pro-inflammatory responses from other innate immune cells we performed co-culture experiments. Indeed, we were able to inhibit the LPS-induced expression of pro-inflammatory cytokines (e.g. TNF-alpha, CXCL-9, CXCL-10 and CXCL-11) from macrophages in co-culture experiments. Interestingly, significant overrepresentations of genes are known to be regulated by TNF-alpha. When we blocked TNF-alpha signalling during LPS-pre-treatment using soluble TNF-receptors, we observed a significant reduction of their capacity to inhibit release of pro-inflammatory mediator by macrophages.

Conclusion:
Our data indicate an important effect of TNF-alpha in the generation of an anti-inflammatory monocyte phenotype during the late phase of monocyte activation by LPS. This may explain the unexpected deleterious effect of TNF-blocking therapeutics in the treatment of sepsis. Improved understanding of this anti-inflammatory feedback mechanism in the response to pathogenic microbes is important for the development of new therapeutic regimes for infections as well as inflammatory disorders.
Comparison the immunogenicity of HIV-1 P24-Nef candidate vaccine conjugated to Flc protein of pseudomonas aeruginosa formulated in Montanide ISA 70 using subcutaneous and intramuscular routes injection in BALB/c mouse model

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Background:
Today, HIV infection is referred to as a serious threat to humanity. Many efforts have been made to prepare an effective vaccine, but has remained inconclusive. Therefore, new strategies in order to increase the immune response to vaccines using immunological adjuvant such as TLR agonists such as the Pseudomonas aeruginosa flagellin (Flc) were presented. In this study, given the effect of immunization route on the immune response induced by vaccination, candidate vaccine HIV-1p24-Nef conjugated to Flc molecule, was injected in different routes and the immune responses were evaluated.

Materials and Methods:
BALB/c mice were distributed into 6 groups and immunized with 20 μg/100μl of HIV-1 p24-Nef conjugated to Flc, p24-Nef and Flc which were prepared in Montanide ISA70, subcutaneously and intramuscular three times under the same conditions. Two weeks after the final boosting, lymphocyte proliferation was measured by Brdu method, the response of IL-4 and IFN-γ cytokines, as well as the level of total antibodies and their isotypes were evaluated using ELISA method. Also IFN-γ ELISPOT was performed to detect the memory T cells frequency.

Results:
Results show that, in compare with control groups, the conjugated HIV-1p24-Nef-Flc significantly increased lymphocyte proliferation responses, higher levels of cytokines responses and IFN-γ producing lymphocytes in the subcutaneously but the level of humoral immune responses significantly increased in the intramuscular route.

Conclusion:
Flc molecule could be used as adjuvant in combination with vaccines candidate against HIV-1.
Keywords: Adjuvant, HIV-1P24-Nef, Flc molecule
Impaired immune fetal development in mice in response to prenatal stress challenge can be ameliorated by maternal progesterone supplementation

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Fetal programming focuses in the effect of environmental factors, such as prenatal stress challenge, which can trigger the vulnerability to chronic immune diseases in later life. The aim of the present study was to unveil the mechanisms by which stress challenge affects fetal development.

Materials and methods:
DBA/2J-mated BALB/c females were exposed to 24 h sound stress on gestation days (gd) 12.5 and 14.5 and injected with the Progesterone (P) derivative dydrogesterone or vehicle on gd 11.5, 13.5 and 15.5. Non-stressed sham injected pregnant females served as controls. On gd 16.5, dams were sacrificed, serum was collected, fetal weight was recorded and fetal immune development as well as maternal immune adaptation to pregnancy were determined by flow cytometry. Cytotoxicity of CD8+ cells was determined as the difference in the positivity for CD107a after and before ionomycin stimulation. CD8+ cells were separated from uterus draining lymph nodes cell suspensions by magnetic cell sorting and stimulated to quantify the production of cytokines.

Results:
Stress challenge decreased serum levels of P and induced intrauterine growth restriction in fetuses. Further, the frequency and cytotoxicity of maternal CD8+ T cells in uterus-draining lymph nodes were increased in stress challenged dams, as well as the their secretion of inflammatory cytokines TNF-α and IFN-γ. Fetal immune development was significantly impaired, e.g. delayed T cell maturation, low regulatory T cells and smaller thymuses were observed. The effect of prenatal stress challenge on dam and fetus could largely be abrogated by supplementation of P.

Conclusions:
Our results show that prenatal stress affects fetal development, by altering maternal endocrine and immune adaptation to pregnancy. We demonstrate a protective function for P in mouse fetal development. Hence, P is susceptible to macro and microenvironmental factors such as stress, and P supplementation may serve as a pharmacological intervention protecting mother and baby.
Implantation unfavorable position of immune parameters displays immune accentuations that predict IVF failure

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Introduction:
Deviations in NK cell frequency, receptors expression and cytotoxicity, as well as in activation markers expression on T-lymphocytes in patients with multiple reproductive failures were described by many investigators. However, this question is still controversial. Difference in design of clinical groups verification often leads to controversial result in significance of investigated parameter. Possibly it is so, because immune factors have only conditional and indirect influence on implantation and pregnancy outcome. Generally, deviated immune parameters still stay in immune normal ranges and were not a marker of immunopathology, but represented immune accentuations (unfavorable state of parameters). Clinical manifestation of immune accentuation can be grade and compensated by other immune or non-immune factors.

Objective:
Clinical significance of single and multiple immune accentuations.

Patients and Methods:
Blood samples from 123 women with multiple reproductive failures (≥3) undergoing IVF were drawn 5-12 d before embryo transfer procedure. 75 women have not become pregnant after IVF (IVF failure group - IVFf), 48 became pregnant. NKc was studied in 4h flow cytotoxicity assay using FDA labelled K562 cell line. Lymphocyte phenotype was studied on FACScan flow cytometer using BD monoclonal abs.

Results:
We showed that levels of CD56 and CD158a expression on CD3+, HLADR on CD3+CD8+, NK frequency and NKc were increased and levels CD3+CD4+ were decreased in patients with subsequent IVF failure compared to women who became pregnant. We also found that patients with accentuated decrease; CD3+CD4+ cells, expression of CD158a or CD8 on NK and increased NK frequency or NK HLADR expression and T cells-CD56 and CD158a expression, values had reduced implantation rate compared to women with conditionally normal values. Patients with 3 and more accentuated values had significantly decreased pregnancy and birth rate, only 21.4% (9/42) and 9.5% (4/42) compared to group without or with isolated accentuation 50.9% (27/53) and 33.9% (18/53). Individuals with 2 accentuated parameters had intermediate levels of pregnancy and birth rate 42.8% (12/28) and 28.5% (8/25) that was not quite significantly different from group with multiple deviations.

Conclusion:
We showed that immune accentuations exert influence on reproductive outcome. Significance of single accentuation can be compensated by other factors and its possible cases of frequent controversial in reported result. Multiple immune accentuations have increased predictive significance for reproductive failures and potentially for diagnostic utilizing.
Transplacental immune regulation refers to the concept that during pregnancy, significant crosstalk occurs between the maternal and fetal immune system with potential long-term effects for both the mother and child. In this study, we made the surprising observation that there is a strong correlation of peripheral blood regulatory T (Treg) cells between the mother and the fetus. In contrast, there is no significant Treg cell correlation between paternal fetal dyads (pairs), suggesting that the specific context of pregnancy, rather than the genetic parental similarity to the fetus, is responsible for this correlation. Gene microarray analysis of Treg cells identified a typical interleukin-10 (IL-10)-dependent signature in maternal and fetal Treg cells. In addition, a direct correlation of serum IL-10 protein levels between maternal fetal dyads was observed. Furthermore we show that maternal serum IL-10 levels correlate with serum estradiol (E2) and estriol (E3), implicating hormonal involvement in this alignment. Interestingly, we show that Treg cells possess higher expression of IL-10 receptor alpha (IL-10RA) and that Treg cell IL-10RA expression directly correlates with their Bcl-2 expression. Indeed, in vitro data in both humans and mice demonstrate that IL-10 upregulates Bcl-2 specifically in Treg cells, but not non-Treg cells. Our results provide evidence for transplacental regulation of cellular immunity, and suggest that IL-10 may influence Treg cell homeostasis through its effect on Treg cell Bcl-2 expression. These novel findings have important implications on immune tolerance in pregnancy and beyond, in areas of autoimmunity, allergy and transplantation.
Increase severity of allergic asthma among offspring of mice treated with Acetaminophen

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Introduction:
The use of Acetaminophen or Paracetamol, abbreviated as APAP, for acetyl-para-aminophenol, during pregnancy is linked to an increased risk for childhood asthma. APAP is mainly metabolized in the pivotal immune organ, the liver, and freely crosses the placenta, resulting in pharmacologically active levels in the developing fetus. In the fetus, the liver transiently functions as the main hematopoietic organ providing progenitor cells, which seed in the thymus to undergo T cell development. However, the alteration of maternal immune adaptation to pregnancy, the impairment of fetal immune development, and the increased the risk of developing allergic disorders following administration of APAP have not been fully investigated.

Objectives:
More than 20% of pregnant women in Western societies report taking APAP. Additionally, the epidemiological findings suggest that use of APAP in pregnancy is associated with increased asthma symptoms in children. Employing a mouse model of allergic asthma, we have therefore sought to study the potential effects of prenatal APAP administration on the risk of allergic disorders among the offspring.

Methods:
Using an OVA-sensitized mouse model of allergic airway inflammation, the offspring of PBS or single-dose APAP treated mice were sensitized and challenged with OVA and the number of eosinophils in bronchoalveolar lavage (BAL) fluid was detected, using cytospin and flow cytometry.

Results:
We observed that administration of a single dose APAP (250 mg/kg) to pregnant mice is associated with dam’s hepatitis and granulocyte infiltration. Furthermore, the severity of asthma was detected since higher eosinophil infiltration in BAL among offspring of APAP treated mothers than controls was demonstrated. The percentage of Siglec-F⁺ cells in BAL of control animals was 37%, which increased to 71% in offspring of mice administered with APAP during pregnancy. Additionally, the increase in eosinophil infiltration in BAL was confirmed by cytospin preparations. Eosinophil counts in BAL among offspring of APAP-administered dams increased from 34% (SD=11%) to 58% (SD=15%).

Conclusions:
We demonstrated that use of APAP during pregnancy is associated with an increased susceptibility to APAP-induced hepatotoxicity. Moreover, transplacental APAP increases the offspring’s susceptibility towards allergic diseases. Full characterization of liver-mediated immune adaptation to pregnancy in APAP-treated dams and their fetus may clarify the mechanisms responsible for the increase in severity of allergic diseases among the offspring.
Bone marrow-raised CD27\textsuperscript{low} mouse natural killer cells selectively home uterus to regulate pregnancy

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Introduction:
Uterine natural killer (NK) cells are pivotal for successful mammalian reproduction. NK cells have a critical role in the success of a healthy pregnancy by maintaining the balance between placental function and fetal requirements. However, insights on functionally distinct subpopulations of uterine NK cells are largely elusive.

Objectives:
The surface expression of CD27\textsuperscript{high} or CD27\textsuperscript{low} subsets has recently been proposed to allow discrimination of NK cells into functional subsets, due to their differential cytotoxicity or cytokine production. Therefore, the distinction between CD27\textsuperscript{high} and CD27\textsuperscript{low} NK cell subsets, for example in the context of reproduction, may allow to define the functional role of NK cell subsets in the context of translational NK cell research. We have therefore sought to study the role of CD27\textsuperscript{high} and CD27\textsuperscript{low} subsets in mouse pregnancy.

Methods:
DBA/2J-mated CBA/J females were used to investigate the frequency and phenotype of NK cells in virgin or pregnant mice during early gestation. Flow cytometry was used to analyse the phenotype, function, and migration of NK cell subsets. Evaluation of implantations and fetal loss rate were performed in syngeneically-mated RAG2\textsuperscript{-/-}/γc\textsuperscript{-/-} females following adoptively transferred NK cells.

Results:
We demonstrated that the CD27\textsuperscript{low} NK subset has low cytotoxic capacity, produces higher amounts of interferon (IFN)-γ, and expresses functional homologs of human NK cell immunoglobulin-like receptors. We further found that bone marrow CD27\textsuperscript{low} NK cells are selectively recruited to the uterus and ameliorate the rate of fetal loss when adoptively transferred into alymphoid RAG2\textsuperscript{-/-}/γc\textsuperscript{-/-} mice. Additionally, expression of CD27 is down-modulated on NK cells upon migration to the uterus.

Conclusions:
We here provide evidence that a subset of murine NK cells present at the fetomaternal interface is pivotal in maintaining pregnancy. Therefore, we propose the existence of a regulatory NK cell subset, which is licensed toward successful pregnancy maintenance at the fetomaternal interface in mice.
Immune adaptation to pregnancy in mice is impaired by prenatal stress challenge via reduced HO-1 expression

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Introduction:
Environmental challenges can target maternal endocrine and immune adaptation to pregnancy. The placenta is a temporary organ that mediates the interaction between the mother and fetus. Heme oxygenase (HO)-1, enzyme that catabolizes the degradation of heme, is highly expressed in placenta. However, its function and modulation throughout pregnancy are not well understood. HO-1 acts as antioxidant and cytoprotectant through its enzymatic products and plays a regulatory role in a number of inflammatory processes.

Objective:
Our objective was to examine if alteration or lack of HO-1 expression, e.g. upon stress challenges or in HO-1 mutant mice, affects fetal development and maternal immune adaptation. Further, we aimed to unveil if HO-1 was regulated by progesterone, as this hormone is decreased by stress.

Materials and methods:
DBA/2J-mated BALB/c wild type (wt) females were exposed to 24 hour sound stress on gestation days (gd) 12.5 and 14.5. HO-1+/− BALB/c mice were syngeneically mated. On gd 16.5 placental tissue and uterus-draining lymph nodes (LN) were collected, fetal weight was documented. In wt pregnancies, placental HO-1 expression was studied by quantitative real time (qRT)-PCR and immunohistochemistry (IHC). Fetal HO-1 genotype was determined in HO-1+/− pregnancies. Phenotype and frequency of maternal immune cells harvested from uterus-draining LN were identified by flow cytometry. As a model for trophoblast cells, human choriocarcinoma cell line BeWo was incubated with vehicle or progesterone receptor antagonist RU486 (1 µg/ml) for 24 h and subsequently stimulated with hemin (50µM) for 24 h. HO-1 mRNA was measured in BeWo cells by qRT-PCR and iron was determined in the culture media. Placental structure was evaluated in Masson’s trichrome stained placental tissue sections. For statistical analyses, one-way analyses of variance and Mann-Whitney U-tests were used for normally and not normally distributed data, respectively. Significance was set at p< 0.05.

Results:
IHC analysis showed HO-1 expression in all placental tissue layers, in decidua, and in some tissue infiltrating leukocytes. HO-1 placental expression was decreased upon stress, as revealed by qRT-PCR. In vitro blockage of progesterone receptor impaired HO-1 expression and function in BeWo cells, suggesting that stress inhibits HO-1 expression through progesterone decrease. HO-1 reduction challenged maternal immune adaptation to pregnancy, by increasing frequency of CD8+ T cells but decreasing regulatory CD122+ CD8+ T cells in LN from stressed and HO-1+/− dams, compared to wt non stressed controls. Placental function was also affected by decreased HO-1, as labyrinth/junctional zone ratio in placentas from HO-1+/− and wt stress challenged pregnancies were increased when compared to wt and control, respectively. Fetal weight was reduced in stress challenged pregnancies and in HO-1+/− offspring, compared to controls or wt fetuses of the same litter.

Conclusion:
Our results show that reduced HO-1 expression induced by stress or in HO-1+/− mice results in altered immune adaptation to pregnancy, characterized by increased CD8+ T cells. This coincides with altered placental function and signs for fetal growth restriction. Reduced HO-1 expression could thereby have detrimental short- and/or long-term effects on offspring development and health. To prevent these effects pharmacological interventions could target HO-1 to modulate immune tolerance during pregnancy.
Role of Toll-like receptor 2 (TLR2) as a modulator of the Fc$_\text{epit}$,RI-expression and function in human epidermal dendritic cells.

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Background and working hypothesis:
Epidermal Langerhans cells (LC) and other dendritic cells (DC) from patients with atopic dermatitis (AD) express the high-affinity receptor for IgE (Fc$_\text{epit}$,RI). The skin of AD patients is highly colonized with pathogens like Staphylococcus aureus, which can be recognized by pattern recognition receptors such as Toll-like receptor (TLR) 2. Therefore, we speculated that both Fc$_\text{epit}$,RI and TLR2 on LC may be of crucial importance for (i) the epicutaneous sensitization driven by DC and (ii) the control of the chronic inflammation in AD.

Experimental system:
Human LC were generated in vitro from cord blood-derived CD34$^+$ hematopoietic stem cells (CD34LC) which expressed TLR2 and Fc$_\text{epit}$,RI. The impact of TLR2- and Fc$_\text{epit}$,RI-mediated signals on the expression of Fc$_\text{epit}$,RI and known transcription factors of the receptor was analyzed on the protein and RNA level by flow cytometry, western blotting and real-time PCR. Changes in the cytokine profile were investigated by real-time PCR and ELISA.

Summary of results:
In human CD34LC, stimulation of TLR2 resulted in down-regulation of the Fc$_\text{epit}$,RI surface expression at transcriptional level. The analysis of Fc$_\text{epit}$,RI-related transcription factors (TF) suggests a PU.1-dependent regulation. Also, miRNA-155, which is reported to control PU.1 expression, was up-regulated upon TLR2 engagement. Further regulating elements might contribute to the regulation process.

1,25-dihydroxyvitamin D3 (calcitriol) down-regulated Fc$_\text{epit}$,RI in a maturation-independent manner and impacted on the cytokine profile of TLR2-stimulated CD34LC. Analysis of TF indicated a different mechanism than TLR2-induced Fc$_\text{epit}$,RI regulation.

CD34LC treated with a TLR2 ligand showed a switch towards a tolerogenic cytokine profile with up-regulation of IL-10 and IL-12p40 and down-regulation of TNF-alpha, while IL-12p35 expression was never observed. Fc$_\text{epit}$,RI cross-linking induced IL-10 and IL-12p40 and further enhanced TLR2-mediated expression of these cytokines. Furthermore, expression of the tryptophan-degrading enzyme IDO, which is involved in tolerogenic mechanisms, was enhanced by TLR2- or Fc$_\text{epit}$,RI-mediated stimulation.

Conclusion:
Taken together, we show a functional crosstalk of TLR2- and Fc$_\text{epit}$,RI-mediated signals influencing human LC behaviour and present first results pointing to the underlying molecular mechanisms and functional consequences.
Granulocytes, Mast Cells, Immune Response in the Skin

T cell not mast cell derived Interleukin 10 regulates contact hypersensitivity responses

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Introduction:
Contact hypersensitivity (CHS) is the classical murine model of allergic contact dermatitis. While the process of sensitization and elicitation is well investigated, mechanisms that determine the resolution of the inflammatory response are less well understood. Recent studies suggested a regulatory role of mast-cells (MC) and MC-derived Interleukin 10 (IL-10) (Grimbaldeston et al 2007) in the control of CHS, while others reported MC to be required for optimal CHS responses (Dudeck et al 2011).

Objective:
Based on these controversies we analysed the cellular sources of IL-10 during the resolution phase of CHS in the DNFB model using IL-10 transcriptional reporter mice (Vert-X).

Results:
Neither at baseline nor after allergen challenge MCs of the affected ear skin or the draining lymph nodes displayed IL-10 expression.
In contrast, a clear IL-10 signal was observed in the T cell compartment. IL-10 expression was observed in CD4⁺ T cells with a maximum during the resolution phase. In addition, a marked increase of IL-10⁺ CD8⁺ cells was detected in the ear skin and in the regional LN.
Characterization of these IL-10⁺ CD8⁺ T cells revealed an activated phenotype and a hapten specificity which points to a possible regulatory activity in CHS inflammation.
The functional role of CD4⁺ T cells in regulating the CHS response was confirmed using MHC class II⁻/⁻ mice that lack CD4⁺ T cells and displayed an augmented CHS response. Similarly, selective depletion of regulatory T cells prior to challenge by using DEREG mice resulted in an augmented CHS response, suggesting a dominant regulatory role of CD4⁺ T cells in attenuating the immune response in CHS.
Finally, the inhibitory role of T cell derived IL-10 was confirmed in mice with a T cell specific IL-10 deficiency (IL-10⁻/⁻ CD4⁻ Cre⁺). Corresponding to previous reports (Roers et al 2004) these mice displayed an enhanced ear swelling response and a delayed resolution of the CHS response as compared to Cre⁺ controls.

Conclusion:
In conclusion, our results do not support the assumption that mast cell derived IL-10 is involved in limiting the CHS response. Instead they confirm a crucial role of CD4⁺ T cells and suggest that also IL-10 producing CD8⁺ T cells may play an additional regulatory role in the resolution of CHS.
Granulocytes, Mast Cells, Immune Response in the Skin

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Neutrophils are crucial innate effectors in the sensitization phase of contact hypersensitivity

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Allergic contact dermatitis (ACD) is a T cell mediated inflammatory skin disease. The innate immune system plays a crucial role in orchestrating the inflammatory response. The role of innate effector cells is only poorly understood. We investigated the role of neutrophils in the contact hypersensitivity (CHS) model, the mouse model of ACD. Contact allergen treatment causes rapid neutrophil infiltration of the skin. In a model of specific neutrophil depletion and in a strain of genetically neutrophil deficient mice, we demonstrate that both sensitization and elicitation of the CHS response are dependent on neutrophils. During the sensitization phase numerous contact allergen induced signs of inflammation such as ROS production, gelatinase activity and the production of various chemokines and cytokines were significantly decreased in neutrophil depleted or deficient mice. In the absence of neutrophils the allergen induced migration of dendritic cells from the skin to the draining lymph nodes was absent. Restimulation of T cells from mice ex vivo showed that T cell priming is compromised if neutrophils are absent at the time point of sensitization. Among the pro-inflammatory cytokines reduced in the skin during sensitization in the absence of neutrophils interleukin(IL)1β was prominent. Administration of recombinant IL-1β restored the sensitization process in neutrophil depleted mice. Passive CHS experiments underlined the requirement of neutrophils for T cell priming in the sensitization phase and for effector T cell recruitment in the elicitation phase. In summary, we here demonstrate that neutrophils are key elements in the orchestration of the innate inflammatory response in CHS. Without their contribution, sensitization does not occur due to defective dendritic cell emigration from the skin and defective T cell priming. During the elicitation phase, neutrophils are needed to recruit effector T cells to the skin. Modulating neutrophil activation and recruitment might be a future therapeutic target for the treatment of ACD.
Cutaneous OX40-OX40 ligand signaling down-regulates Treg function and prevents UVB-induced immunosuppression

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The skin is constantly exposed to various environmental factors resulting in the induction of immune responses. Previous work revealed that co-stimulatory molecules, like members of the TNF/TNF receptor family, are involved in the regulation of cutaneous immunity since signaling via receptor-ligand-pairs of the TNF family modulates survival, differentiation or activation of immune cells. The TNF receptor OX40 is expressed on T cells whereas its ligand (OX40L) is up-regulated upon inflammation on antigen presenting cells (APC), such as epidermal Langerhans cells (LC). Using transgenic mice with cutaneous overexpression of OX40 (K14-OX40 tg mice) we demonstrated an impact of OX40 ligation on the suppressive activity of CD4⁺CD25⁺Foxp3⁺ regulatory T cells (Treg). During cutaneous inflammation Treg from K14-OX40 tg mice were characterized by a poor suppressor function due to the OX40/OX40L-mediated down-regulation of CTLA-4, IL-10 and Foxp3, all markers which have been implicated in Treg function. Accordingly, in a contact hypersensitivity (CHS) model we observed an increased proliferation of activated effector T cells in K14-OX40 tg mice compared to wildtype (wt) controls, which translated into an elevated ear swelling response. This effect was clearly mediated by the impaired suppressor function of Treg since adoptive transfer of Treg from wt donors normalized the ear swelling response in sensitized/challenged K14-OX40 tg recipients to wt levels. Treg are developing in the thymus as a separate T cell subset. However, besides thymic-derived Treg, induced Treg, which can be generated in the periphery from conventional CD4⁺ T cells by direct contact to tolerogenic APC, are involved in the regulation of cutaneous immune responses. In particular UVB irradiation is known as an environmental factor promoting the peripheral induction of Treg. To assess the role of OX40/OX40L signaling on the proliferation and suppressive activity of induced Treg we irradiated wt and K14-OX40 tg mice with UVB light before sensitization to the contact allergen oxazolone. As expected, UVB irradiation induced Treg in wt mice resulting in the inhibition of effector T cell proliferation and the suppression of contact allergy. Notably, UVB irradiation did not suppress CHS in K14-OX40 tg mice. This effect was attributable to the impaired development of induced Foxp3⁺Helios⁺ Treg in irradiated K14-OX40 tg mice resulting in an up-regulated proliferation of activated effector T cells like Th1 or Th9 cells. Interestingly, OX40/OX40L activated LC seemed to be critical for the inhibitory effects on the peripheral induction of Treg in tg mice since ablation of LC in K14-OX40 tg x Langerin-DTR double mutants normalized the generation of UVB-induced Treg and the suppression of CHS to wt levels. Together, these data indicate that OX40/OX40L signaling is crucially involved in the regulation of the suppressive activity of thymic-derived Treg as well as the peripheral induction of Treg upon UVB-irradiation.
The role of IL-6 and IL-17A in IMQ-induced psoriasis-like skin disease

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Question:
Psoriasis is the most common autoimmune disease of the skin, which affects up to 4% of the population [1]. It is characterized by a strong interplay of epidermal keratinocytes, T cells and other leukocytes [2], but not yet completely understood. Recently, it turned out that the IL-17/IL-23 axis plays a critical role in the development of psoriasis [3,4] and also IL-6 [5]. We aim to further investigate the inflammatory processes in psoriasis, especially focusing on IL-6, as a foundation for future treatment options.

Methods:
A common mouse model to induce psoriasis-like skin disease is the application of Imiquimod (IMQ), a Toll like receptor (TLR) 7/8 ligand, which leads to dermal damage similarly to human psoriasis [3]: By IMQ, we induce psoriasis-like skin disease in mice which have a deficiency of the IL-6 receptor on macrophages and granulocytes (LysM-Cre IL6Rfl/fl) and on mice overexpressing IL-6 in macrophages and granulocytes (LysMCre IL-6ind/+). Besides, our lab provides a mouse model of skin-specific overexpression of IL-17A (K14-IL-17Aind/+). Skin, Spleen, bone marrow as well as blood are analyzed by Flow Cytometry, ELISA and RT-PCR.

Results:
Mice that skin-specifically overexpress IL-17A in keratinocytes show an increased population of CD11b+, F4/80+ and Gr-1+ in the skin and all mentioned organs, which indicates a high invasion of granulocytes and macrophages. This is also true for effector T cells. Besides IL-17A, IL-6 is elevated in the skin and the blood of these mice. By anti-IL-6 treatment a reduction of the psoriatic phenotype was detectable as described by us before (Croxford et al, submitted to JID). In mice with a deficiency of the IL-6 receptor on the LysM-positive cells, the IMQ-induced skin inflammation seems to be slightly reduced in comparison to IMQ-treated control mice. This remains to be further investigated also turning attention to the IL-6 trans-signaling. In comparison, mice overexpressing IL-6 in the LysM-positive cells will be analysed.

Conclusion:
Besides IL-17A, IL-6 seems to be highly important in the development of psoriasis. Whereas anti-IL6 treatment significantly improves psoriasis in our genetic mouse model, the knockdown of the IL-6 Receptor in macrophages and granulocytes only slightly milders IMQ-induced psoriasis. For further investigating the role and influence of IL-6, further experiments will be needed also with mice overexpressing IL-6 in macrophages and neutrophils.

Reference:
Proteomic identification of contact allergen regulated proteins in human monocytes

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Question:
Nickel (Ni) represents the most common human contact allergen. In order to characterize molecular events underlying Ni-associated innate and/or adaptive immune responses of T cell mediated human allergic contact dermatitis (ACD), a proteomic approach was chosen to identify Ni-regulated proteins in human monocytes. Monocytes, which are known precursors of dendritic cell, were stimulated with increasing concentrations of Ni and subsequent regulation of proteins was investigated using differential gel electrophoresis (DIGE) technology.

Methods:
Differentially Ni-regulated proteins were detected by Delta-2D software (Decodon, GE) and spots were identified by mass spectrometry (MALDI-TOF), followed by bioinformatics analysis, functional clustering and functional cellular assays (FACS, confocal microscopy).

Results:
More than 30 allergen-regulated proteins were detected by Delta2D examination and subjected to MALDI-TOF analysis. Functional clustering revealed novel and known Ni-associated clusters such as metal-binding and cytoskeletal organization. Moreover, confocal microscopy and functional FACS analysis confirmed distinct Ni-specific differential protein regulation and clustering results.

Conclusion:
The proteomic identification and analysis of monocyte proteins, which are affected by the most common contact allergen Ni, is an important step in increasing the understanding of molecular mechanisms involved in the development and pathophysiology of human ACD. Furthermore, functional analyses indicate involvement of cellular signaling responses, which may support predictive assay development to identify allergen-specific versus non-allergen specific pathways.

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The Ying & Yang of p40 in psoriatic plaque formation in mice

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Cytokines are mediators as well as regulators of inflammatory immune responses. The myeloid cell derived cytokine IL-23 is a critical player in psoriatic pathogenesis. Monoclonal antibody directed against the common subunit p40, targeting both IL-12 and IL-23, is currently used therapeutically with impressive efficacy in treating psoriasis.

Recently, it was shown that Aldara™ cream, a drug used in the treatment of basal cell carcinoma, is a potent inducer of psoriatic plaque formation in mice and (inadvertently) human patients. Its mode of action in mice implicates TLR7 dependent induction of proinflammatory cytokines IL-17A, -17F and -22.

In accordance to the successful therapeutic regime in most human patients, anti-p40 mAbs treatment dramatically reduced psoriatic plaque formation in this mouse model, yet a mild degree of lesion formation still remained. Since anti-p40 mAbs blocks pathogenic IL-23 as well as IL-12, we hypothesised, a conflicting role of the two sister cytokines in psoriatic plaque formation.

While IL-23p19 mutant mice are resistant to induction of disease, we found that skin inflammation was highly increased in mice defective in IL-12 signalling (IL-12null or IL-12Rb2null). These more severe lesions exhibit an even more prominent IL-17 cytokine signature and a change in the composition of infiltrating innate lymphocytes. The effect was IL-12 specific as local injection of recombinant IL-12 during disease development reverted the alterations in IL-12null mice.

Taken together, our findings suggest a protective role of IL-12 in psoriatic inflammation and stress the need of specificity, when targeting the IL-23/IL-17 axis therapeutically.
MicroRNAs (miR) play substantial roles in T cell biology as depletion of the enzyme Dicer, that is indispensable for miRNA processing, results in massive functional T cell failures. However, whether multiple miRNAs or a single miRNA are responsible for this phenotype is still unclear. In this study, we aim to analyze the role of miR183 in CD4+ T cell function. For this purpose, we first cloned the miR183 precursor sequence into a retroviral expression vector. This vector expresses functional miR183 molecules as shown by luciferase assays. The resulting viral particles were used to obtain miR183 over-expression in CD4+CD25- T effector cells. Functional analysis of these retrovirally transduced T cells revealed no suppressive activity in vitro and no induction of the regulatory T cell-related transcription factor FoxP3 upon miR183 over-expression. However, we observed an increased proliferative activity of stimulated miR183 over-expressing T cells compared to control-vector transduced T cells in vitro. In line with these results we detected a more rapid up-regulation of the early activation marker CD69 and also an earlier drop of CD69 surface expression in the course of T cell re-stimulation in vitro. This phenotype might result from Egr-1 down-regulation, a known target of miR183, which mRNA has also been down-regulated in miR183 transduced T cells. These results suggest that miR183 has a stimulatory function during T cell activation resulting in increased T cell proliferation.
Impaired functionality of antiviral T cells in G-CSF mobilized stem cell donors: Implications for the selection of CTL donor

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Cellular therapies have become a powerful tool to complement impaired immune reconstitution in patients after hematopoietic stem cell transplantation (HSCT). Recent publications suggest that G-CSF-mobilized donors might be a source for antiviral T cells for adoptive therapy. As information on number and functionality of antiviral T cells is scarce, we aimed to (1) identify the immunomodulatory effects of G-CSF on antiviral T cells, (2) detect influence of the apheresis procedure and (3) discuss optimal time points to collect antiviral T cells for transfer.

We assessed (1) numbers of CMV-, EBV- and ADV-specific T cells using 14 HLA-matched multimers, (2) functionality by IFN-γ ELISpot in response to overlapping peptide pools, (3) immunophenotyping using bio-plex, Granzyme B ELISA and multicolour flow cytometry for T-effector and memory cell subsets in order to characterize the influence of G-CSF and enrichment procedure.

Absolute numbers of antiviral T cells detected after G-CSF stimulation and compared to G-CSF-untreated samples and in vitro expansion rate upon antigenic stimulation was not influenced. However, functionality as expressed by mean reduction in IFN-γ (75% in vivo, 40% in vitro) and Granzyme B secretion (32% in vitro) was significantly impaired in response to all tested antigens tested.

Our results suggest that antiviral T cells from mobilized stem cell grafts might not be the optimal source for adoptive therapies due to their functional impairment. For patients with early viral complications whose donors is still under the influence of G-CSF or those with seronegative donors, third party T cells might be an attractive alternative.
**Modulation of heme oxygenase (HO)-1 enzyme activity by metalloporphyrins affects the antiviral T-cell response**

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Adoptive transfer of virus-specific cytotoxic T cells (CTLs) can prevent reactivation of latent viruses such as cytomegalovirus (CMV) and Epstein-Barr virus (EBV) and have been demonstrated to lead to a sustainable and rapid reconstitution of antiviral immunity. *In vitro* stimulation and expansion of virus-specific T cells is required for clinical applications. In the present study we asked whether modulation of the immunomodulatory enzyme heme oxygenase-1 (HO-1), which is the inducible isoform of enzymatic heme degradation, might have effects on *in vitro* T cell activation. To this end we investigated whether (1) induction by cobalt-protoporphyrin (CoPP) or inhibition by tin-mesoporphyrin (SnMP) can affect expansion of virus-specific CD8\(^+\) T cells *in vitro*, (2) modified HO-1 activity modulates proliferation and functional activity, (3) *in vitro* application influences other cell populations within the group of PBMCs (DCs, Tregs, natural killer cells) mediating effects on proliferating T cells, (4) HO-1-modulated antigen-specific T cells would be suitable for the purpose of adoptive immunotherapy. Inhibition of HO-1 via SnMP in peptide-pulsed PBMCs results in a significant enhanced virus-specific CD8\(^+\) T-cell proliferation, whereas increase of HO-1 activity by CoPP did not impaired T-cell responses. Transcription and secretion of IFN-\(\gamma\) in response to viral peptides is up-regulated upon inhibiting HO-1. Inhibition of HO-1 has no effect on mDCs and NK cells, but depletion of Tregs and additional application of SnMP multiplies virus-specific T-cell frequencies. Taken together we found, that SnMP-treatment may be suitable for the purpose of adoptive T-cell therapy.
Peripheral prepositioning and local CXCL9 chemokine-mediated guidance orchestrate rapid memory CD8+ T cell responses in the lymph node

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After an infection, the immune system generates long-lived memory lymphocytes whose increased frequency and altered state of differentiation enhance host defense against reinfection. Recently, the spatial distribution of memory cells was found to contribute to their protective function. Effector memory CD8+ T cells reside in peripheral tissue sites of initial pathogen encounter, in apparent anticipation of reinfection. Here we show that within lymph nodes (LNs), memory CD8+ T cells were concentrated near peripheral entry portals of lymph-borne pathogens, promoting rapid engagement of infected sentinel macrophages. A feed-forward CXCL9-dependent circuit provided additional chemotactic cues that further increase local memory cell density. Memory CD8+ T cells also produced effector responses to local cytokine triggers, but their dynamic behavior differed from that seen after antigen recognition. These data reveal the distinct localization and dynamic behavior of naive versus memory T cells within LNs and how these differences contribute to host defense. Immunity. 2013 Mar 21;38(3):502-13.
T cells specific for CMV and HPV differ substantially in prevalence, phenotype and functionality after expansion for adoptive T cell transfer

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Background:
Adoptive T cell transfer (ACT) consists of in vitro expansion of patient-derived antigen-specific T cells and transfer of these cells back into respective cancer patients. The capacity of antigen-specific T cells to detect and eliminate even single residual tumour cells gives ACT a considerable advantage over less specific and sensitive conventional therapeutic modalities. Key to therapeutic success is the T cell expansion protocol, as anti-cancer functionality has to be maintained. Therefore, a reliable platform is needed for functional testing of in vitro expanded antigen-specific T cells. In this study, we established an expansion platform for CMV-specific T cells and tested whether this protocol can be applied for expansion of HPV-specific T cells.

Methods:
Peripheral blood mononuclear cells (PBMC) were isolated from healthy donors (for CMV) and head and neck cancer patients (for HPV) by density gradient centrifugation. Enrichment or deletion of specific cells from PBMC was performed by positive or negative selection of magnetically-labelled cells. We tested the effect of the activation method (antigen-loaded APC vs. soluble peptide), media (generic vs. specific T cell expansion medium) and supplemented cytokines (e.g., IL-2, IL-21, etc.) on prevalence, phenotype and functionality of T cells, respectively.

Results:
A reliable expansion protocol without requirement for IL-2 could be established for CMV. The method of antigen delivery did not influence expansion. Specific medium was significantly better in expanding CMV-specific T cells (3 times more efficient compared to generic and 11 times more efficient compared to immediate ex vivo isolation).
Applying the same expansion protocol to HPV-specific T cells showed striking differences, most notably the reduction of cell numbers. While the degree of reduction could be attenuated by IL-2, an expansion similar to that driven by CMV was not achieved yet.
The numbers of CD3+CD8+ T cells were increased for CMV, but were decreased in the HPV model. In the CMV-driven expansion the majority of T cells showed an effector memory T cell phenotype. HPV-driven expansion displayed a mixed phenotype primarily consisting of effector memory and effector memory RA T cells.
Antigen-specific functionality (tested by IFN-γ ELISpots) showed a great disparity, as HPV-expanded T cells did not secrete IFN-γ upon specific restimulation, unlike CMV-expanded T cells. These differences might result from expansion of an immunosuppressive cell subpopulation in the HPV model. Our initial studies suggest that by selectively deleting CD4 T cells these differences might be overcome.

Conclusions and outlook:
T cells specific for CMV and HPV show striking differences in their requirements for expansion as well as their prevalence, phenotype and functionality after expansion by similar protocols. Further studies need to establish a reliable and strong expansion protocol for HPV-specific T cells from cancer patients.
Human peripheral Vδ1+ γδ T cells can develop into αβ T cells

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Background: Thymic activity declines with adolescence, can be impaired by treatment with anti-cancer drugs, transplant preconditioning regimen or removed early in life, yet human individual develop and maintain an immune system with an almost normal T cell pool. T lymphocyte maturation independent of the thymus has been suggested for several extrathymic tissues such as liver, skin, lamina propria, peyer’s patches and tonsils and has been supported by the detection of markers specific for early T lymphocyte development in the thymus: namely RAG1/-2 and preTCRα at extrathymic sites. This suggests that pre-T cells are trafficking from the bone marrow or the thymus to other tissues to continue differentiation and selection. The peripheral T cell compartment in human can be divided in two large classes: αβ and γδ T cells. It is believed that they develop in the thymus from a common progenitor and that the choice between αβ and γδ T cell fate is the first lineage decision made by progenitors after they commit to the T-cell lineage.

Observation: Here we show that peripheral Vδ1+ CD4+ γδ T cells in an inflammatory environment can transdifferentiate into αβ T cells. Upon their extrathymic route of differentiation, that resembles well-characterized molecular program of thymic αβ lineage development, Vδ1+ T cells upregulate CD4+ coreceptor, transcribe RAG, preTCRα and express a particular Vβ chain on their cell surface. Simultaneously inflammation confers the controlled initiation of rearrangement in the TCRα locus thereby following precisely the spatial order of thymocyte development. Molecular and functional analysis reveals that the generated T-cell pools have a complex TCR repertoire and are functional.

Conclusion: Differentiation of Vδ1+ CD4+ T cells, identified on clonal and on bulk-culture level, into functional CD4+ or CD8+ αβ T cells via a Vδ1 TCR/αβ TCR and CD4+/CD8+ double positive state suggests that, Vδ1+ T-cells upon inflammatory stimuli, contribute to homeostasis with the induction of adaptive immune responses. Identifying an innate T cell as an αβ T-cell progenitor and showing that its developmental steps are thymus-independent but triggered by inflammatory stimuli is of utmost academic and clinical relevance and will deeply impact our understanding of immune responses in infection, malignancy and autoimmune processes.
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Notch controls generation and function of human effector CD8+ T cells

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The generation of effector CD8+ T-cells with lytic capacity is crucial for the control of tumor growth. Dendritic cells (DC) provide important signals to promote naive CD8+ T-cell priming as well as activation of effector T-cells. Here, we report that the Notch pathway has an important role in both these processes in human CD8+ T-cells. Activated moDC express the Notch ligands Jagged1 and Delta-like4, while naive CD8+ T-cells express Notch2. The role for Notch signaling in CD8+ T-cell priming was determined using an ex-vivo model system in which tumor-antigen-specific primary CD8+ T-cell responses were measured. Inhibition of Notch using either a g-secretase inhibitor or soluble Delta-like4-Fc during activation reduced expansion of antigen-specific CD8+ T-cells, which was mirrored by decreased frequencies of IFNγ-, TNFα- and GranzymeB-producing CD8+ T-cells. Moreover, we found that T-cells primed when Notch signaling was prevented are functionally low avidity T-cells. In addition, Notch partially regulates established CD8+ effector T-cell function. Activation-induced Notch signaling is needed for IFNγ release but not for cytolytic activity. These data indicate that Notch signaling controls human CD8+ T-cell priming and also influences effector T-cell functions. This may provide important information for designing new vaccination strategies for the treatment of cancer.
The transcription factor Interferon Regulatory Factor 4 is required for the generation of protective effector CD8\(^+\) T cells

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Robust cytotoxic CD8\(^+\) T cell response is important for immunity to intracellular pathogens. Here, we show that the transcription factor Interferon Regulatory Factor 4 (IRF4) is crucial for the protective CD8\(^+\) T-cell response to the intracellular bacterium *Listeria monocytogenes* (*Lm*). IRF4-deficient (*Ir4*\(^{-/-}\)) mice could not clear *Lm* infection and generated decreased numbers of *Lm*-specific CD8\(^+\) T cells with impaired effector phenotype and function. Transfer of wild-type CD8\(^+\) T cells into *Ir4*\(^{-/-}\) mice improved bacterial clearance suggesting an intrinsic defect of CD8\(^+\) T cells in *Ir4*\(^{-/-}\) mice. Following transfer into wild-type recipients, *Ir4*\(^{-/-}\) CD8\(^+\) T cells became activated and showed initial proliferation upon *Lm* infection. However, these cells could not sustain proliferation, produced reduced amounts of IFN-\(\gamma\) and TNF-\(\alpha\), and failed to acquire cytotoxic function. Forced IRF4 expression in *Ir4*\(^{-/-}\) CD8\(^+\) T cell restored the defect. During acute infection, *Ir4*\(^{-/-}\) CD8\(^+\) T cells demonstrated diminished expression of Blimp-1, Id3 and T-bet, transcription factors programming effector-cell generation. IRF4 was essential for expression of Blimp-1, suggesting that altered regulation of Blimp-1 contributes to the defects of *Ir4*\(^{-/-}\) CD8\(^+\) T cells. Despite increased levels of Eomes, Bcl6 and Id2, *Ir4*\(^{-/-}\) CD8\(^+\) T cells showed impaired memory-cell formation indicating additional functions for IRF4 in this process. As IRF4 governs B- and CD4\(^+\) T-cell differentiation, the identification of its decisive role in peripheral CD8\(^+\) T-cell differentiation, suggests a common regulatory function for IRF4 in adaptive lymphocytes fate decision.
Tc9 cells, a new subset of CD8+ T cells, support Th2-mediated airway inflammation

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Similar to T-helper (Th) cells, CD8+ T cells also differentiate into distinct subpopulations. However, the existence of IL-9-producing CD8+ T (Tc9) cells has not been elucidated so far. We show that murine CD8+ T cells activated in the presence of IL-4 plus TGF-b develop into transient IL-9 producers characterized by specific IFN-g and IL-10 expression patterns as well as by low cytotoxic function along with diminished expression of the cytotoxic T lymphocyte (CTL)-associated transcription factors T-bet and Eomesodermin. Similarly to the CD4+ counterpart, Tc9 cells required for their differentiation STAT6 and IRF4. Tc9 cells deficient for these master regulators displayed increased levels of Foxp3 that in turn suppressed IL-9 production. In an allergic airway disease model, Tc9 cells promoted the onset of airway inflammation, mediated by sub-pathogenic numbers of Th2 cells. This support was specific for Tc9 cells because CTLs failed to exert this function. We detected increased Tc9-frequency in the periphery in mice and humans with atopic dermatitis, a Th2-associated skin disease that often precedes asthma. Thus, our data point to the existence of Tc9 cells and to their supportive function in Th2-dependent airway inflammation, suggesting that these cells might be a therapeutic target in allergic disorders.
Sortilin controls the exocytic trafficking of interferon-γ and granzyme A in T cells


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Introduction:
Cytotoxic T lymphocytes (CTLs) play an important role during the elimination of virus-infected and tumorigenic cells. Their cytotoxic activity depends on the release of perforin and granzymes inducing target cells to undergo apoptosis. In addition, CTLs release cytokines like Interferon-γ (IFN-γ) that contribute to host defense in several ways. The release of these effector molecules occurs on two different routes. On the one hand, CTLs release the cytolytic agents perforin and granzymes via the regulated secretory pathway, a process that involves the storage in and release from secretory lysosomes. On the other hand, the release of IFN-γ depends on the constitutive secretory pathway. While the sorting receptors and transport molecules of IFN-γ are not known, considerable insight into mechanisms that allow transport and release of granzymes exists. The intracellular transport of granzymes from the Golgi complex to secretory lysosomes is believed to be carried out by the Cl-M6PR which exhibits structural and functional similarity to the Vps10p-receptor Sortilin, previously reported to be involved in neurotrophin sorting in neurons and in lipoprotein metabolism in hepatocytes.

Objectives:
In this study, we asked whether the sorting function of Sortilin has any immunoregulatory effect on the cytolytic competence in CTLs. We focused on a cell biological analysis to characterize individual components and stages within the secretory pathway of CTLs that regulate their activity and cytolytic efficiency. Herein, we addressed how a constant supply of the newly synthesized effector molecules granzymes A and IFN-γ is maintained.

Material & Methods:
To examine the function of Sortilin during an immune response, we analyzed the release of cytotoxic mediators and cytokines from purified CTLs and T helper type 1 (Th1) cells derived from a wild type and a Sortilin knockout mouse strain. Furthermore, we determined the cellular cytotoxicity of mixed lymphocyte reaction-stimulated CTLs against allogeneic target cells in vitro. Cell biological analysis of the secretory pathway in CTLs from wild type and Sortilin-deficient mice was performed. Using confocal microscopy, the trafficking pathway of granzyme A and IFN-γ was analyzed in more detail. Moreover, we performed in vivo studies to assess systemic consequences of Sortilin deletion with regard to adaptive immune responses in infection and autoimmunity.

Results:
Sortilin negatively regulates the release of Granzyme A. The enhanced Granzyme A release correlates with a lower VAMP7 availability and therefore tempers lymphocytes killing activity in vitro. Simultaneously, Sortilin is required for IFN-γ exit from the Golgi complex to early endosomes and promotes its secretion. The loss of Sortilin leads to a reduced secretion of IFN-γ in CTLs, Th1 and NK cells. Systemically, reduction of IFN-γ release is associated with enhanced liver damage and correlates with higher bacterial titers upon adenovirus and L. monocytogenes infection, respectively. Due to the reduced IFN-γ release, the disease course in inflammatory bowel disease is milder.

Conclusion:
Our results suggest that Sortilin modulates systemic immune responses through exocytic sorting of the immunological effector molecules granzyme A and IFN-γ.
The transcription factors Blimp-1 and c-Maf cooperatively control interleukin-10 expression by Th1 cells

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Secretion of the immunosuppressive cytokine interleukin(IL)-10 by effector T cells is an essential mechanism of self-limitation and containment of adaptive immune responses in a variety of infections. However, the transcriptional regulation of IL-10 expression particular in pro-inflammatory T helper(Th)1 cells is insufficiently understood. In order to identify transcription factors that control IL-10 expression in Th1 cells, we performed gene-expression profiling of IL-10 secreting cells. Here we report the crucial role for the transcriptional regulator Blimp-1 as a Th1 specific co-factor for c-Maf mediated IL-10 production, independent of the aryl hydrocarbon receptor (AhR) which acts as a c-Maf co-factor in T regulatory 1 cells and potentially in Th17 cells. Blimp-1 deficient Th1 cells fail to express IL-10 both in vitro and in vivo and mediate enhanced immunopathology and inflammation during T. gondii infection. Blimp-1 expression in Th1 cells is downstream of c-Maf and both factors bind to the Il10 locus and synergistically induce IL-10. Our data further support the idea that c-Maf is a central regulator of IL-10 production which relies on cell-type specific co-factors. We propose a dual model of transcriptional regulation of IL-10, in which c-Maf cooperates with Blimp-1, when IL-10 is induced under strong pro-inflammatory conditions, whereas IL-10 expression downstream of TGF-b relies on c-Maf/AhR.

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**figure 1**

**figure 2**
Liver-Primed Memory T Cells Generated under Noninflammatory Conditions Provide Anti-infectious Immunity


Development of CD8+ T cell (CTL) immunity or tolerance is linked to the conditions during T cell-priming. Professional antigen-presenting cells such as dendritic cells (DC) matured during inflammation generate effector/memory T cells whereas immature DC cause T cell deletion or anergy. We identify a third outcome of T cell-priming in absence of inflammation enabled by cross-presenting liver sinusoidal endothelial cells. Such priming generated memory T cells that were spared from deletion by immature DC. Similar to central memory T cells, liver-primed memory T cells differentiated into effector CTLs upon antigen re-encounter on matured DC even after prolonged absence of antigen. Their re-activation required combinatorial signaling through the TCR, CD28 and IL-12R and controlled bacterial and viral infections. Gene expression profiling identified liver-primed memory T cells as a distinct Neuropilin-1+ memory population. Generation of liver-primed memory T cells may prevent pathogens that avoid DC-maturation by innate immune-escape from also escaping adaptive immunity through attrition of the T cell-repertoire.
Cytokine expression level is a stable feature of differentiated T helper cells

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A major effector function of CD4⁺ T helper (Th) cells is the secretion of selected sets of cytokines. Upon stimulation, a certain percentage of antigen-experienced T cells within a population produces and secretes different types of cytokines. Up to now, insight into the kinetics of cytokine production by individual T cells is lacking. It is a long-standing question whether individual cells produce cytokines for extended time periods of several hours or whether cytokine production by a given T cell is a rather short-term phenomenon. In the latter case, continuous recruitment of reactivated Th cells into the pool of cytokine-producing cells could be envisaged, resulting in a constant replacement of former short-term active cytokine producers. To address this question, we combined sequentially two techniques for the identification of individual cytokine producers: First, we labeled IFN-γ- or IL-17-producing cells alive by cytokine secretion assay technology, followed by intracellular staining of the respective cytokine after different culture periods. Our findings indicate the presence of a stable population of cytokine-producing T cells within the pool of stimulated cells, and only a fraction of stimulated Th cells appears to switch on cytokine production later than the majority. Thus, a population of activated T cells can be divided into cytokine-producing and non-producing cells, with individual Th cells producing cytokines only with a certain probability and not necessarily in a synchronous manner. However, most cytokine producers can be continuously active for several hours during recall stimulation.

We also found the cells to be stable in their amount of cytokine expression during the course of a recall stimulation. Mathematical modeling indicated that the variability of cytokine expression amounts is due both to stochastic fluctuations in the rate of gene expression and more stable differences in expression capacity between individual cells. Our findings define the kinetics of cytokine production in Th cells at the single-cell level and demonstrate a stable cytokine production behavior throughout an antigenic challenge.
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**IL-12-mediated STAT4 signalling and T-cell receptor (TCR) signal strength cooperate in induction of CD40L in human and mouse CD8^+ T cells**

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CD40L is one of the key molecules bridging the activation of specific T cells and the maturation of professional and non-professional antigen-presenting cells (APCs) including B cells. Originally, CD4^+ T cells have been regarded as the major T-cell subset that expresses CD40L upon cognate activation. However, we could demonstrate that CD40L^+ CD8^+ T cells are generated in a wide range of human and murine immune responses and that CD40L^+ CD8^+ T cells could demonstrate helper functions like activation of APCs in vitro as well as in vivo. Furthermore we analysed which conditions during priming of CD8^+ T cells lead to the induction of CD40L on CD8^+ T cells. STAT4-mediated IL-12 signalling proved to be the major instructive cytokine signal to boost the ability of CD8^+ T cells to express CD40L in vitro and in vivo after immunisation with peptide loaded DCs. Furthermore, modulation of T-cell receptor (TCR) signalling strength using stimulation of OT1 CD8^+ T cells with SIINFEKL or altered peptide ligand loaded APCs revealed a correlation of TCR signalling strength during priming with CD40L induction in CD8^+ T cells in vitro as well as in vivo. The induction of CD40L in CD8^+ T cells regulated by IL-12 and TCR signalling may enable CD8^+ T cells to respond autonomously of CD4^+ T cells. Thus, we propose that under proinflammatory conditions a self-sustaining positive feedback loop could facilitate the efficient priming of T cells stimulated by high affinity peptide-displaying APCs.
Binary IL-2 expression of CD4+ T cells ensures IL-2 availability for its paracrine action *in vivo*

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IL-2 and IL-2 receptor expression have to be tightly balanced *in vivo* because they are key players of opposite immune functions, in fact immunity and tolerance. Using an adoptive transfer approach, cytokine secretion assays, and single cell analysis of transferred CD4⁺ T cells after immunization enabled us to quantify *ex vivo* IL-2 production and IL2Ra expression per cell. We demonstrated that the *in vivo*-induced amounts of IL-2 and IL2Ra in naive CD4⁺ T cells correlated with the amount of antigen used. However, IL-2 is expressed per cell in a binary (all-or-none fashion) and IL2Ra in a graded manner *in vivo*. We hypothesize that binary IL-2 expression might be important to ensure the paracrine action of IL-2 within the peripheral lymphatic organs, which is crucial for the expansion of both, effector and regulatory T cells, as well as differentiation of Th cells.
Higher sensitivity of restored human CD8 T-cells to virus- and tumor-derived antigens

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Research in human immunobiology is mainly based on working with peripheral blood mononuclear cells (PBMC). However, recent investigations have shown that circulating mononuclear cells are less sensitive to several T-cell activating agents in comparison to mononuclear cells located in lymphoid tissues that contain a high cell density of immune cells. Interestingly, the impaired responsiveness of circulating T-cells can be restored by a short-term preculture of PBMC at a high cell density (HDC PBMC).

Here we report that interferon-gamma ELISpot assays of HDC PBMC can be used as a sensitive in-vitro test for detection and characterization of antigen-specific CD8 T-cell responses in clinical and basic research. Recall responses to various virus- and tumor-derived antigens significantly increased in sensitivity if CD8 T-cells were allowed to interact with matured monocytes during a preculture of PBMC. Depletion of monocytes from PBMC before HDC completely abrogated the increased sensitivity of CD8 T-cells. Under defined long-term culture conditions, HDC even positively affected the responsiveness of expanded antigen-specific CD8 T-cells, whereas unspecific CD8 T-cells were unaffected.

Investigations on a molecular level showed significant differences in the RNA expression pattern of precultured CD8 T-cells compared to circulating CD8 T-cells. Changes in the gene expression of cell adhesion molecules, co-stimulatory molecules and the glycolysis might facilitate cell survival and frequent cell-cell interactions. This result supports the hypothesis that cell-cell interactions, especially between T-cells and matured monocytes, induce sub-threshold T-cell signals, which are responsible for an increase in sensitivity of T-cells towards antigens.
IL-17A deficiency is associated with Inflammatory Bowel Disease in children

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Background:
Over the last two decades, a significant increase of pediatric patients with Inflammatory Bowel Diseases (IBD), including ulcerative colitis (UC) and Crohn’s Disease (CD) was observed. In particular, the pediatric population of western industrialized countries appears to be affected.
Since the manifestation of IBD in childhood differs clinically from adult patients, research of children who develop IBD may enable treating the disease in an earlier stage. Moreover, the treatment can be adjusted to the particular needs of the affected children.

Methods:
To investigate childhood-onset IBD, we performed colonoscopy in a pediatric cohort with gastrointestinal disorders. RNA from Colon-biopsies of 50 patients, including 14 UC, 15 CD and 21 negative control patients without disease pattern of IBD was analyzed. Therefore, RNA was isolated, transcribed into cDNA and analyzed in real-time PCR-Arrays. Furthermore, epidemiological data including patient history, living environment and treatment with antibiotics were collected via questionnaires from all patients.

Results:
Our data from a pediatric cohort of IBD showed that Interferon regulating factor (IRF-) 4 was significantly induced in patients with UC but not MC. IRF-4, known for its positive impact on IL-17A, had no increasing effect on IL-17A. Moreover, the expression of anti-inflammatory cytokines like IL-10 and IL-22 were induced but their receptors were inhibited.

Conclusions:
In adult-onset IBD, TH1, TH2 and particularly TH17 cells contribute significantly to the course of disease. Our data from a pediatric cohort of IBD indicated that IL-17, the main TH17 cell specific cytokine was absent, suggesting that IL-17A could regulate/maintain intestinal immune homeostasis. Its absence in both forms of disease could be a significant trigger for IBD in children.
The adapter protein SLy1 (SH3 domain protein expressed in lymphocytes) is a member of a family of three highly homologous proteins. SLy1 contains two protein-protein interaction domains, namely an SH3 and an SAM domain, and is exclusively expressed in lymphocytes. It was shown that SLy1 is phosphorylated on Ser27 upon TCR stimulation, implicating a potential role in TCR signaling. The importance of SLy1 in T cell development was demonstrated in SLy1 knockout (KO) mice, which showed a massive reduction of lymphoid organ cell numbers compared to wildtype littermates. Especially thymocyte numbers were reduced by approximately 50 percent. To examine thymocyte development \textit{ex vivo}, we purified double negative (DN) thymocytes and seeded them onto OP9 cells, which express Delta-like 1 ligand. Analysis of thymocyte development revealed an impaired proliferation in SLy1 KO mice, as well as a reduced differentiation. A higher apoptosis in DN cells from SLy1 KO cells compared to wildtype was suggested to be the reason for reduced cell counts in thymus. However, the overexpression of Bcl2, an anti-apoptotic protein, could not restore the wildtype phenotype. Interestingly, we observed overall reduced proliferation in Bcl2 overexpressing mice, pointing out an anti-proliferative function for Bcl2. The Bcl2 expression analyses revealed higher Bcl2 levels in SLy1 KO DN thymocytes and mature T cells compared to wildtype, providing a potential mechanism behind impaired thymocyte proliferation and differentiation in SLy1 KO mice. In summary our data involve SLy1 in the regulation of early T cell development and reveals the anti-proliferative protein Bcl2 as a relevant mediator.
The role of transcription factors NFATc1 and NFATc2 in CTL function

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CD8+ T cells play a major role in killing virus-infected and transformed cells as they discriminate between self and non-self antigens. After development from naive CD8+ T cells to cytotoxic T lymphocytes (CTLs), these cells gain the potential to lyse target cells and to secrete effector cytokines.

The differentiation from naive CD8+ T cells towards CTL effector cells including later CTL function depends on TCR-mediated Ca2+ signals. The calcium sensitive phosphatase calcineurin controls NFAT (Nuclear factor of activated T cells) dephosphorylation and translocation into the nucleus.

Different NFAT family members have overlapping and distinct functions in various processes in the immune response. Here, we are investigating the role of NFATc1 and c2, the most prominent NFATc proteins in peripheral T cells, in CD8+ T cell physiology and function using NFAT deficient mice.

Several key functions of CTLs, like cytokine release upon (re)stimulation and effector cell expansion are directly regulated by NFAT factors. Our results show impaired CTL function - cytokine release, degranulation and target cell killing capacity - in NFATc1 deficient CD8+ T cells upon in vitro restimulation whereas NFATc2 deficiency alone does not affect CTL physiology. In co-culture with an allogeneic plasmacytoma cell line, we observed significantly decreased target cell killing capacity of NFATc1 deficient CTLs. One interesting finding shows that NFATc1 is not only involved in signal transduction to the nucleus but affects also the secretory pathway of lytic granules upon restimulation of effector cells.

To further understand the role of NFAT in CTL biology, we reinforce these results in a tumor mouse model in vivo. This will help to gain a better understanding of NFAT transcription factors to including the development of potential therapeutic strategies in tumor biology.
Human CD4+CD25+ regulatory T cells are highly sensitive to low-dose cyclophosphamide compared to CD4+ T helper cells and CD8+ cytotoxic T cells

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Cyclophosphamide is a widely used anticancer drug and immunosuppressive agent. The effects are dose dependent. Thus, high-dose cyclophosphamide leads to immunosuppression whereas low-dose cyclophosphamide results in an ameliorated immune response. The reason for this is unclear and it is important to understand these effects because immunostimulation is desired in cancer therapy whereas immunosuppression is often required in the treatment of inflammatory diseases that are related to autoimmune response. Since cyclophosphamide treatment lead to a depletion of regulatory T cells (Treg) in mice resulting in immunostimulation, we decided to analyse the sensitivity to cyclophosphamide of human Treg, which play an important role in controlling the immune system by inhibiting the T cell function, and compared them with other T cell populations. We isolated CD4+CD25+ regulatory T cells, CD4+ T helper cells (Th) and CD8+ cytotoxic T cells (Tcyt) from peripheral blood of healthy human volunteers by using magnetic bead coupled antibodies and determined the frequency of apoptosis by annexinV flow cytometry following in vitro treatment and cultivation. We show that Treg are more sensitive to low-dose mafosfamide, which is the activated form of cyclophosphamide, compared to Th and Tcyt. Mafosfamide treated Treg also showed a decrease in their suppressive capacity, which was tested in cocultivation experiments with stimulated Th. Maf is a genotoxicant that gives rise to interstrand crosslinks (ICL) by alkylating the N7-position of guanine. Treating Treg with mafosfamide resulted in a high level of ICL, which were repaired more slowly than in Th and Tcyt. During the repair of these DNA lesions DNA double-strand breaks (DSB) are formed that trigger phosphorylation of histone 2AX (forming γ-H2AX). Thus, the formation and reduction of γ-H2AX can be attributed to DNA repair processes. Mafosfamide treated Treg showed lower levels of γ-H2AX foci within the first 6 hours and a higher level after 24 hours compared to Th and Tcyt. Based on this data we hypothesize that Treg are impaired in the repair of DNA crosslinks induced by cyclophosphamide. Our data describe human Treg as sensitive to cyclophosphamide as a result of a DNA repair defect, which might cause Treg depletion and stimulation of the immune response in patients treated with low-dose cyclophosphamide.
Aggravation of liver damage in CEACAM1-deficient mice in the murine model of concanavalin A-induced liver injury

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Objectives:
The T cell mitogenic plant lectin concanavalin A (ConA) induces acute immune-mediated liver injury after a single intravenous injection into mice. Hallmarks of liver injury are increased plasma transaminase activities and the release of pro-inflammatory cytokines. The cellular adhesion molecule CEACAM1 is a member of the immunoglobulin superfamily with different isoforms that determine its signaling and regulatory capacities on epithelia, endothelia and leukocytes. CEACAM1 has been originally identified as an intercellular, homophilic adhesion molecule on hepatocytes. The CEACAM1 isoform with a long cytoplasmic domain contains an immune receptor tyrosine-based inhibition motif (ITIM) that is pivotal in for the negative regulation of leukocyte activation, more specifically, CEACAM1-long suppresses the activity of NK-cells, T cells and myeloid cells, such as granulocytes and monocytes/macrophages. This negative regulation is important in modulation of innate immunity in both infection and sterile inflammation. Thus, elucidation of CEACAM1-dependent regulatory mechanisms in the murine ConA model might be useful to evaluate novel therapeutic approaches in human liver disorders.

Material and Methods:
C57/BL6 wildtype and CEACAM1-deficient mice were injected with a sublethal dose of ConA (5mg/kg) or saline, respectively. Mice were sacrificed 24 hrs post ConA challenge. Subsequently, plasma transaminase activities and the expression of IL-2, IL-10, IL-6, IL-17, TNFalpha and IFNgamma were determined by ELISA and RT-PCR. To evaluate the relative distribution of CEACAM1+ and CEACAM1− T cell populations, FACS analyses of single cell suspensions from liver and spleen were performed.

Results:
ConA-induced liver damage was exacerbated in CEACAM1-deficient mice in contrast to wild type mice, evident by significant elevation of plasma transaminase activities and an exaggerated Th1-cytokine response. Interestingly, we observed a marked increase of CEACAM1-expression on CD4+ T cells, CD4+Foxp3+ regulatory T cells (Tregs) and well as CD8+ T cells after ConA treatment. Furthermore, we observed higher abundance of CD4+Foxp3+ regulatory T cells in both livers and spleens of naïve wild type mice in contrast to CEACAM1-deficient mice.

Conclusions:
Aggravation of liver damage in CEACAM1-deficient mice suggests an involvement of CEACAM1 in T cell mediated attenuation of liver damage during immune-mediated liver diseases. We suggest that CEACAM exerts its immune-modulatory function in the ConA model by regulating hepatic and splenic CD4+ and CD8+ T cell abundance and polarization. Further studies are under way to characterize the molecular basis for the immune modulatory role of CEACAM1+Th1 effector cells, CEACAM1+ Tregs, and CEACAM1+CD8+ T cells in ConA-mediated acute liver injury. Using this model, we will be able to describe the functional role of T cell activation and the immunosuppressive capacity of CEACAM1+ Tregs following a Th1-polarized immune response yielding liver protection.
Impact of the prosurvival Bcl-2 family member A1 on T cell immunity

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Introduction:
Apoptosis is a key mechanism to prevent the development of autoimmunity and cancer. It is induced either by death receptor ligation on the cell surface (extrinsic pathway) or by stress factors involving proteins of the Bcl-2 protein family (intrinsic pathway). A1/Bcl2A1 is an anti-apoptotic member of the Bcl-2 family that is mainly expressed in the hematopoietic system. Altered expression of A1 has been reported in context with autoimmune disorders as well as in different cancers in humans. A1 has been shown to be involved in myeloid differentiation and lymphocyte development as well as in and activation of mast cells and macrophages during inflammatory events. In T cells A1 is thought to play a role during early T cell development and after TCR ligation upon activation. However, since no antibodies and knock-out models are available for the analysis of A1 function are available, the physiological function of A1 remains to be clarified.

Objectives: Due to quadruplication in the mouse genome generation of A1 knockout mice is not feasible. Therefore, our laboratory has generated a mouse model that targets all functional A1 isoforms using an RNAi approach leading to a stable A1 knockdown in the hematopoietic system. Using this approach we are able to investigate the impact of diminished A1 expression on T cell maturation, differentiation and effector function.

Materials & methods:
Different T cell subsets were isolated from wild type mice and analyzed for A1 mRNA expression by qRT-PCR. The impact of A1 knockdown on T cell development was investigated in vivo by T cell subset distribution analysis, by using in vitro T cell development assays (OP9-DL1 differentiation system as well as by using TCR-transgenic mice in which a mi-shRNA targeting A1 was expressed under control of the Vav-gene promoter. Furthermore, we analyzed the abundance of naive, memory and Treg cells in the thymus and spleen and used Experimental Autoimmune Encephalomyelitis (EAE) as an in vivo model to study the role of A1 in T cell mediated autoimmunity.

Results:
We confirmed strong A1 mRNA induction in T cells upon TCR-ligation and T cell activation. Additionally, A1-mRNA levels were found elevated in memory T cells when compared to naive T cells. Although no impaired T cell development and T cell distribution pattern were observed in A1-knockdown mice under steady state conditions, we found a delayed onset of EAE, indicating an involvement of A1 in the development of this form of autoimmunity.

Conclusion:
Diminished expression of A1 does not seem to grossly impair T cell development and T cell subset distribution under steady state conditions. This may be due to insufficient knockdown efficiency or counter-selection phenomena in our model system. However, we observed a delayed development of EAE in A1 knockdown mice compared to control mice or mice expressing a mi-shRNA targeting firefly luciferase. This strongly points towards an involvement of A1 in inflammatory responses that we are currently investigating in more detail.
A new pathogenic CD4+ T cell subset in Patients with Rheumatoid Arthritis is characterized by TNFR1 Expression

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Introduction:
The infiltration of the synovial membrane by immune cells, in particular CD4+ T cells, is one hallmark of Rheumatoid Arthritis (RA). The underlying pathology leading to the massive increase of CD4+ T cell migration to and retention in the rheumatoid synovial membrane is unclear.

Objectives:
Aim of the study was the identification of a surface marker which allows discrimination of tissue infiltrating CD4+ T cells from non-infiltrating CD4+ T cells. Therefore a novel in vitro migration assay was developed which allows the characterization of synovial tissue infiltrating cells.

Methods:
Vital tissue sections from rheumatoid synovium were generated using a horizontally oscillating microtom, and were co-incubated with fluorescence-labelled CD4+ T cells. Migrating T cells were recovered from the tissue using enzymatic digestion and phenotypically analyzed by FACS. Peripheral TNFR1+ CD4+ T cells from RA patients were characterized by flow cytometry. Therefor the expression of naive and memory T cell marker (CD45RA and CD45RO), marker for T cell activation (CD25, CD71 and CD154) and expression of ICAM-1 were determined. To identify the T helper cell signature of TNFR1+ CD4+ T cells intracellular staining of the Th1, Th2 and Th17 master transcription factors T-bet, GATA-3 and ROR-γt, respectively, was accomplished.

Results:
It has been shown previously that infiltrating CD4+ T cells differ from non-infiltrating ones in their increased expression of TNFR1. Furthermore, TNFR1 is expressed on a fraction of circulating CD4+ T cells from RA patients, but not from healthy controls. Peripheral TNFR1+ CD4+ T cells of RA patients have neither a preferential naive nor a memory phenotype, but showed increased expression of the activation marker CD25, CD71 and CD154 compared to TNFR1- CD4+ T cells. TNFR1+ CD4+ T cells of the peripheral blood express higher frequencies of T-bet and ROR-γt than TNFR1- CD4+ T cells. There is no difference in GATA-3 expression between TNFR1 positive and negative CD4+ T cells. Functionally, it has been shown that the cytokine TNF acts as chemokine to attract CD4+ T cells to the rheumatoid joints. Beside this direct effect of TNF, there are known indirect effects of TNF including the upregulation of cell adhesion molecules like ICAM-1. Therefore, ICAM-1 expression of migrating TNFR1+ T cells was investigated. The results show, that migrating TNFR1+ T cells recovered from synovial tissue are more frequently ICAM-1 positive than non-migrating ones.

Conclusion:
TNFR1+ discriminates CD4+ T cells with a synovial tissue infiltrating phenotyp. The pathogenic infiltration is ICAM-1 dependent. Taken together the results show that the TNFR1 expression characterizes a pathogenic subset of activated CD4+ T cells with Th1 and/or Th17 signature in patients with Rheumatoid Arthritis.
A potent and highly selective inverse agonist for RORγt suppresses Th17 differentiation and shows benefit in autoimmune therapy

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Introduction:
CD4+ T helper cells sustain the immune reaction by releasing cell type specific cytokines with variant functions. One of these subsets are Th17 cells, which protect against extracellular bacteria and fungal infections particularly at epithelial and mucosal barriers, through their production of IL-17A, IL-17F and IL-22. However, excessive amounts of these cells are thought to play a key role in several autoimmune diseases. The nuclear receptor RORγt acts as the major transcription factor for Th17 differentiation and promotes proliferation and maintenance of this T cell lineage and directly induces the production of Th17 marker cytokines. Therefore, inhibition of RORγt should be of therapeutic benefit for treatment of autoimmune diseases.

Objectives:
To identify an inverse agonist of RORγt with minimal toxicity, which specifically inhibits Th17 differentiation with therapeutic potency in Th17 dependent pathology.

Methods:
After compound library screening we developed different series of potent and selective RORγt small molecule inhibitors. Compounds were tested on purified CD4+ cells, which were cultured under Th17 or under Th1 conditions. Inhibition of Th17 differentiation was quantified through flow cytometry or by ELISA. In vivo efficacy of PX22554 was evaluated in the mouse model of imiquimod (IMQ) induced psoriasis. IMQ is a TLR7 ligand and a potent immune activator, which induces a dermatitis closely resembling human psoriasis, a chronic inflammatory skin disorder, critically dependent on the IL-23/IL-17 axis. The RORγt inhibitor was orally administered and the mice were scored daily for the severity of the disease and analyzed at peak of disease by histology, flow cytometry and quantitative real-time PCR on different organs.

Results:
We report here the properties of the RORγt inhibitor PX22554. We present experiments testing its effects on the differentiation of Th17 and other T cell lineages in vitro. We found that PX22554 specifically inhibited Th17 differentiation. We detected less IL-17 production and also less differentiation into RORγt+ cells, without an effect on viability and without a shift to other T cell subtypes. Application of PX22554 in the psoriasis model strongly reduced the severity of the disease. This was reflected in less thickness, redness and scaling of the skin, less infiltration of proinflammatory cells and lower expression of disease associated genes including Th17 signature genes IL-17A, IL-17F and IL-22.

Conclusion:
We present here data using a small molecule RORγt inverse agonist, which specifically inhibits Th17 differentiation. Furthermore, we show that this inhibitor significantly reduces the severity of imiquimod induced psoriasis-like skin inflammation in mice. RORγt inhibitors potentially represent a future therapeutic option for Th17 driven human inflammatory and autoimmune diseases such as psoriasis, rheumatoid arthritis, inflammatory bowel disease and others.
CD8$^+$ cytotoxic T cells (CTL) are capable of releasing TNF and inducing apoptosis of infected hepatocytes after cross-presentation of viral antigen via liver sinusoidal endothelial cells (LSEC). This novel non-canonical CTL effector function accounts for about 40\% of the antiviral effector function. CD4$^+$ T cells in turn are reported to be necessary for an efficient licensing of dendritic cells and priming of CD8$^+$ T cells. But since especially T$_{h}1$ cells are a source of inflammatory cytokines like TNF, they could also be able to induce liver damage after viral infection. To investigate if CD4$^+$ T$_{h}1$ cells also possess a non-canonical effector function, we used a similar model. Mice were infected with a recombinant hepatotropic adenovirus, which primarily infects hepatocytes and expresses ovalbumin, followed by adoptive transfer of in vitro differentiated CD4$^+$ T cells. Protein fragments of ovalbumin can be presented to the OVA-specific H2-Ab1 (I-Ab)-restricted T helper cells. Preliminary findings so far support the ability of T$_{h}1$ differentiated cells to induce liver damage, whereas T$_{h}2$ differentiated cells, incapable of producing TNF, could not induce a comparable ALT elevation. This potential non-canonical effector function of T$_{h}1$ cells is furthermore dependent on the amount of transferred cells. Future experiments will also elucidate if other T helper cells with the potential to produce inflammatory cytokines like TNF, e.g. T$_{h}17$ cells, are also capable of inducing liver injury and possess an antiviral activity.
CD152 (CTLA-4) unleashes a Tc17 differentiation program with sustainability

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CD8⁺ T-cells that produce IL-17 (Tc17 cells) have been linked to host defense and anti-tumor immunity. Tc17 cells show low levels of IFN-γ and granzymeB expression resulting in diminished cytotoxicity. Upon adoptive transfer into mice infected with Listeria monocytogenes (LM) or with B16 melanoma, Tc17 cells convert partially towards Tc1 phenotype while retaining certain Tc17 characteristics such as increased persistence or survival. Nevertheless, the factors involved in Tc17 lineage plasticity and stability is not known yet. Studies have shown that Tc1 cells lacking Eomes and T-bet show Tc17 characteristics such as enhanced IL-17 and RORγT with reduced IFN-γ. This implies that Tc17 differentiation program is a default pathway in the absence of Tc1 differentiation program.

CTLA-4 (CD152), a glycoprotein of the Ig superfamily is one of the most effective players in the attenuation of T-cell effector responses. Blockade of CTLA-4 gives promising results for better clearance of LM and tumor rejection. Studies have shown that CTLA-4 selectively reduces production of IFN-γ, Granzyme B and Perforin by down-regulating Eomes, ultimately leading to diminished cytotoxicity of Tc1 cells. Even though the striking impact of CTLA-4 in Tc1 cells is well appreciated - its role in Tc17 cell differentiation is not known so far.

To address the issue of the role of CTLA-4 in differentiation of Tc17 cells we analyzed the differentiation program of CTLA-4⁺/⁻ and −/− CD8⁺ T cells under Tc17 condition and evaluated their quality in vitro and in vivo.

Naïve CTLA-4⁺/⁻ and −/− CD8⁺ cells were stimulated under Tc17 condition and analysed for expression of Tc17 characteristics like IL-17 and RORγT. mRNA levels of different Tc17 hallmarks were analysed by qPCR and Pepscan PepChip kinomics array was performed to identify phosphorylation levels of different proteins and the kinases involved in their phosphorylation. Characteristics of Tc17 cells were analysed upon adoptive transfer into mice infected with LM and B16 melanoma.

Our analysis revealed a reduced cell intrinsic ability of CTLA-4⁻/⁻ to differentiate into Tc17 cells after antigen-specific primary activation in vitro. Our results indicate that CTLA-4 controls Tc17 differentiation by sustaining the levels of IL-17 inducing factors like RORγT, IRF4, Runx1 and pSTAT3. Even though CTLA-4 did not have any impact on TNF-α production but altered its coproduction with IFN-γ or IL-17 upon recall response in vivo. CTLA-4⁻/⁻ Tc17 cells had a poorer quality in clearing LM infection or controlling tumor growth whereas CTLA-4⁺/⁻ Tc17 cells were nearly as effective as Tc1 cells.

Our results indicate that CTLA-4, which sustains Tc17 differentiation, might therefore be a novel target for Tc17 cells to convert into Tc1 like cells, with increased persistence associated with enhanced immunity in clearance of LM infection and controlling tumor growth.

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Thymic antigen presenting cells induce active demethylation of the foxp3 locus in developing regulatory T cells

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Stability of Foxp3 expression in regulatory T cells (Tregs) depends on DNA demethylation at the Treg-specific demethylated region (TSDR), a conserved CpG-rich region within the foxp3 locus. The TSDR is selectively demethylated in ex vivo Tregs purified from secondary lymphoid organs, but it is unclear at which stage of Treg development demethylation takes place. Here we show that TSDR demethylation is initiated in Tregs during their thymic development, continues as they mature, and is finalized after thymic egress. TSDR demethylation did not depend on cell division, and enrichment of 5-hydroxymethylcytosine within the TSDR in thymic Tregs implicated an active mechanism of DNA demethylation. Thymic antigen presenting cells (APCs) were superior to splenic APCs in the generation of stable Tregs with a demethylated TSDR in vitro, and ex vivo thymic Tregs, regardless of maturity, expressed inherently stable Foxp3, suggesting that commitment to a stable Treg lineage takes place during early stages of Treg development in the thymus and is supported by signals derived from thymic APCs.

figure 1
Generation of stable, alloantigen-specific Foxp3+ regulatory T cells

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Introduction:
CD4+Foxp3+ regulatory T cells (Tregs) are currently being investigated as a potential immunotherapy to prevent graft rejection, however their therapeutic use is still a concern due to stability issues. Our previous studies have revealed that the stability of Foxp3 expression in Tregs correlates with DNA demethylation at the Treg-specific demethylated region (TSDR). Recent in vitro co-culture experiments have displayed that TSDR demethylation in thymic Tregs is initiated only by thymic and not by peripheral antigen-presenting cells (APCs).

Objective:
Generation of stable alloantigen-specific Tregs and to understand the molecular mechanisms enabling stable Foxp3 expression in Tregs.

Materials and method:
Established in vitro co-culture assays to generate alloantigen-specific Tregs. These alloantigen-specific Tregs are currently being tested in an in vivo skin transplantation model for their functionality.

Results:
We could successfully generate stable alloantigen-specific Tregs displaying a demethylated TSDR. These in vitro generated allo-Tregs by different thymic APCs including thymic dendritic cells (DCs) and medullary thymic epithelial cells (mTECs), exhibited different cytokine profiles. The mTEC-induced allo-Tregs showed higher IFNg production while the DC-induced allo-Tregs showed higher IL-10 production.

Conclusion:
Stable alloantigen-specific Tregs could be generated with thymic APCs, displaying unique cytokine profiles. Hence the results obtained so far indicated the uniqueness of each APC in relation to not only their ability to initiate epigenetic modifications at the Foxp3 locus but also induce different types of allo-Tregs.

In order to further investigate the contribution of APCs in TSDR demethylation in Tregs, we have performed Treg induction cultures using conditioned media from thymic APCs. Furthermore, RNA microarrays will be performed on different APC subsets from the thymus and periphery. The identified factors will be exploited to better understand the remodeling of the Foxp3 locus in developing thymic Tregs using gene silencing or over-expression techniques. In the long-term perspective, our investigations might have a potential to facilitate the usage of alloantigen-specific Tregs in clinical transplantation therapy.

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Antigen-specific Tregs can induce tolerance in solid organ transplantation without further chronic immunosuppression

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Regulatory T cells (Tregs) play an important role in the induction of allospecific tolerance. However, tolerance in solid organ transplantation by mere transfer of Tregs has been difficult. Besides this, the stability of the differentiation phenotype of Tregs has recently been questioned.

We therefore aimed at generating large numbers of stable allospecific Tregs from naive T cells by retroviral transduction with Foxp3. These were tested in an immunogenic skin transplantation model.

We established a system of transduction of mouse T cells with ecotropic retroviruses expressing Foxp3 and Thy1.1 as a surface marker to follow up transduced T cells. Alloantigen-specific T cells were isolated and transduced with Foxp3. Alloantigen-specific Foxp3 T cells showed high expression for the Treg markers Foxp3, CTLA-4 and GITR. They could suppress a MLR in an alloantigen-specific manner and could be expanded in vitro while maintaining their Treg phenotype. These Tregs prevented skin graft rejection without the need for chronic immunosuppression and recipients showed allospecific tolerance. Alloantigen-specific Tregs were far more potent than polyspecific Tregs. Mechanisms of tolerance were graft specific homing, expansion and long-term persistence of Tregs within the graft. In fact, tolerance could be transferred with re-transplantation of the tolerant graft onto secondary recipients while third party grafts were readily rejected.

The results prove that large numbers of stable alloantigen-specific Tregs can be generated from a polyclonal repertoire of naive T cells. This is the first time that allotolerance was achieved in a non-lymphopenic transplant model using skin grafts in an immunogenic strain combination. Therefore, antigen-specific Tregs might have a huge therapeutic potential after solid organ transplantation.
Regulatory T cells inhibit allergen-induced gut inflammation in humanized mice

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Recently, we have developed a humanized mouse model of allergen-induced IgE-dependent gut inflammation in PBMC-engrafted immunodeficient mice. In the present study we investigated the role of regulatory T cells (Treg) in this model. Therefore, NOD- scid-γc−/− mice were injected intraperitoneally with human PBMC from allergic donors together with the respective allergen or with NaCl as control in the presence or absence of different concentrations of CD4+CD25+FoxP3+ Treg of the same donor. After an additional allergen boost one week later, mice were challenged with the allergen rectally on day 21 and gut inflammation was monitored by a high resolution video mini-endoscopic system. Allergen-specific human IgE in mouse sera, which was only detectable in PBMC plus allergen-treated mice, was strongly inhibited by co-injection of Treg at a ratio of at least 1:10. The presence of Treg also reduced allergen-specific proliferation and cytokine production of human CD4+ T cells recovered from spleens at the end of the experiment. Furthermore, the allergen-induced endoscopic score evaluating translucency, granularity, fibrin production, vascularity, and stool after rectal allergen challenge was significantly decreased by Treg. Activation of Treg prior to injection further increased all inhibitory effects. These results demonstrate that allergen-specific gut inflammation in human PBMC-engrafted mice can be avoided enhancing the numbers of autologous Treg in these mice which is of great interest for therapeutic intervention of allergic diseases in the intestine.
Epicutaneous and oral tolerance protects from Th1/Th17-mediated colitis

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Inflammatory bowel diseases in humans like Crohn’s disease are complex chronic autoimmune disorders, which affect the entire gastrointestinal tract and each intestinal layer. The mouse model of trinitrobenzene sulfonic acid (TNBS)-induced Th1/Th17-mediated colitis resembles human Crohn’s disease and, hence, is suitable for investigations of cellular and immunological mechanisms. Previously, we have demonstrated that a contact hypersensitivity reaction (CHS), a CD8+ Tc1-mediated skin inflammation, is prevented through induction of a low zone tolerance (LZT) by applications of subimmunogenic amounts of haptens (e.g. TNCB) prior to sensitization. In the current study, we have analyzed the impact of orally and epicutaneously induced LZT on the outcome of the TNBS-induced colitis. Notably, we found that repeated applications of oral as well as of epicutaneous subimmunogenic doses of the hapten TNCB affected the course of the TNBS-induced colitis as demonstrated by significantly reduced inflammation of the gut in vivo. These results were evaluated by use of mini-endoscopy (in vivo) to assess a panel of inflammatory parameters (vascularity, granularity, translucency of the gut wall, fibrin, consistency of stool) and histology (decreased infiltration of inflammatory cells, vessel density, colon wall thickness, loss of goblet cells). In addition, a diminished hapten-specific T cell-proliferation and decreased Th1/Th17-cytokine production (IFN-γ, IL-2, IL-17) were observed in vitro after both, epicutaneous and oral tolerization, indicating an inhibition of the Th1/Th17-mediated colitis by LZT. Furthermore, we put into question the role and function of CD4+CD25+ regulatory T cells (Tregs) in LZT inhibition of colitis. Here, mice were treated with an anti-CD25 mAb for Treg depletion prior to oral or epicutaneous tolerization and subsequent colitis induction. In the absence of CD4+CD25+ Tregs, a pronounced colitis response was observed, indicating a failed LZT development. These results were determined by a significantly increased colitis score and strong hapten-specific T cell responses (vigorou T cell proliferation, Th1/Th17-cytokine pattern) as compared to control tolerized colitis mice, indicating a pivotal role for Tregs in colitis prevention by LZT. In summary, this study demonstrates that LZT to haptens mediated by CD4+CD25+ Tregs results in an abrogation of a CD4+ Th1/Th17-mediated colitis and is independent of the site of tolerance induction.
Mechanisms leading to Treg homeostasis in mice - a combined \textit{in vivo} and \textit{in silico} approach

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Introduction:
Regulatory T cells (Tregs) are a specialized immunosuppressive cell population that is essential for maintenance of immune homeostasis and prevention of autoimmunity.

Material and Methods:
In order to investigate the mechanisms contributing to Treg homeostasis in mice, we made use of targeted depletion of Tregs and monitored the kinetics of Treg replenishment and naive CD4$^+$ T cell (Tnaive) activation. These high-resolution data were applied for the development of the first multi-organ mathematical model for T cell homeostasis and recirculation in mice.

Results:
The quantitative analysis revealed organ-specific differences between peripheral lymph nodes and spleen. We provide evidence that, upon depletion, Treg proliferation alone cannot account for Treg replenishment, which has to be supported by \textit{de novo} conversion of Tnaives to Tregs as well as by Treg-dependent changes in trans-organ migration of Tnaives. The quality of the data allowed an estimation of the relevance of Treg peripheral conversion in steady-state. Conversion was found to play a relevant role for Treg homeostasis in peripheral lymph nodes but only to a small extent in spleen.

Conclusions:
Besides of giving the first estimation of the conversion rate of Tnaives to Tregs \textit{in vivo} under steady state conditions, this study suggests new aspects of immune regulation by Tregs which might have not been noticed before. Applying such an combined \textit{in vivo} and \textit{in silico} approach to models of infection, organ transplantation or cancer, for example, will further improve our understanding of Treg biology.
Mechanism of Tolerance induced by T Cells

Antigen-targeting to DEC-205 leads to diminished intestinal inflammation by affecting migratory properties of Th1 T cells

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Introduction:
Crohn’s Disease and Ulcerative Colitis are the two major types of inflammatory bowel disease in humans. The loss of tolerance towards commensal gut bacteria seems to be an initial event for the manifestation of disease. Apart from anti-inflammatory drugs there are no causative therapies available for IBD making research in this field essential. A promising approach for the prevention of IBD is the induction of tolerance in the gut. Antigen-targeting to DEC-205 expressed on the surface of dendritic cells was reported to induce tolerance in different experimental disease settings. However, whether this approach is sufficient to also induce tolerance in the gut is currently unknown. Therefore, we evaluated the potential of antigen-targeting to DEC-205 for the induction of tolerance in the gut.

Methods:
To study the effect of DEC-205 antigen-targeting in a CD4+ T cell dependent manner a MHC class II restricted epitope of the model antigen hemagglutinin (HA110-120) was cross-linked to an α-DEC-205 antibody (DEC-HA) and used for in vivo applications. Furthermore, we established a new antigen-specific mouse model for intestinal inflammation. In this model the adoptive transfer of HA-specific, Th1 polarized CD4+ T cells into transgenic VILLIN-HA mice expressing the HA protein under the gut-specific VILLIN promotor leads to severe inflammation in the intestine.

To determine the potential of DEC-205 mediated antigen-targeting in this mouse model, VILLIN-HA mice were treated with DEC-HA or control compounds followed by adoptive transfer of HA-specific, Th1 polarized CD4+ T cells. Mice were observed for five days, sacrificed and analyzed for clinical and immunological parameters, such as weight loss, histopathology and expression of pro-inflammatory cytokines in the gut.

For the evaluation of DEC-HA mediated mechanisms of tolerance induction gene expression profiling of re-isolated, HA-specific CD4+ Th1 T cells from VILLIN-HA mice was performed using Gene Array analysis. Furthermore, migratory properties of DEC-HA treated Th1 T cells were analysed in transwell migration assays.

Results:
We demonstrate that antigen-targeting to DEC-205 under steady state conditions leads to a significant abrogation of inflammation in the intestine of DEC-HA treated mice. This is represented by the reduction of weight loss, histopathology and secretion of pro-inflammatory cytokines like IFN-γ and IL1-β in the gut. Gene expression profiling of re-isolated HA-specific CD4+ T cells revealed differential regulation of genes responsible for T cell migration in adoptively transferred Th1 cells treated with DEC-HA. In chemotaxis assays we could demonstrate that Th1 polarized, DEC-HA re-stimulated cells show diminished migration towards different chemokines.

Conclusion:
Antigen-targeting to DEC-205 represents a potential tool for the induction of tolerance in the gut. Less severe inflammation in an antigen-specific model of intestinal inflammation was achieved by the interference of migratory properties of antigen-specific, Th1 polarized CD4+ T cells.
Regulatory FoxP3+ CD4+ T cells (Tregs) are essential for immune homeostasis and mice that lack Tregs develop spontaneous and fatal autoimmunity. The mechanisms of Treg mediated suppression as well as their target cells are diverse and include both cell-contact dependent and independent suppression. Inhibition of Dendritic cells (DCs) plays a key role for Treg mediated suppression since DCs are central in regulating the adaptive immune response. We have recently shown that DCs in the immunological steady state need to be constantly suppressed by regulatory T cells to maintain the tolerizing capacity of the DC. Unsuppressed DCs prime autoreactive T cells resulting in severe autoimmunity rather than inducing peripheral T cell tolerance. However, the mechanisms and molecules involved in Treg mediated suppression of steady state DCs are not fully understood. We have established a model system that allows us to investigate the mechanisms that are critical for DC suppression in vivo without disturbing the immunological steady state. To this end, we have generated bone marrow chimeric mice, in which we can induce antigen expression selectively on DCs while having deficiencies for various molecules in the Treg compartment. Using this model we demonstrate that suppression of DC function in the steady state involves cell-contact dependent mechanisms and molecules such as LFA-1, CTLA-4 and LAG-3 but is independent of the anti-inflammatory cytokine IL-10 and cytotoxic activity of Tregs.
Impaired IL-6 production by T cells of multiple sclerosis patients correlates with their insensitivity to regulatory T cell function


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T cell-dependent autoimmune diseases are either caused by dysfunctional regulatory T cells (Treg) or autoaggressive T effector cells (Teff) that are inefficiently controlled by the immune system. Here we demonstrate that Teff of multiple sclerosis (MS) patients are unresponsive to Treg-mediated suppression caused by their accelerated IL-6 synthesis, increased IL-6R expression and enhanced phosphorylation of protein kinase B (PKB)/c-Akt. IL-6 when present at early processes of Teff activation rendering respective T cells insensitive to Treg control. Blockade of IL-6R signaling or direct inhibition of PKB/c-Akt phosphorylation restored Treg susceptibility of these Teff. Early IL-6 synthesis especially by activated CD8+ Teffector cells from MS patients also conveyed Treg insensitivity to surrounding T cells, a process we described as “bystander resistance”. IL-6 itself triggers a positive feedback loop resulting in accelerated IL-6 production. To confirm these observations in vivo we used a humanized experimental autoimmune encephalomyelitis (EAE) mouse model. After immunization with MOG protein, human CD34+ stem cell engrafted NOD/Scid/gc- mice developed EAE symptoms associated with neuroinflammation and IL-17-producing Teff. Furthermore, sick mice showed similar IL-6-mediated Treg unresponsiveness of Teff as observed before in MS patients, demonstrating the impact of IL-6 as a modifier of early Treg-T cell communication in autoimmunity.
Treg-specific GARP expression is dependent on epigenetic modifications and Foxp3

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Background:
Glycoprotein A repetitions predominant (GARP) has been recently identified to be specifically expressed on regulatory T cells (Tregs) in response to activation. Diminution of GARP expression attenuates the suppressive capacity of Tregs, indicating its important role for Treg function. Here, we investigated in detail the molecular mechanisms underlying Treg-specific GARP transcription.

Methods:
Taqman gene expression assay was used to analyze GARP expression in purified human CD25+ and CD25- CD4 T cells. To delineate transcriptional mechanisms of GARP expression, human GARP promoter sequences and a 7.9 kb upstream CNS region were cloned into luciferase reporter vectors. Their transcriptional activity in response to different stimuli was assessed in mouse EL-4 or primary human CD25+ and CD25- CD4 T cells. To analyze the chromatin configuration state of the GARP promoter and the -7.9kb CNS region, chromatin immunoprecipitation was performed, and lysine acetylation of histone 3 (H3ac), trimethylation of histone 3 at lysine 4 (H3K4me3), and trimethylation of histone 3 at lysine 27 (H3K27me3) were assessed. DNA methylation levels were assessed by bisulfite sequencing. To confirm the role of DNA methylation for GARP transcription, the GARP promoter regions were cloned into a CpG-free luciferase vector, methylated by SssI methylase in vitro and analyzed by luciferase assay.

Results:
Two different transcript variants under the control of different promoters are encoded by the human GARP gene locus. In CD25+ Tregs, both transcript variants were expressed and upregulated upon TCR stimulation for 24 hours. Luciferase expression under the control of the GARP promoter was dependent on TCR stimulation and the presence of Forkhead box protein 3 (Foxp3). Addition of the NF-kB inhibitor, JSH-23, decreased transcription from both GARP promoters in a concentration dependent manner. Treatment with TGF-ß or retinoic acid failed to induce or to reduce the promoter activity. When inserted upstream of the GARP promoters, the -7.9 kb CNS region enhanced the luciferase activity significantly. Bisulfite sequencing of the GARP promoters revealed decreased DNA methylation in CD25+ Treg cells in comparison to CD25- T cells, B cells, and monocytes. DNA methylation was critical for transcriptional activity, as already partial methylation of the GARP promoter region dramatically decreased the activity. Moreover, histone modifications in both promoter regions and in the upstream CNS changed towards a more accessible chromatin configuration upon T cell activation in CD25+ Tregs.

Conclusion:
GARP expression in CD25+ CD4 T cells, but not in CD25- CD4 T cells, was initiated by coordinate action of TCR stimulation, Foxp3 and NF-kB from two promoters and was enhanced by the -7.9 kb CNS region, confirming Treg specificity of GARP expression. In addition, DNA methylation of the GARP promoter region limits the expression to CD25+ Treg cells and prevents its transcription in other cell types. The transcriptionally active status of the GARP gene in activated CD25+ Treg cells was supported by histone modifications. Thus, epigenetic modifications and the Treg-specific transcription factor, Foxp3, determine Treg-specific GARP expression.
The role of the Casein Kinase 2 in Treg-mediated control of Th2 response

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Introduction:
CD4⁺CD25⁺Foxp3⁺ regulatory T cells (Treg) display a subpopulation of T cells, with crucial regulatory features. Several animal models and patient studies have proven their suppressive function in the maintenance of peripheral tolerance. Impaired Treg function results in the development of tremendous autoimmune diseases like multiple sclerosis, diabetes type 1, arthritis as well as allergic diseases. Additionally Tregs are able to inhibit efficient anti-tumor-immunity. Hence, Tregs display a promising target for therapeutic intervention in several diseases.

Objectives:
Despite 20 years of research on Tregs the knowledge about the underlying molecular features and functions of these cells is limited and has to be greatly increased, in order to develop novel and innovative Treg-based therapeutic strategies. Albeit multiple analyses of the transcriptome of Tregs, little attention has been payed on distinctive signalling pathways cardinally employed by Tregs. To achieve this, we ran comparative kinome arrays with Tregs and conventional CD4⁺ T cells. Our analyses showed that the Casein Kinase 2 (CK2) is one of the most abundantly active kinases in Tregs upon T cell receptor-mediated activation. Hence, the aim of this project was, to characterize the role of the CK2 in Treg function.

Materials & Methods:
To investigate the Function of the CK2 in Treg-mediated suppression we used the CK2 inhibitor DMAT in classical in vitro proliferation assays. Furthermore we generated mice with conditional genetic ablation of the regulatory CK2 β subunit in Tregs. FACS analyses were used to characterize the phenotype of these animals. Additionally we performed asthma experiments to support our data in an in vivo model.

Results:
The Results of our kinome array identify the CK2 as one of the most abundantly active kinases in Tregs upon T cell receptor-mediated activation. Pharmacological inhibition of the CK2, by CK2 inhibitor DMAT, demonstrated a crucial contribution of this enzyme to the suppressive properties of Tregs (unpublished data). Apart from that, conditional genetic ablation of the regulatory CK2 β subunit underlined the importance of this kinase in Treg-mediated suppression under homeostatic conditions in vivo. Mice with CK2 β deficient Tregs showed moderate, spontaneous Th2 mediated immune response. Additionally mice with CK2 β deficient Tregs showed severely impaired ability to control Th2-mediated airway inflammation, in an asthma model in vivo. Independently, Foxp3 expression in CK2 β deficient Tregs was strongly diminished, indicating a role of this kinase in Foxp3 stabilisation.

Conclusion:
Taken together, our data identify the CK2 as an important kinase essentially involved in the molecular mechanisms underlying Treg-mediated suppression. In particular, we could show a crucial contribution of the CK2 in the Treg-based control of Th2-mediated immune responses.
Human regulatory T cells rapidly suppress T cell receptor-induced calcium, NF-κB, and NFAT signaling in conventional T cells

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Question:
CD4+CD25hiFoxp3+ regulatory T cells (Tregs) are critical mediators of self-tolerance, which is crucial for the prevention of autoimmune disease, but Tregs can also dampen antitumor immunity. Tregs inhibit the proliferation of CD4+CD25− conventional T cells (Tcons), as well as the ability of these cells to produce effector cytokines. However, the molecular mechanisms of contact-dependent rapid suppression of cytokine expression in Tcons by Tregs, as well as the signaling events in suppressed Tcons, remain largely unknown.

Methods:
To elucidate the molecular mechanisms of rapid Treg-mediated cytokine suppression in Tcons, we employed in vitro cocultures of primary human Tcons with HLA-A2-disparate primary, pre-activated human Tregs. Due to the short time period required to induce cytokine suppression, T cell receptor (TCR)-induced signaling events in Tcons might be directly influenced by Tregs. Therefore, TCR-induced signaling events in Tcons upon suppression by Tregs, compared to control Tcon:Tcon cocultures, were analyzed after short-term TCR and costimulation by Western Blot, Luminex, calcium measurements, inositol phosphate and signaling lipid analysis, Phosflow and Q-RT-PCR.

Results:
We show that human Tregs rapidly suppressed the release of calcium ions (Ca2+) from intracellular stores in response to TCR activation in Tcons. The inhibition of Ca2+ signaling resulted in decreased dephosphorylation, and thus decreased activation, of the transcription factor nuclear factor of activated T cells 1 (NFAT1) and reduced the activation of nuclear factor κB (NF-κB). In contrast, Ca2+-independent events in Tcons, such as TCR-proximal signaling and activation of activator protein 1 (AP-1), were not affected during coculture with Tregs. Despite suppressing intracellular Ca2+ mobilization, coculture with Tregs did not block the generation of inositol 1,4,5-trisphosphate (IP3) in TCR-stimulated Tcons. The Treg-induced suppression of the activity of NFAT and NF-κB and of the expression of IL-2 was reversed in Tcons by increasing the concentration of intracellular Ca2+, suggesting Ca2+ suppression to be a causative event for these Treg-mediated effects.

Conclusions:
Our results elucidate a previously unrecognized and rapid mechanism of Treg-mediated suppression. Ongoing and future studies aim at deciphering new molecules causative for this rapid suppression by next-generation proteomics studies, pathway analyses and computational data integration. This increased understanding of Treg function and Tcon resistance to suppression may be exploited to generate possible therapies for the treatment of autoimmune diseases and cancer.
Mechanism of Tolerance induced by T Cells

**FIGURE 1** | Mechanism of Treg-mediated Tcon suppression. Tregs have been described to suppress Tcons by different mechanisms, depending on the experimental setup, site and type of immune response. Tregs can generate immunosuppressive adenosine or transfer cAMP to Tcons. Tregs can rapidly suppress TCR-induced Ca\(^{2+}\), NFAT, and NF-κB signaling. Tregs can also produce immunosuppressive cytokines (IL-10, TGF-β, IL-35), and they can suppress by IL-2 consumption or induce effector cell death via granyma and perforin. Furthermore, Tregs can suppress Tcons indirectly by downregulating costimulatory molecules on APCs such as DCs via CTLA-4. Details are described in the text.

**FIGURE 2** | Tregs suppress particular TCR signaling pathways in Tcons. Activated Tregs rapidly suppress cytokine expression in Tcons via inhibition of Ca\(^{2+}\) signals and consequently reduced NF-κB and NFAT activation. Suppression by Tregs requires at least 30 min of coculture, is favored by cell contact and sustained after Treg removal.

Red indicates Treg-mediated suppression of TCR-induced activation in Tcons. Molecules displayed in green were not inhibited during Treg-mediated rapid suppression of cytokine transcription. Molecules in gray were not analyzed or TCR-induced activation was not detectable.
CD4+Foxp3+ regulatory memory T cells are the dominant population within the *Aspergillus fumigatus* specific T cell pool in human peripheral blood

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Question:
Despite chronic exposure to fungal pathogens immunocompetent individuals are usually fully protected from fungal infections without developing any signs of chronic inflammation. How the immune system achieves this balance is unclear.

Methods:
To better understand the contribution of T cells for protection against this bidirectional challenge we used two recently developed techniques to simultaneously identify antigen-specific regulatory and conventional T cells via a converse expression pattern of the activation markers CD154 and CD137 with ultra-high sensitivity, even at the level of naive T cells through magnetic pre-enrichment. We used these methods to characterize the full human naive, memory and regulatory CD4+ T cell repertoire directed against two major fungal pathogens, the airborne fungus *Aspergillus fumigatus* and the gastrointestinal/epithelial commensal *Candida albicans*.

Results:
We show that despite chronic fungus exposure, up to 80% of the *A. fumigatus*-reactive T cells are still in a fully naive state, whereas *C. albicans*-reactive cells have almost completely acquired a memory phenotype. Surprisingly, we identified *A. fumigatus*-reactive regulatory T cells (CD4+CD25+CD127-Foxp3+) to exceed conventional naive and memory T cells in number. Treg depletion strongly increases the frequency of *A. fumigatus*-reactive but not *C. albicans*-reactive T cells. *A. fumigatus* Tregs express Helios, display a memory phenotype, possess demethylated TSDR, do not produce cytokines and efficiently suppress *in vitro* T cell activation in a fully antigen-dependent fashion.

Conclusions:
Our data reveal fundamental differences in the physiologic T cell responses against *A. fumigatus* and *C. albicans*, i.e. Treg versus conventional memory formation, respectively. The capacity of the airborne fungus *A. fumigatus* to selectively generate Treg responses may protect against chronic stimulation and allergies but increases the risk for fatal *Aspergillus* spp. infections in immunocompromised individuals.
Mechanism of Tolerance induced by T Cells

Characterization of the role of hydroxyprostaglandin dehydrogenase in regulatory T cells

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Regulatory T cells (Treg cells) are indispensable for self-tolerance and immune homeostasis. The forkhead transcription factor FOXP3 is essential for Treg-cell development and function. We could identify hydroxyprostaglandin dehydrogenase (HPGD) as significantly higher expressed in human Treg cells compared to conventional CD4⁺CD25⁻ T cells. HPGD fulfills an important function in the metabolism of eicosanoids and prostaglandins and is one of the major prostaglandin E₂ (PGE₂) metabolizing enzymes. Notably, HPGD expression was specific for naturally occurring Treg cells (nTreg cells) as HPGD was not upregulated during CD4⁺ T-cell differentiation and even induced Treg cells. Furthermore, exclusively in nTreg cells, HPGD expression could be specifically modulated by Interleukin-2. Minuscule amounts of IL-2 were sufficient to strongly upregulate HPGD expression. Blockade of JAK3/STAT5 signaling inhibited HPGD upregulation in Treg cells. Moreover, the dependency of HPGD expression on IL-2-dependent signaling could be confirmed using reporter assays. Interestingly, FOXP3 ChIP experiments showed binding of FOXP3 to the HPGD promoter and loss of function experiments as well as reporter assays revealed that FOXP3 modulates rather than suppresses expression of HPGD. Furthermore, the dependency of HPGD expression on the extracellular microenvironment also indicates a tissue-specific expression and potential function of HPGD within Treg cells. Taken together, the present study demonstrates that HPGD represents a novel gene, which is specific for human nTreg cells. Although the relevance of HPGD for Treg-cell function remains to be elucidated, the present study indicates a tissue-specific role of HPGD in Treg cells and provides a basis for further research to determine the role of HPGD for Treg-cell function.
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Stable Foxp3 expression in regulatory T cells - transcriptional regulation and manipulation by natural compounds.

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Introduction:
Regulatory T cells (Tregs) are known as prominent players in the regulation of immune responses. Tregs obtain immunosuppressive capacity by the continuous expression of the transcription factor forkhead box protein 3 (Foxp3). We have previously reported that stable Foxp3 expression is tightly associated with epigenetic modifications in the CpG-rich Treg-specific demethylated region (TSDR) of the Foxp3 locus. Whereas a demethylated TSDR correlates with stable Foxp3 expression, cells that have a methylated TSDR only transiently express Foxp3.

Objectives:
Identification of signal transduction pathways as well as transcription factors which are critically involved in the induction and maintenance of Foxp3 expression.
Identification of substances that interfere with Foxp3 expression.

Methods:
Primary Tregs or RLM-11 cells were transfected with the reporter gene of interest. Cells were stimulated with PMA and PMA/Ionomycin when necessary and dual luciferase assays were performed. Currently, luciferase assays are being established to identify substances that can interfere with the expression of Foxp3.

Results:
We demonstrated that the methylation status of the TSDR controlled its transcriptional activity, but that TSDR activity did not require Treg-specific transcription factors. One of the four CpG motifs within the TSDR, which were found to be critical for full transcriptional activity, was part of a postulated NF-κB binding site. However, we could demonstrate that this site did not act as a NF-κB responsive element. Moreover, blocking the NF-κB signaling pathway did not critically influence TSDR enhancer activity indicating that the TSDR functions in a NF-κB-independent manner. In line with this, the NF-κB subunit c-Rel revealed to be dispensable for stable Foxp3 expression.

Conclusions:
We therefore propose that NF-κB signaling is not involved in the TSDR-mediated stabilization of Foxp3 expression. Currently, we are exploiting our knowledge about Foxp3 gene regulation by establishing luciferase reporter assays allowing the identification of small molecules and natural compounds that may interfere with the expression of Foxp3. Transient inhibition of Foxp3 expression has the potential to enhance the efficacy of vaccinations against tumors and infections.
Role of the NK cells in the genesis of angiotensin II-induced vascular dysfunction

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Hypertension is a common disease characterized by vasoconstriction, vascular inflammation and remodeling. Recruitment of immune cells into the vessel walls plays an important role in the development and maintenance of the disease. A role for NK cells in the pathology of vascular inflammation and dysfunction remained unknown. Here, we report that NK cells control the aortic recruitment and IL-12 production of CD11b⁺Gr-1low monocytes through the proinflammatory cytokine IFN-γ. IFN-γ⁻/⁻ mice and Tbx21⁻/⁻ mice showed reduced aortic NK cell infiltration and were largely protected from angiotensin II-induced vascular ROS production and vascular endothelial and smooth muscle dysfunction, whereas mice overexpressing IFN-γ showed constitutive vascular dysfunction. Thus, NK cells play a crucial role in hypertension-associated vascular dysfunction by mutual activation of vascular infiltrating monocytes. These findings disclose NK cells and the IFN-γ pathway as attractive new targets in the development of prospective therapeutic regimen in cardiovascular disease.
Distinct roles of NK cells in viral immunity during different phases of an acute retroviral infection

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Introduction:
In many virus infections natural killer (NK) cells are critical for the rapid containment of virus replication. Polymorphisms in NK cell receptors as well as viral escape from NK-cell responses are associated with pathogenesis and viral loads in HIV-infected individuals, emphasizing their importance in retroviral immunity. In contrast, NK cells of LCMV-infected mice dampened virus-specific T-cell responses resulting in impaired virus control. Thus, the exact role of NK cells during different phases of viral infections remains elusive.

Aim of the study:
In this study we wanted to characterize NK-cell responses at different time points. Thus, we analyzed the effector function of NK cells in the initial phase of FV infection (3 to 4 days post infection), where only innate immune cells are activated. In the medium phase of infection (7 to 15 days) FV infection is mainly controlled by T cells. In the later phase of acute FV infection (30 days post infection) mice susceptible to FV infection develop a lethal erythroleukemia. At this time mice harbor virus-infected as well as transformed cells which might be target cells for cytotoxic T or NK cells.

Methods:
We analyzed NK cell effector function of FV-infected or naïve mice via flow cytometer. To further analyze their role during acute FV infection we performed NK cell depletion experiments. In addition, NK cells were isolated to determine their cytotoxic potential in an in vitro cytotoxicity assay. Viral loads were observed by infectious center assays.

Results:
NK cell depletions during the initial phase of FV infection (3 to 4 days post infection) resulted in increased viral loads, which correlated with enhanced target cell killing and elevated NK cell effector functions. At days 7 to 15 post infection, NK cells did not contribute to anti-retroviral immunity. In the transition phase between acute and chronic infection (30 days post infection), NK cells exhibited an inhibitory role. Here, depletion of NK cells resulted in reduced viral loads and significantly improved FV-specific CD8⁺ T-cell responses.

Discussion:
Our results demonstrate an opposed activity of NK cells during retroviral infection. They were protective in the initial phase of infection, when adaptive T-cell responses were not yet detectable, but were dispensable for viral immunity after T cell expansion. At later time points they exhibited regulatory functions in inhibiting virus-specific CD8⁺ T-cell responses.
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NK Cell Activation in Visceral Leishmaniasis is Dependent on Neutrophils

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Introduction and objectives:
In experimental visceral leishmaniasis caused by the protozoan parasite *Leishmania (L.) infantum* natural killer (NK) cells get rapidly activated, which leads to expression of interferon (IFN)-γ and an increase in cytotoxicity. Full NK cell activity requires Toll-like receptor 9-triggered release of interleukin (IL)-12 by infected CD8+DEC205+ dendritic cells (DCs) as well as the presence of T cell-derived IL-2 and of IL-18. As interaction between neutrophils (PMNs) and NK cells was previously reported to contribute to NK cell maturation and functionality under homeostatic conditions, we tested whether PMNs play a role in NK cell activation in response to *Leishmania* parasites and how these two cell populations interact with each other during the infection.

Methods:
To investigate the effect of PMNs on NK cells, depleting antibodies were injected intraperitoneally in C57BL/6 mice 24 h prior to infection with *L. infantum*. Intracellular IFN-γ protein expression in splenic NK cells was determined ex vivo by FACS analysis 12 and 24 h p.i., whereas the NK cell cytolytic activity was measured by 51Cr release assay against tumor target cells. To analyse direct interactions between PMNs and NK cells, splenic sections from *L. infantum*-infected mice were stained for both cell types using confocal laser scanning fluorescence microscopy (CLSM). The role of different factors for the PMN-NK cell interaction was evaluated by RT-PCR gene expression, cell transfer experiments and the use of neutralizing antibodies and/or respective knock out mice.

Results:
Depletion of PMNs in *L. infantum*-infected C57BL/6 mice reduced the NK cell response by ca. 50%. CLSM revealed that PMNs accumulated in the splenic red pulp during the first hours of infection and showed direct cell contact to NK cells localized in the same area. PMNs neither produced NK cell-stimulating IL-12 in response to *L. infantum* alone nor did they modulate the release of IL-12 by DCs during infection. In addition, PMNs were not the source of IL-18 which we previously found to be necessary for full NK cell activity. As PMN-derived IFN-α/β inducible gene 15 (ISG15) was described to directly activate NK cells, we also analysed the role of ISG15. Although ISG15 expression was upregulated in sorted splenic PMNs of *L. infantum*-infected mice during the first day of infection, we observed no difference in the NK cell activity between WT and ISG15−/− mice.

Conclusions:
Following *L. infantum* infection PMNs are in close contact with NK cells thereby supporting the activation of NK cells. The NK cell-stimulating effect of PMNs does not result from their modulation of DC functions or from the release of IL-12, IL-18 or ISG15.
**MicroRNA Expression in Ex Vivo-Activated Human Peripheral Natural Killer Cells under Normoxia versus Hypoxia**

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**Introduction:**
Natural killer (NK) cells integrate cytokine-receptor signals, mediate cytotoxicity against infected and transformed cells, and thus operate in low oxygen environments. Transcriptome analysis suggests roles for microRNA regulation of NK cell survival and effector functions. Soluble interleukin 15 (IL-15) promptly promotes these functions in vitro and in vivo.

**Objectives:**
To detect short-term microRNA profile changes in mature NK cells exposed in vitro to IL-15 under normoxia versus hypoxia.

**Materials & Methods:**
Two male and two female healthy volunteers between 48 and 53 years of age were included in this study. Normal blood counts and leukocyte activation markers were ascertained by flow cytometry at six standardized samplings per donor over a period of 5 weeks. Peripheral untouched NK cells were isolated from each donor within this period by magnetic cell separation. Obtained CD56+/CD3- NK cells from single preparations were split into four cultures. Two cultures each were incubated for 22 h under normoxia and hypoxia (1% oxygen). For the last 6 h of this period, IL-15 or vehicle was added to one of the two cultures, respectively. Total RNA from these cultures was DNase treated, preamplified, and profiled by RT-PCR using low density arrays that assemble 755 small RNAs and include both endogenous and negative controls. Data were analyzed by the delta-delta CT method using U6 snRNA as endogenous control.

**Results:**
Upregulation of miR-27a-5p, a marker of IL-15 stimulation in NK cells, by IL-15 and upregulation of HIF1A induced miR-210 by low oxygen confirmed that cytokine treatment and hypoxia, respectively, elicited expected cellular responses. Among 115 putatively regulated small RNAs from this screen, 14 showed highly consistent responses across all four donors. Among these, the following 8 were changed on average more than 2-fold: Co-regulated oncogenic miR-221 and -222 were induced by IL-15. MiR-193b-3p was induced by IL-15 only under normoxia and miR 574-3p by hypoxia only in the presence of IL-15. Both these miRNAs had previously been assigned tumor suppressor functions. Reductions by IL-15 were found for miR-638 and 601 under normoxia and for tumor suppressor miR148b-5p under hypoxia. Hypoxia reduced oncogenic miR-590-3P in the presence of IL-15.

**Conclusion:**
We identified regulation of 8 miRNAs in mature NK cells that had so far only been investigated for solid tumors where they regulate cell proliferation, migration, and invasion. It remains to be determined whether they control similar functions in NK cells, and whether hypoxia modulates IL-15 induced changes in microRNA expression, which may play a role in impaired NK cell effector function in infection and in the tumor environment.
Surface expression of ‘activation-induced C-type lectin’ (AICL) tags cytokine-stimulated human NK cells

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Introduction:
The ‘activation-induced C-type lectin’ (AICL) is encoded in the Natural Killer Gene Complex (NKC) adjacent to its receptor, the activating NK cell receptor NKp80. AICL is expressed by myeloid cells and NKp80/AICL interaction was shown to promote the cross-talk between NK and myeloid cells.

Objectives:
Unexpectedly, we recently observed substantial AICL expression by human NK cell lines and subsequently investigated regulation of expression, cellular localization, and functional recognition of AICL by resting and activated human NK cells.

Materials and Methods:
AICL expression by NK cells was assessed using qRT-PCR, immunoblotting and flow cytometry. Confocal microscopy revealed subcellular localization of AICL in NK cells. Functional assays included cytokine and degranulation assays. Hybrids of AICL and KACL proteins, the latter being a close AICL relative, were generated to map domains involved in intracellular AICL retention in 293 transfectants.

Results:
Resting human NK cells contain intracellular stores of AICL glycoproteins associated with the Golgi complex. As evident from mutational analyses, the C-type lectin-like ectodomain of AICL determines intracellular AICL retention. However, upon exposure of freshly isolated NK cells to inflammatory cytokines, AICL expression is upregulated resulting in a substantial and prolonged cell surface expression. AICL on cytokine-stimulated NK cells is functionally recognized by resting autologous NK cells triggering their effector functions in an NKp80-dependent manner.

Conclusion:
Our results show that AICL glycoproteins predominantly localize in the Golgi complex. While resting NK cells are devoid of surface AICL, there is substantial AICL cell surface expression following exposure to inflammatory cytokines facilitating recognition by autologous NK cells via NKp80. Hence, cytokine-stimulated NK cells become tagged by AICL surface expression for recognition by bystander NK cells.
Murine listeriosis is ameliorated in the absence of NK cells and NKT cells

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NKR-P1B/C (NK1.1)+ cells have been considered to play a protective role in various bacterial infections, because they secrete large quantities of IFN-γ immediately after infection. Yet, evidence exists arguing against a protective role of NK1.1+ cells in some bacterial infections. Because NK1.1+ cells comprise two major populations, namely NK cells and NKT cells, it is possible that the discrepancy is caused by different contributions of each cell population to bacterial infections. To re-evaluate the relative contributions of these NK1.1+ cell populations to bacterial infection, we compared the susceptibility to Listeria monocytogenes infection of mice lacking NKT cells and/or NK cells with that of control mice. Jα18+/mice were more resistant to L. monocytogenes infection than Jα18−/mice. In-vivo depletion of total NK1.1+ cells enhanced resistance to L. monocytogenes infection not only of C57BL/6 and Jα18−/mice, but also of Jα18−/mice, RAG-1−/− and β2m−/− mice, despite the fact that serum levels of IFN-γ and frequencies of IFN-γ-secreting cells in the liver and spleen were diminished. Similarly, the resistance of C57BL/6 and Jα18−/mice to L. monocytogenes infection was enhanced by NK cell depletion. Inflammation in the liver and spleen following L. monocytogenes infection was also ameliorated by depletion of total NK1.1+ cells or NK cells. Numerical increase of Gr-1+ cells and CD8α+ cells was found in NK1.1+ cell-depleted mice immediately after L. monocytogenes infection, and depletion of Gr-1+ cells and to a lesser degree of CD8α+ cells exacerbated listeriosis in NK1.1+ cell-depleted mice. Our results not only indicate that both NK cells and NKT cells play a detrimental role in L. monocytogenes infection, but also suggest that IFN-γ secreted from these cell populations is negligible.
Immune Senescence and Immune activation of Natural Killer cells in HIV-1 infection after ART

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Objective:
HIV-1 infection is known to have a detrimental impact on natural killer (NK) cell phenotype and functions. In this study, we investigated the effect of antiretroviral therapy (ART) on NK cell subsets distribution, activation and senescence of NK cell subsets in a longitudinal as well as cross-sectional study.

Materials and Methods:
We analyzed NK cell subsets by multi colour flow cytometry from cryopreserved peripheral blood mononuclear cells in 35 untreated HIV-infected individuals, 25 treated HIV-infected individuals and 15 healthy subjects. 15 HIV-infected patients were longitudinally followed before and after one year of initiation of ART.

Results:
We found that frequencies as well as absolute counts of CD56bright NK cells in HIV+ blood donors were similar compared to uninfected controls. However, CD56bright NK cells exhibited higher activation states compared to uninfected controls (P<0.0001). Patients on ART had a substantial decrease in activated CD56bright NK cells as compared to untreated patients and activation of CD56bright NK cells were restored after one year of anti-retroviral treatment. CD56dim NK cells were decreased in untreated HIV-infected patients as compared to uninfected controls. Treated patients had higher frequencies of CD56dim NK cells as compared to untreated patients. The decrease in the frequencies of CD56dim NK cells in untreated patients was restored after ART and the frequencies of CD69+/CD56dim NK cells decreased after ART initiation (P<0.0001). Expression of immune senescence marker CD57 on CD56dim NK cells was similar before and after one year of ART. In addition, we observed an increase in the frequency of CD56+/CD16+ NK cells in untreated patients as compared to uninfected controls. Lower frequencies of CD56-/CD16+ NK cells were found in patients on ART compared to untreated patients. The increase in the percentage of CD56-/CD16+ NK cells in untreated patients was restored after ART longitudinally and the frequency of CD69+/CD56-/CD16+ NK cells decreased after ART (P<0.0001). However, CD56-/CD16+ NK cell show higher CD57 expression after one year of ART in a longitudinal study.

Conclusion:
Our data indicate that disturbed NK subsets restored after 1 year of ART and this restoration was associated with a decreased immune activation as indicated by CD69 expression. In contrast to CD56-/CD16+ NK subset, immune senescence of CD56dim NK cell subset was not affected by ART, as indicated by expression of CD57. (This study is supported by Hannover Biomedical Research School(HBRS), MHH, Hannover, Germany)
Proteomic analysis of distinct developmental stages of CD56<sup>pos</sup> NK cells

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**Background:**
NK cells realize an important part of the innate immune response and defense against viral infections and cancer. CD56<sup>bright</sup> and CD56<sup>dim</sup> NK cells constitute functionally distinct subsets. Former studies confirmed immune regulatory CD56<sup>bright</sup> NK cells as progenitors of cytotoxic CD56<sup>dim</sup> NK cells. Furthermore, recent studies differentiated CD57<sup>+</sup> and CD57<sup>-</sup> NK cells among the CD56<sup>dim</sup> NK cell population and suggested a fully mature developmental status for CD57<sup>+</sup> NK cells.

**Question:**
Which protein functions and cellular processes define different NK cell maturation steps?

**Methods:**
Distinct developmental stages of primary NK cells were isolated from blood of 10 healthy human donors by using FACS-based sorting. CD56<sup>bright</sup>, CD56<sup>dim</sup>, CD56<sup>dim</sup>CD57<sup>-</sup> and CD56<sup>dim</sup>CD57<sup>+</sup> NK cells (1x10E6) were comparatively analyzed by using quantitative peptide sequencing (iTRAQ<sup>TM</sup>-LC-MS/MS). Statistical evaluation revealed donor-independent and subset-specifically protein regulations. Selected novel candidates were functionally analyzed, including co-localization studies.

**Results:**
This proteomic study generated relative quantitative data for 3400 proteins, whereof 98% were identified in all four NK cell subsets, suggesting the presence of a CD56<sup>pos</sup> NK cell core proteome. Signaling pathway annotation allowed comprehensive insight into relevant cellular processes, such as cytoskeletal rearrangement, NK activation and cytotoxicity, as well as differentiation. Comparative analysis of CD56<sup>dim</sup> developing into CD56<sup>bright</sup> NK cells exhibited both more regulations and a higher dynamic range of regulation. In contrast, the terminal maturation step from CD56<sup>dim</sup>CD57<sup>-</sup> to CD56<sup>dim</sup>CD57<sup>+</sup> NK cells showed only a limited number of significant regulations. The list of significantly regulated proteins provides proof of concept data as well as so far un-described NK cell proteins, like S100A4 (Calvasculin). Together with Myosin Ila this protein is actively and time-dependently recruited and accumulates in highest intensity 15 minutes after activation at the NKIS in tumor cell-activated primary human NK cells. By using super-resolution imaging, especially dSTORM technologies this interaction was confirmed on the nano-molecular level. Further knock down experiments will clarify its contribution to the formation of the NK cell immune synapse and development of NK cell cytotoxicity in general.

**Conclusion:**
We provide here novel insights into developmental stages of NK cells at the proteome level (Scheiter et al., 2013). The data basically support the recent differentiation model. Significant regulations were observed in both steps of maturation. Whereas the comparative view on CD56<sup>dim</sup>CD57<sup>+</sup> NK cells and the involved processes of differentiation significantly extends our knowledge, the limited number of regulated proteins gives a clear advice for perspective NK-CD57<sup>+</sup>-research. Furthermore, functional analysis of novel NK cell proteins will help to find the missing links leading to NK cell cytotoxicity.
Critical role for miR-181a/b-1 in agonist selection of invariant NKT cells

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T cell receptor (TCR) signal strength determines selection and lineage fate at the CD4⁺CD8⁺ double positive (DP) stage of intrathymic T cell development. Members of the miR-181 family comprise the most abundantly expressed miRNA at this stage of T cell development. Here, we show that deletion of miR-181a/b-1 reduced the responsiveness of DP thymocytes to TCR signals and virtually abrogated early iNKT development, resulting in a dramatic reduction in iNKT cell numbers in thymus as well as in the periphery. Increased concentrations of agonist ligand rescued iNKT cell development in miR-181a/b-1⁻/⁻ mice. Our results define a critical role of miR-181a/b-1 in early iNKT cell development and show that miR-181a/b-1 sets a TCR signaling threshold for agonist selection.
High-throughput sequencing of the γδ T cell receptor repertoire


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γδ T cells are potentially able to express more unique T cell receptors (TCRs) than αβ T cells. However, knowledge about their TCR repertoire is very limited. Therefore, we established high-throughput sequencing to investigate the TCR diversity of γδ T cells.

1) We sorted Vγ4+ γδ T cells from peripheral lymph nodes and spleen of adult γδ T cell reporter mice (TcrdH2BeGFP). Then, we amplified abundant Tcrd chains such as Vδ5-Jδ1 from this cell pool, and analyzed complementarity determining region 3 (CDR3) sequences. We observed an invariant dominant subset of Vδ5’-Dδ2’-Jδ1’ γδ T cells. Our previous study showed that generation of IL-17-producing γδ T cells is restricted to embryonic development. Hence, we sorted Vγ4+ cells in two functional subsets of IL-17-producing and IFN-γ-producing cells according to specific surface markers, CCR6 and CD27, respectively. We found that the invariant dominant clone belongs to the IL-17-producing γδ T cells. Furthermore, we applied an inducible Rag1 knock-out mouse model (Indu-Rag1) crossed with TcrdH2BeGFP mouse. Using this model we showed that the dominant clone was not generated after induction of the Rag1 production in adult mice. Our results suggest that IL-17-producing γδ T cells possess a different TCR repertoire than IFN-γ-producing γδ T cells. In other words, functionally different subsets of γδ T cells generate different TCR repertoires.

2) We sorted Vγ7+ γδ T cells from the small intestinal epithelium of adult TcrdH2BeGFP mice in germ-free (GF) and specific-pathogen-free (SPF) conditions. Then, we amplified Vγ7-Jγ TCR chains from these cells, and compared their TCR repertoires. We observed a significantly more diverse repertoire of γδ TCRs in GF condition. This suggests that commensal bacteria affect the TCR repertoire shaping. However, further experiments are needed to verify this finding.
CCR7-mediated migration into the thymic medulla is crucial for normal gammadelta T cell development

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T cell development is a multistep differentiation pathway including controlled thymocyte migration to mediate interactions with different regions of the thymic microenvironment. However, the interplay of intrathymic migration and thymic development of gammadelta T cells are poorly understood. Here, we used CC chemokine receptor (CCR)7- and CCR9-deficient mice that were crossed to TcrdH2BeGFP reporter background to investigate the impact of thymic localization on gammadelta T cell development.

For gammadelta T cell localization in medullary or cortical thymic regions, our data showed that intrathymic expression of CCR7 and CCR9, respectively, is important. To address whether intrathymic gammadelta T cell migration was altered in CCR7- or CCR9-deficient mice, we analyzed thymic slices by two-photon microscopy.

In lymph nodes and spleen, we observed that frequencies and absolute numbers of gammadelta T cells in CCR7-deficient mice were decreased. This was in sharp contrast to an increase of gammadelta T cells in CCR9-deficient mice. Co-culture of wildtype (WT) and CCR7-deficient early thymic progenitors together with OP9-DL1 cells excluded a cell intrinsic defect of CCR7-deficient cells as they showed normal in vitro gammadelta T cell development. However, adoptive transfer of CCR7- or CCR9-deficient bone marrow into irradiated C57BL/6 WT recipients reproduced the phenotype of altered gammadelta T cell development in chimeric mice. Further, intrathymic injection of a reactive biotin derivate allowed us to identify a decreased number of gammadelta T cells among recent thymic emigrants in CCR7-deficient mice.

In summary, our results underline the importance of intrathymic migration and localization for proper gammadelta T cell differentiation. We demonstrate impaired gammadelta T cell development in CCR7-deficient mice, thus propose that the thymic medulla promotes gammadelta T cell development. The exact mechanisms how the thymic medulla supports positive selection, proliferation or final export of gammadelta T cells into the periphery need to be further investigated.
Evolution of Vγ9Vδ2 TCR

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Introduction

Vγ9Vδ2 T cells carry TCR with eponymous V gene products and Vγ9Jγ1.2 rearrangements, which recognize pyrophosphate containing metabolites of host and microbial isoprenoid synthesis (phosphoantigens) such as IPP and HMBPP. It is generally perceived that these T cells are restricted to higher primates including humans.

Objectives:

The aim of this study is to test for the existence of such cells in other mammalian taxa and to use this knowledge to understand the molecular basis of their TCR-mediated activation.

Materials & methods:

Human Vγ9 and Vδ2 genes were taken as the query sequences to search across public databases. The blast search was performed against whole genomic sequences of eutherian mammals using the discontinuous Megablast algorithm, in order to find species carrying sequences which are similar to the query sequence. Further sequence analysis was performed with cDNA from peripheral blood mononuclear cells (PBMCs) of horse and alpaca (Vicugna pacos) and genomic bovine DNA. Human and alpaca Vγ9 and Vδ2 like chains were amplified by RT-PCR, cloned and analysed. Full length Vγ9 and Vδ2 like chains of Alpaca were cloned into mammalian expression vectors and transduced into murine TCR negative T cell hybridoma.

Results:

Analysis of public databases revealed fragments of Vγ9 and Vδ2 like genes with 70 - 80% nucleotide sequence identity to their human counterpart in at least one clade of the Eutheria subgroups (Euarchontoglires, Laurasiatheria, Xenarthra, Afrotheria) suggesting the common emergence of Vγ9 and Vδ2 genes and placental mammals but also the loss of these genes in many phylogenetic groups including muridae. In six species full length Vγ9 and Vδ2 like sequences including RSS were found: 1. Horse 2. Cow 3. Alpaca 4. Sloth 5. Bottlenose dolphin 6. Grey mouse lemur. The first three were further analyzed by (RT)-PCR and sequencing. CDNA analysis of horse revealed the presence Ig domain disrupting mutations in Vγ9 & Vδ2 like genes and analysis of BoMAC showed the presence of stop codons in Vγ9 like sequence at the genomic level. Whereas alpaca blood mononuclear cells revealed the presence of Vγ9 & Vδ2 containing TCR chains with a dominance of functional Vγ9Jγ1.2 rearrangements and typical features of CDR3d found in phosphoantigen-specific human TCR. Somatic hypermutation (SHM) reported for few TCR γ and δ genes of the old world camelid Camelus dromedarius could not be found for human Vγ9 and Vδ2 like genes of the new world camelid alpaca but for other alpaca TCR γ and δ genes. Finally, a TCR-negative but CD3 positive murine T-cell hybridoma was transduced with full length Vγ9Vδ2 TCR chains of alpaca. Analysis of expression and the function of this TCRs is in progress.

Conclusion:

Analysis of public databases suggests the common emergence of Vγ9 & Vδ2 genes and eutherian mammals. Among horse, cow and alpaca, alpaca is the one which possesses human Vγ9 & Vδ2 like chains and possess a high degree of identity at the sequence level. It also shows a preference towards the usage of functional Vγ9Jγ1.2 rearrangements. In contrast to other gd TCR, Vγ9Vδ2 TCR bear no SHM. All the above results suggest that alpaca could possibly carry Vγ9Vδ2 T cells and may be a promising non-primate species to study the physiological function of Vγ9Vδ2 T cells and reasons for differential SHM of camelid γδ TCR.
γδ T cells react against the murine cytomegalovirus

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The cytomegalovirus (CMV) is a large beta herpesvirus and various layers of the innate and adaptive immune system provide control in CMV-infected individuals. In this study we focused on the protection mediated by γδ T cells against this virus.

Using the murine model we studied protective capacities of γδ T cells after infection with the murine cytomegalovirus (MCMV). We furthermore started to characterize γδ T cells reacting and expanding after CMV infection.

Adoptive transfer of γδ T cells purified from spleens and lymph nodes of infected donor mice protected immunocompromised hosts (Rag⁻/⁻ mice) from the lethal course of disease and virus titers were markedly decreased 14 days after infection in several organs.

To examine, whether there is a role for γδ T cells during MCMV infection in immune competent mice as well, we infected wildtype C57BL/6 and TCRδ⁻/⁻ mice and compared viral titers on days 3 and 5 after infection. TCRδ⁻/⁻ mice showed significant higher viral titers in several organs. This indicates that γδ T cells also play a role in immune competent mice during the early stage of infection.

We further characterized the γδ T cells reacting and expanding after CMV infection regarding their expression of certain surface molecules using flow cytometry. The percentage of CD44 expressing γδ T cells increased after infection, what points to an activated state of these cells. In addition we observed a long-lasting increase in NKG2D expressing cells after infection, which is concomitant with down modulation of CD27.
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Dose-dependent effect of HDAC inhibitor on functional responses of human γδ T lymphocytes

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Histone deacetylase inhibitors (HDACi) have been shown to possess therapeutic potential in cancer, autoimmune diseases and lymphoproliferative disorders. Valproic acid (VA) is an epigenetic modulator, which has potent histone deacetylase inhibitor activity and also acts as inducer of demethylation. VA has been studied with respect to its influence on immune cells such as dendritic cell differentiation and NK cell cytotoxicity. However, the potential functional impact of VA on human γδ T lymphocytes remains unknown. In our study, we investigated dose-dependent effects of VA on proliferation and the immunophenotype of human γδ T cells. ³H-thymidine incorporation-based proliferation assay revealed significant inhibition on day 7, when Ficoll-Hypaque-separated PBMC were stimulated with gd T-cell activating phosphoantigen BrHPP or aminobisphosphonate zoledrionic acid in the presence of VA. Similarly, potential inhibition was also noted when purified human γδ T cells were restimulated with BrHPP. In parallel to inhibition of 7 day cultured γδ T cell proliferation, comparable reduction in Vγ9 and Vδ2 immunophenotype was found in the presence of VA treatment. Using quantitative ELISA, we determined that VA decreased the interferon-γ cytokine secretion. By combined annexin V - propidium iodide staining, we found that VA induces early apoptosis in freshly isolated PBMC and also following BrHPP stimulation. Our investigations focusing on the impact of HDACi on human γδ T lymphocytes will be helpful in understanding its safety profile in clinical application for tumor immunotherapy and aminobisphosphonate-based therapies.
Evaluation of assays for detection of cellular immunity towards CMV and *M. tuberculosis* in samples from deceased donors

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Assays for cell-mediated immunity may be valuable for risk assessment of organ donors, but no data exist on their feasibility in deceased donors. Therefore, we comparatively evaluated the performance of an ELISA, an ELISPOT and a flow-cytometric assay (FACS) for the determination of T-cell immunity towards *M. tuberculosis* and CMV in deceased donors. 100 donors (51.8±17.0 yrs) were screened at the time of organ procurement. A CMV-IgG ELISA was used as a gold standard for CMV infection. Specific stimulation was performed using a CMV lysate, PPD, and ESAT-6/CFP-10 in combination with commercial assay formats (QuantIFERON QFT-CMV/TB for ELISA, T-SPOT.TB for ELISPOT). PHA or SEB were used as positive controls. Indeterminate results were defined as positive control reactions below the cut-off in the absence of antigen-specific reactivity. Indeterminate results were observed in 49.0% of ELISA, 13.3% of FACS and 0% for ELISPOT assays. CMV-specific immunity was detected in 26.0%, 46.9%, and 54.1% of ELISA (QFT-CMV), FACS, and ELISPOT-samples, respectively. Agreement with serology was highest for FACS (93%, K=0.85), followed by ELISPOT (81%, K=0.61), and ELISA (81%, K=0.62). Agreement between ELISA and serology increased if the CMV lysate was used as stimulus (94%, K=0.86), although the rate of indeterminate results remained high (44.4%). Agreement between ELISPOT and FACS was substantial (K=0.80), and moderate between QFT-CMV and ELISPOT (K=0.42) or QFT-CMV and FACS (K=0.53). Again, agreement among assays increased, if ELISA was performed with the CMV lysate (90-94%). The percentage of PPD-positive results differed between assays (27.3% for ELISA, 27.6% for FACS, and 48.9% for ELISPOT). Among PPD-positive samples, 8.3% were QFT-TB positive, 16.7% were positive in an ESAT-6/CFP-10-specific FACS-assay, and 25.6% were positive in the T-SPOT.TB test. In the routine setting of this study, 85.4% of donors had received steroids and 56.3% of ELISA samples, 97.0% of FACS samples and 92.9% of ELISPOT samples could be processed within the respective recommended time frame. Nevertheless, neither delayed processing nor steroids had a significant effect on indeterminate results.

In conclusion, cellular immunity may be analysed from samples of deceased donors, although the rate of indeterminate results is higher than that of healthy individuals.
In vitro-generated myeloid-derived suppressor cells (MDSCs) inhibit graft-versus-host disease (GVHD) while preserving the graft-versus-tumor effect (GVT)

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Allogeneic bone marrow transplantation (BMT) is a curative treatment modality for hematologic malignancies, since alloantigen-specific donor T cells eliminate residual tumor cells (graft-versus-tumor (GVT) effect). However, these T cells are also responsible for the induction of graft-versus-host disease (GVHD) by attacking alloantigen expressing recipient tissue. Therapeutic goal in BMT is the inhibition of GVHD while maintaining the GVT-effect. Myeloid-derived suppressor cells (MDSCs), a heterogenic population of myeloid precursors, suppress T cell activation and might therefore be a putative therapy in GVHD prophylaxis.

MDSCs were generated by culturing BM cells in the presence of GM-CSF and G-CSF. These in vitro-generated CD11b+Gr-1+ MDSCs suppressed allogeneic T cell proliferation in vitro. To test, whether MDSCs inhibit GVHD, B6-derived MDSCs were co-transplanted with B6-derived allogeneic BM and spleen cells (SC) into lethally irradiated B6.bm1 or B6D2F1 mice. In a dose dependent manner, MDSCs significantly suppressed clinical and histological GVHD and improved survival up to 70% - 100%, depending on the BMT-model used. Although transplanted MDSCs homed into lymphoid and GVHD target organs, they did not interfere with allogeneic T cell numbers, phenotype, proliferation and cytotoxicity. Importantly, co-transplantation of MDSCs did not interfere with the GVT-effect, since syngeneic tumor cells were still efficiently eradicated after MDSC co-transplantation.

In summary, transplantation of in vitro-generated MDSCs efficiently prevented clinical and histological GVHD while preserving T cell-mediated tumor cytotoxicity, suggesting that MDSC co-transplantation represent a useful cellular GVHD-therapy.

Figure 1: MDSC co-transplantation prevents clinical and histological GVHD, while MDSCs do not interfere with the GVT effect. (A) Survival of lethally irradiated B6.bm1 mice transplanted with B6-derived T cell depleted bone marrow (TCD-BM) alone or in combination with B6 spleen cells (SC) in the presence or absence of 1*10⁷ MDSCs. (B) Histological scores from ileum and colon, liver and skin (* p<0.05; ** p<0.01). (C) Survival of B6.bm1 animals which received the thymoma cell line JM6-61-18 and were transplanted with TCD-BM, TCD-BM + SC and TCD-BM + SC + MDSCs.
Topical application of soluble CD83 induces IDO-mediated immune modulation, increases Foxp3+ T cells and prolongs allogeneic corneal graft survival


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Introduction:
Modulation of immune responses is one of the main research aims in transplant immunology.
Objectives: Here we investigate the potential use of soluble CD83 (sCD83) for immune-modulatory therapy at the graft-host interface using the high-risk corneal transplantation model.

Methods:
In this model, which mimics the inflammatory status and the preexisting vascularization of high-risk patients undergoing corneal transplantation, allogeneic donor corneas are transplanted onto sCD83-treated recipient animals. This model allows the direct and precise application of the immune modulator at the transplantation side.

Results:
Soluble CD83 was able to prolong graft survival not only after systemic, but therapeutically even more relevant, also after topical application. The therapeutic effect was accompanied by an increase in the frequency of regulatory T cells and was mediated by the immune-regulatory enzyme IDO as well as TGF-β. In vitro, sCD83 induced long-term IDO expression in both conventional and plasmacytoid dendritic cells via autocrine/paracrine production of TGF-β, a cytokine previously shown to be an essential mediator of IDO-dependent, long-term tolerance.

Conclusions:
Our data indicate that sCD83 opens promising novel treatment avenues for local immune modulation after organ and tissue transplantation.
Recipient MHC determines the strength of induced primary antibody responses to porcine xenoantigen: Studies in congenic rat strains

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Objective:
A particular high number of foreign (xeno)peptides, which can trigger the immune system of a recipient will be generated after xenotransplantation. It is very likely that different MHC class-II haplotypes differ in their capability to present xenopeptides resulting in strong or weak anti-graft reactivity in an individual. Systematic analyses defining “high- or low-risk” MHC constellations for potential recipients of xenografts have not been performed. In this study we used a genetically defined rat strain system to address this question.

Methods:
LEW, LEW.1A, LEW.1F, and LEW.1U rats were immunized with porcine L23 cells (B-cell line). These congenic strains have identical genetic background (LEW) but differ exclusively in MHC-haplotypes (RT1\textsuperscript{l}, \textsuperscript{a}, \textsuperscript{f}, \textsuperscript{u}). The level of anti-L23-antibodies was assessed in the serum of individual rats at day 7 after immunization by flow cytometry analysis. Furthermore, the B-cell population in draining lymph nodes was characterized.

Results:
High levels of anti-L23 antibodies were found in sera from LEW, LEW.1F, and LEW.1U rats. In contrast, immunization of rats from the LEW.1A strain with L23 cells induced only weak anti-L23 antibody responses. Thus, the RT1\textsuperscript{u} MHC class-II haplotype seems to be less efficient in presentation of porcine xenopeptides. To further define the differences in B-cell activation/differentiation induced by xenoantigen between low and high antibody producer strains, we analyzed the size of the B-cell subset with the phenotype MHC\textsuperscript{+} Ig\textsuperscript{-} most probably resembling antibody secreting plasma cells in draining lymph nodes of immunized LEW.1A and LEW.1U rats. These experiments revealed a low frequency of antibody secreting cells in LEW.1A lymph nodes (12.5\% ± 1.2\%), whereas a significantly larger population was observed in LEW.1U animals (20.4\% ± 3.18\%; p=0.0023).

Conclusion:
These data suggest an important role of the recipient’s MHC class-II haplotype in regulating/controlling antibody responses to xenoantigen. Genetically defined model systems as described here may help to define “high and low risk” MHC class-II haplotypes also in the human, which can be expected to be associated with strong or weak immune responses to xenografts.

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Pre-formed cell-mediated alloreactivity is dominated by CD8 T cells and is more frequently observed in renal transplant candidates as compared to controls

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Detailed knowledge on the preformed donor-specific T cell repertoire in transplant recipients prior to transplantation would allow guidance on individualized immunosuppressive drug treatment early after transplantation. As current tests for the detection of alloreactivity are not suitable for rapid and repeated use in a clinical setting, we now developed a simple flow-cytometric assay to simultaneously quantify alloreactive CD4 and CD8 T cells directly from whole blood samples after an incubation period of only 6h. This assay is based on the intracellular accumulation of IFN-γ and the induction of CD69 and was evaluated in a total of 966 alloreactivity tests (632 in healthy controls and 334 in dialysis patients awaiting renal transplantation) that were performed by pair wise combinations of whole blood from 35 controls and 30 dialysis patients. Respective autologous combinations served as negative controls. In addition, 114 pairs (73 from patients and 41 from controls) were re-tested after 3 months.

The detection limit was 0.0081% among CD4 and 0.0143% among CD8 T cells. Alloreactivity in the cytotoxic T-cell compartment was more common, as 17.49% of all paired combinations had detectable alloreactive CD8 T cells compared to only 7.66% of combinations with alloreactivity among CD4 T cells (p<0.0001). Alloreactivity among both CD4 and CD8 T cells was detectable in 2.69% of combinations. Interestingly, when comparing dialysis patients and controls, CD8 T-cell alloreactivity was significantly more frequent in dialysis patients than in controls (28.74% versus 11.55% of combinations, p<0.001). Of note, individual CD8 T-cell frequencies may reach 6.9%, and the magnitude of alloreactive CD8 T cells was significantly higher in dialysis patients (median 0.22%, IQR 0.05-1.61) compared to controls (0.04%, IQR 0.02-0.08, p<0.0001). Re-testing of 114 pairs after 3 months showed that 80% of all cases with alloreactive CD8 T cells remained positive on follow-up. In addition, the frequency of alloreactive CD8 T cells observed at the two time points showed a strong correlation (r=0.82; 95% CI 0.72-0.88, p<0.0001).

In conclusion, preformed cellular alloreactivity is dominated by CD8 T cells and can be detected from whole blood samples within one day. Together with published evidence based on more laborious assays, the substantially higher burden of T-cell alloimmunity among transplant candidates and the stability over time may indicate increased risk for graft rejection and feasibility to identify the risk arising from preformed alloreactivity for each individual recipient-donor pair in solid organ transplantation.
Basophils as regulators of CD4 T cells in graft-versus-host disease

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Questions:
T cell proliferation is an important part of immune regulation, as well as in malignant disease. We investigated if basophils influence CD4+ T cells in a full MHC mismatch model of GvHD.

Methods:
The in vitro proliferation of CD4+ T cells was determined in mixed lymphocyte reactions (MLR) using CFSE. Furthermore, the cytokine profile was analysed by ELISA to measure the influence of basophils in vivo a full HMC mismatch model of graft-versus-host disease (GvHD) was used. In these experiments, mice were treated with the basophil-depleting antibody MAR-1 or isotyp control. Mouse survival was monitored in long-term experiments, in short-term experiments mice were sacrificed at day 14 and the T cell phenotype determined using flow cytometry.

Results:
We have found that non-activated and activated basophils (DX5+, IgE+, FcεR1+) are able to inhibit T cell proliferation in syngenic and allogenic MLRs in vitro. This effect is independent of Fas and MHCII, but dependent on the soluble factors IL-4 and IL-6. Based on these findings, we analyzed the role of basophils in the model of GvHD and detected a significant increase in the GvHD score of mice after depletion of basophils. Adoptive transfer of basophils led to a significant reduction in the GvHD score compared to control. Mice depleted of basophils showed an increased CD4+ T cell count in spleen and mesenteric lymph nodes as well as an increase in total CD45+ cells in the GvHD model. Depletion of basophils also led to more IL-17+ CD4+ T cells in the mesenteric lymph nodes but not in the spleen compared to controls. To analyse the in vivo effects of IL-4 and IL-6 in the GvHD model we administered the cytokines or PBS. IL-4 treated mice showed a significant lower GvHD score and better survival compared to control mice. In contrast, administration of IL-6 significantly increased the GvHD score and reduced survival.

Conclusions:
These data suggest that secretion of IL-4 and IL-6 by basophils might have beneficial as well as harmful effects on the outcome of a GvHD. In conclusion, basophils might be an interesting target to control an acute GvHD.
Collagen-producing hematopoietic cells (fibrocytes) in experimental heart transplantation and their regulation by basophils

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Introduction:
Fibrotic organ remodelling of cardiac allografts represents a major factor for progression of heart failure after the first post-transplantation year. Mechanistically, cardiac graft fibrosis presents as a complex interplay between the immune response against the graft and various collagen-producing cells. So far, it is unclear which cells contribute to production of collagen in allograft fibrosis and how inflammatory cells influence this process.

Objectives:
We investigated whether collagen producing hematopoietic cells (fibrocytes) are present in fibrotic allografts and how basophils are involved in modulation of fibrocytes following organ fibrosis leading to chronic cardiac rejection.

Material and Methods:
Bm12 donor hearts were transplanted into wild-type MHC-class-II-mismatched C57BL/6j recipient mice and Balb/c donor hearts were transplanted into completely mismatched wild-type B6 mice and transiently depleted of CD4⁺ lymphocytes. Graft function was assessed by palpation of the abdomen and rejection was defined as cessation of cardiac contractility. Before transplantation basophils were depleted with mAb against FcεR1, the high affinity IgE receptor. Basophils and fibrocytes (CD11b⁺ Collagen type 1⁺ cells) were identified by immunohistochemistry and flow cytometry. Collagen deposition was determined by Masson’s trichrome staining and qPCR.

Results:
In both models, progressive allograft rejection of donor hearts with decreased organ function, severe vasculopathy and interstitial fibrosis was evident within four weeks, as demonstrated by histologic evaluation of the grafts. Allograft rejection and fibrosis was associated with increased infiltration of large numbers of collagen producing hematopoietic cells (fibrocytes). Furthermore, depletion of basophils resulted in a reduction of infiltrating CD4⁺ lymphocytes as well as fibrocytes and inhibits collagen expression in allografts.

Conclusion:
Our results demonstrate an important role of basophils during cardiac allograft rejection in two chronic rejection models and describes for the first time the presence of collagen producing hematopoietic cells in allografts.
Histamer-based selection and separation of functionally active ADV-specific CD8+ T lymphocytes

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Multimers of soluble peptide-major histocompatibility complexes (pMHC) allow a specific visualization, phenotype characterization and reversible isolation of antigen-specific T cells from ex vivo samples. Adoptive transfer of antigen-specific T cells sorted by pMHC multimers is an effective therapeutic strategy for treatment of patients with malignancies or infectious diseases after transplantation.

The reversible pMHC Histamer technology was developed enabling a specific detection and isolation of antiviral T cells from peripheral blood mononuclear cells. The HLA-A01/ADV5_Hexon Histamer was generated by coupling 6xHis-tagged pMHC molecules onto cobalt-magnetic beads. The specificity and sensitivity of the magnetic bead-based Histamer was evaluated by flow cytometry. Sorting of ADV-specific CD8+ cytotoxic T cells (CTLs) was performed by immunomagnetic separation, followed by the monomerization of the pMHC Histamer in the presence of L-histidine. Sorted T cells were analyzed in phenotype and function.

The reversible Histamer showed high specificity and sensitivity (up to 99.5%). Antigen-specific T cells were isolated by this technology with a high purity of up to 99.6%. A rapid and complete disassembly of the T-cell surface-bound pMHC Histamer followed by the subsequent dissociation of the pMHC monomers from CD8+ CTL receptors was achieved using 100mM L-histidine. The function of antiviral T cells enriched by Histamer staining did not differ from CTLs induced by standard T-cell assays.

This reversible T-cell staining procedure preserves the functionality of antigen-specific T cells and can be adapted to GMP conditions. The pMHC Histamer technology offers full flexibility and fulfills all requirements to generate clinical grade T lymphocytes.
Predictive value of pneumococcal and HLA antibodies for kidney transplant outcome

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Introduction:
Pneumococcal vaccination is recommended in kidney transplant recipients as it reduces mortality. However, there have been concerns that immune activation after vaccination could lead to (subclinical) rejection.

Objectives:
The aim of the present study was to define (I) if pneumococcal vaccination induced HLA antibodies using highly sensitive methods and (II) if pneumococcal/HLA antibodies were predictive of patient outcome.

Patients & methods:
Forty-nine kidney transplant recipients were immunized with Pneumovax 23. The median interval between transplantation and vaccination was 6.5 years, the median serum creatinine concentration 1.3, 1.3 and 1.4 mg/dL pre-vaccination, at month 1 and 15 post-vaccination, respectively. In none of the patients rejection occurred within 5 years post-vaccination. Pneumococcal antibodies were determined by Luminex™ assay and HLA antibodies by lymphocytotoxicity test and Luminex™ mixed beads technology (HLA class I and II and MICA).

Results:
While pneumococcal antibodies were significantly higher at month 1 and 15 post- vs. pre-vaccination (P<0.0001 each), HLA/MICA antibodies remained unchanged. The mean value for panel reactive antibodies (PRA) was 4.0, 4.0 and 2.3% and positive Luminex™ reactions were present in 63, 67 and 63% (HLA class I), 47, 47 and 55% (HLA class II) and 29, 29 and 29% (MICA) pre-vaccination, at month 1 and 15, respectively. Kaplan-Meier analysis indicated that inferior patient survival was significantly associated with high concentrations of pneumococcal antibodies pre-vaccination [hazard ratio (HR)=3.7, P=0.04] but not with pneumococcal antibodies post-vaccination. Similarly, HLA class I antibodies pre-vaccination were associated with patient survival (HR=3.6, P=0.04). Pneumococcal and HLA class I antibodies pre-vaccination were positively correlated (r=0.26, P=0.07).

Conclusion:
Presumably, “natural” antibody formation (without vaccination) could be a surrogate marker for decreased patient survival.

Figure:
Patient survival (Kaplan-Meier plots) in 49 kidney transplant recipients stratified by antibody status. The presence of HLA class I antibodies and high pneumococcal antibodies determined by Luminex™ pre-vaccination was both significantly associated with decreased patient survival.
Heme oxygenase-1 modulates the expression of pro-inflammatory adhesion molecules in anti-HLA antibody-activated endothelial cells


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Chronic antibody-mediated rejection (ABMR) is the key limiting factor for long-term graft survival after kidney and heart transplantation. The endothelium of allografts plays a major role in the pathogenesis of ABMR, because it is directly targeted by antibodies (Abs) against anti-human leukocyte antigens (HLA). While it is well established that endothelial cell (EC) injury is mediated via complement-dependent effects of anti-HLA Abs, complement-independent effects of such Abs by signal transduction in ECs are less well understood. To investigate the regulatory role of anti-HLA Abs in ECs, cell cultures of human umbilical ECs (HUVECs), human aortic ECs (HAECs) and human dermal microvascular ECs (HDMVECs) were treated with the monoclonal anti-HLA class I Ab w6/32. Exposure of ECs to w6/32 markedly up-regulated gene expression of the pro-inflammatory adhesion molecule vascular cell adhesion molecule-1 (VCAM-1) in a time-dependent manner. Moreover, intercellular adhesion molecule-1 (ICAM-1), monocyte chemoattractant protein-1 (MCP-1) and IL-8 were induced by w6/32. Up-regulation of these pro-inflammatory genes by w6/32 was mediated via a signaling cascade that involves activation of the PI3K/Akt pathway and NF-kB. To examine the role of the inducible anti-inflammatory endothelial enzyme heme oxygenase (HO)-1 in this regulation, HO-1 was specifically modulated by either pharmacological compounds or knockdown with small interfering (si)RNA. Blocking of HO-1 activity by zinc-protoporphyrin (PPIX) and siRNA-mediated knockdown markedly increased w6/32 dependent induction of VCAM-1 gene expression. In contrast, up-regulation of HO-1 by cobalt-PPIX inhibited w6/32-mediated VCAM-1 induction. Finally, w6/32 increased the interaction of THP-1 monocytes with ECs in an in vitro adhesion assay, which was counteracted by pharmacological up-regulation of HO-1. In conclusion, anti-HLA class I Ab-dependent induction of pro-inflammatory adhesion molecules in human ECs is inhibited by HO-1 suggesting that targeted modulation of endothelial HO-1 may serve as a therapeutic target in ABMR after kidney and heart transplantation.
Inducible depletion of hematopoietic stem cells \textit{in vivo} challenges niche availability as the critical determinant for bone marrow engraftment.

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Pre-conditioning for bone marrow (BM) or hematopoietic stem cell (HSC) transplantation is accomplished by irradiation or cytoreductive drug treatment of the recipient, resulting in loss of hematopoietic stem and progenitor cells (HSC/P). Current belief is that pre-conditioning empties HSC niches and thereby allows engraftment of donor HSC. Here we report a mouse model allowing inducible Cre/lox\textsuperscript{P}-mediated depletion of primitive HSCs \textit{in vivo}. A strain expressing tamoxifen-inducible HSC-specific Cre (HSC-scl-CreERT) was mated to Rosa26-loxP-STOP-loxP-Diptheria toxin A (R-DTA) mice, in which diphtheria toxin (DT) is expressed upon Cre-mediated deletion of the loxP-flanked stop element. Tamoxifen induction resulted in 100-fold reduction of primitive HSC (Kit\textsuperscript{+}Sca\textsuperscript{-}1\textsuperscript{+}linCD48\textsuperscript{-}CD150\textsuperscript{-}CD135\textsuperscript{-}CD34\textsuperscript{-}) but only a mild reduction of more mature HSC/P in the BM of Cre\textsuperscript{R-DTA} animals. Hematopoietic niche cells including mesenchymal stem/progenitor cells, CXCL12 abundant reticular cells and osteoblasts were not directly altered by the depletion procedure. Prolonged maintenance of these mice on tamoxifen allowed for efficient suppression of HSC numbers for at least ten weeks. Competitive transplantation of HSC depleted and control BM into irradiated recipients confirmed the reduction of functional HSC. Thus, most HSC niches should be empty in induced Cre\textsuperscript{R-DTA} mice. Contrary to expectation, congenic BM transfer into (un-irradiated) HSC-depleted Cre\textsuperscript{R-DTA} recipients did not result in improved engraftment of donor HSC. Even long-term tamoxifen-treatment of Cre\textsuperscript{R-DTA} recipients (before and after donor BM transfer) did not result in engraftment. These findings demonstrate that vacating HSC niches is not sufficient to allow engraftment of donor HSC and stimulate research into additional factors determining the success of HSC engraftment.
Sequence variants (c.503T>C) of the human CD161 receptor differ in glycosylation patterns and the capacity to inhibit NK cell activation

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Background: Genetic variability in immunologically relevant genes contributes to the individual risk for rejection and/or infection after solid organ transplantation. CD161 is a glycosylated C-type lectin-like receptor that inhibits the cytolytic activity of natural killer (NK) cells, thus playing an important role in the regulation of this subset. A genetic variation (c.503T>C) in the human KLRB1 gene leads to an amino acid exchange in a ligand-binding loop-structure of the receptor. We have previously shown that about 40% of healthy individuals are homozygous for the TT genotype; 14% carry CC, and 46% are CT heterozygotes.

Question: In this study we asked whether there are structural alterations between CD161 molecules from CC and TT carriers which could affect the receptor-ligand interaction of CD161 molecules.

Methods: HEK293 transfectants expressing either the CC or TT CD161 receptor variant were created. Structural analysis of CD161 molecules was performed by immunoprecipitation and Western-Blot experiments. Receptor-ligand interaction of TT and CC CD161 receptors was examined by flow-cytometric assessment of their capacity to inhibit activation (LAMP-1) of NK cells in response to LLT1 (CD161 ligand) expressed by allogeneic cells or ligand-like antibody.

Results: Analysis of immunoprecipitated CD161 molecules revealed a slightly higher electrophoretic mobility of the CC receptor compared to TT. Further characterization using glycan-specific lectins and Western-Blotting suggested glycosylation patterns of the CC receptor were altered compared to TT. In line with this assumption was the observation that triggering of CD161 by LLT1 or antibody in cells from CC individuals inhibited NK cell activation less effectively than in cells from TT individuals (antibody: 59.0 ± 5.1% inhibition (TT) vs. 43.5 ± 5.1% inhibition (CC), p< 0.05; LLT1: 43.6 ± 4.6% inhibition (TT) vs. 29.7 ± 7.0% inhibition (CC), p= 0.11). As NK cells are being discussed to promote operational tolerance after liver transplantation, a possible role of the 503T>C polymorphism on the clinical course ought to be assessed. We recently started genotyping a cohort of liver grafted patients. Preliminary data indicate a certain accumulation of the C allele in stable patients as compared to patients suffering from one or more rejection episodes in the early course after LTx.

Conclusion: Our results suggest that the c.503T>C sequence variation in the human KLRB1 (CD161) gene leads to structural alterations in the CD161 receptor of the CC-type. This might result in CD161 hypo-responsiveness observed as reduced inhibitory capacity in NK cells of CC homozygous individuals.

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Studying human Dendritic Cells in humanized mice

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Introduction:
In the context of targeted therapies, antibodies and small molecules, murine like other preclinical animal models are often hampered by the species-specificity of these approaches. Humanized mice models provided new insights into the human immune system and might help to partially close this gap. Our focus is the role of persisting antigen presenting cells (APC) in human immunity, especially in regulatory processes (e.g. acute Graft-versus-host disease (GvHD)).

Objectives:
We are interested in the development and differentiation of human myeloid cell subsets, especially dendritic cells (DC) in a humanized mouse model with the comparison to their human counterparts. Moreover we focus on particular DC subsets regarding tissue-distribution and function.

Material & Methods:
5 to 9 week-old NOD-scid/gamma chain (NSG) mice were irradiated and up to 5x10⁶ HSC were injected intravenously (i.v.). Bone marrow (BM), spleen and peripheral blood (PB) were analyzed by flow cytometry. Spleen as well as murine skin were also examined by immunohistochemistry. Application of different cytokines was done i.v. To test functionality of the engrafted APC in vivo, allo-reactive T cells generated from a third party donor by mixed-lymphocyte culture (MLC) were injected into mice that had been reconstituted with human HSC 3 months earlier.

Results:
We established 16 models with a reconstitution rate of 76 %. From week 6 on, we found engrafted human CD45-positive cells in the BM, PB and spleen. With regard to the reconstitution of APC, we observed CD14-positive monocytes, CD11c-positive DC and plasmacytoid DC. Additionally, we detected myeloid precursors, which, together with the DC subsets, exhibit characteristics of human myeloid cells. To check for tissue resident DC, we stained biopsies and found human Langerhans cells (LC) in murine skin in approximately 25% of the mice. These LC showed no proliferative activity. The APC subsets could be found for up to 36 weeks post transplantation, whereas T cells could not be detected before week 18.
To optimize the reconstitution of DC, we applied IL 7 and Flt3L, respectively. As seen before in different models, the application of Flt3L led to an increase of the myeloid compartment, whereas B cells showed a marked reduction in BM and spleen. The application of IL 7 showed no impact on the reconstitution.
Following i.v. injection of MLC-generated allo-reactive third party T cells, their in vivo proliferation was enhanced in the presence of human hematopoiesis. B cells seemed to be the main target of allo-reactivity, but first experiments using mice later after humanization also showed depletion of DC and monocytes.

Conclusion:
Our humanized mice enable studies of the development and differentiation of human APCs in vivo. The engrafted DC subsets showed characteristics comparable to human myeloid cells. First in vivo tests support the idea that DC obtain their full functionality at later time points during humanization compared to the engrafted B cells. Moreover we saw differences in the functionality for particular DC subsets as a function of time.
However further experiments are needed to evaluate the biology of the engrafted DC in regard to their human counterparts. It is not clear how far the engrafted DC resemble the in vivo situation concerning their development, function and tissue distribution. Factors that are essential for DC differentiation and specific functions (e.g. LC proliferation) need to be explored further.
Conventional T cells polyclonally stimulated \textit{in vitro} do not induce acute graft versus host disease and display regulatory activity \textit{in vivo}

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The treatment of hematological malignancies with allogeneic bone marrow transplantations is often hampered by acute graft versus host disease (aGvHD) induced by mature T cells contained in the graft. Apart from inducing aGvHD the alloreactive T cells also mediate the desired graft versus tumor (GvT) effect crucial for curing the patient from his malignancy.

Selective suppression of aGvHD without abrogating the GvT effect can be achieved by enriching the graft for CD4\textsuperscript{+} CD25\textsuperscript{+} Foxp3\textsuperscript{+} regulatory T cells (T\textsubscript{reg} cells) by \textit{in vitro} culture of lymph node cells of mice with a superagonistic anti-CD28 monoclonal antibody (CD28-SA) prior to transplantation. As the common source for T\textsubscript{reg} cells in humans, peripheral blood, contains much lower numbers of these cells than mouse lymph nodes we evaluated whether CD28-SA stimulation of conventional CD4\textsuperscript{+} T cells \textit{in vitro} would also be suitable for selective suppression of aGvHD.

The CD28-SA D665 we used for polyclonal activation of CD4\textsuperscript{+} T cells from C57BL/6 mice was immobilized on paramagnetic beads as were the anti-CD3 and the conventional anti-CD28 mAb (co-stimulation) used for comparison. Both stimulatory protocols efficiently protected lethally irradiated BALB/c recipient mice from aGvHD. Protection from aGvHD did not require \textit{de novo} induction of Foxp3\textsuperscript{+} regulatory T cells (T\textsubscript{reg} cells) as co-stimulation did not induce Foxp3 expression and blocking induction of Foxp3 upon CD28-SA stimulation \textit{in vitro} with the neutralizing anti-TGF-\beta mAb 1D11 did not increase aGvHD severity.

Mechanistically, tracking previously CD28-SA-activated carboxyfluorescein succinimidyl ester diacetate (CFSE)-labeled allogeneic conventional CD4\textsuperscript{+} T cells in the recipient animals \textit{in vivo} revealed that the vast majority of them continued to divide \textit{in vivo} while after transplantation of unmanipulated conventional CD4\textsuperscript{+} T cells only the 5-10\% of truly alloreactive cells proliferated. \textit{In vivo} proliferation was particularly efficient after CD28-SA pre-stimulation in the presence of 1D11. Co-transplantation experiments of unmanipulated and CD28-SA plus 1D11 pre-activated allogeneic conventional CD4\textsuperscript{+} T cells, further, showed that the pre-activated conventional CD4\textsuperscript{+} T cells are capable of inhibiting alloreactive CD4\textsuperscript{+} T cells from inducing aGvHD as did \textit{bona fide} T\textsubscript{reg} cells transplanted in parallel.

As far as the GvT effect is concerned, total, i.e. CD4\textsuperscript{+} and CD8\textsuperscript{+}, T cells pre-stimulated with the CD28-SA \textit{in vitro} displayed greater cytotoxic activity \textit{in vivo} than co-stimulated T cells and allowed for long-term protection of the allogeneic BALB/c recipient mice from the BCL-1 lymphoma.

Polyclonal stimulation of conventional T cells with a CD28-SA \textit{in vitro}, thus, constitutes an efficient means for preventing aGvHD while maintaining the desired GvT effect.

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Role of toll like receptors in sensing endogenous danger signals released during hepatocyte cold storage or upon warming-up

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Introduction:
Cell death and injury often leads to release or exposure of the intracellular molecules called damage-associated molecular patterns (DAMPS) that are recognized by the innate immune system by pattern recognition receptors (PRRs) - the same receptors that detect pathogen-associated molecular patterns which, reveals similarities between pathogen induced and non-infectious inflammatory responses. Toll like receptors (TLRs) play an important role in the innate immune response initiated by DAMPS that are released by stressed and dying cells. Ischemia/reperfusion injury elicits inflammatory response which lacks a microbial constituent yet poses a potentially lethal threat to the host in solid organ allografts, with profound influence on acute as well as long-term graft function. Increasing evidence suggests that TLRs are involved in mediating ischemia-induced tissue damage in several organs. Hypothermic stress induces cell death which is a major cause of primary graft non-function following liver transplantation.

Objectives:
Our objective is to evaluate the stimulatory potential of homogenates of cold stored or/and heat shocked primary hepatocytes by challenging macrophages of various genotypes and identify endogenous DAMPs that are involved.

Materials and methods:
ELISA was performed to measure the cytokine release in the macrophage culture supernatants 16 hours post challenge with pretreated primary rat hepatocyte homogenates. ROS production from conditional Hoxb8 neutrophils upon challenge with hepatocyte homogenates was measured by lucigenin chemiluminescence assay.

Results:
We observed that neither overnight hypothermic (4°C) preservation nor rewarming of the hepatocytes induced a substantial stimulatory capacity as detected in our experimental system. ROS production from conditional Hoxb8 neutrophils upon challenge with hepatocyte homogenate was increased by IFN-g priming. In contrast, 45°C heat shock for 10 minutes followed by incubation at regular cell culture conditions (HS-cult) resulted in profound cell stimulation upon hepatocyte homogenate challenge. Moreover, lack of activation of MyD88/TRIF⁻⁻ macrophages in response to HS-cult treated hepatocytes suggested the involvement of TLRs in cell activation.

Conclusion:
Our results indicate involvement of TLRs in sensing endogenous DAMPs released from the dying hepatocytes during the hypothermic ischemic phase.
Treatment with a Heat shock protein 90 inhibitor is protective in a mouse model of acute Graft versus Host Disease

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Introduction:
Acute Graft versus Host Disease (aGvHD) represents a major cause of morbidity and mortality in patients with hematologic malignancies undergoing allogeneic hematopoietic stem cell transplantation (ASCT). Recently we have shown that heat shock protein 90 (Hsp90) inhibitor 17-(dimethylaminoethylamino)-17-demethoxygeldanamycin (DMAG) selectively depletes human alloreactive T cells upon in vitro stimulation.

Objectives:
We assessed, whether our in vitro findings would translate into a therapeutic effect of Hsp90 inhibition in a mouse model of aGvHD in vivo, putatively identifying Hsp90 inhibition as a novel therapeutic strategy for the prevention of aGvHD after ASCT.

Material and methods:
To study the effect of Hsp90 inhibition on activated T cells of mice in vitro, we stimulated lymph node cells from C57/BL6 mice with 0.5 µg/ml anti-mouse CD3ε monoclonal antibody and 30U/ml IL-2. Activated lymph node cells were incubated for the last 24 h of culture or for the whole culturing period with different concentrations of DMAG or DMSO only as a control.

BALB/c mice were conditioned for BM transplantation by total body irradiation with 8 Gy as a single dose. Approximately 24 hours after irradiation the mice received 107 T cell depleted (TCD) bone marrow (BM) cells and 5 x 10⁴ or 5 x 10⁵ CD4⁺ T cells from CD90.1 congenic C57/BL6 mice intravenously. In short-term experiments CD90.1 CD4⁺ T cells were labeled with CFSE before being transferred intravenously. Mice then received 10µg DMAG/day or DMSO resolved in PBS/TWEEN from days 0 to +2 post-BM transplantation (BMT). During the experiment, incidence and severity of aGvHD were assessed by measuring body weight and scoring the clinical appearance of the mice. Percentages of donor CD4⁺Foxp3⁺ and CD4⁺Foxp3⁻ T cells were determined in secondary lymphoid organs and liver 3 and 7 days after transplantation by fluorescence-activated cell sorting analysis (FACS).

Results:
Similar to human T cells, inhibition of Hsp90 by DMAG reduced proliferation of lymph node responder T cells in vitro in a concentration-dependent manner. Having identified the mouse as a relevant model for Hsp90 inhibition in humans, we next assessed the effect of DMAG in vivo in the C57BL/6 into BALB/c mouse model of aGvHD. Here, DMAG showed a clear protective effect after transplantation of 5 x 10⁴ allogeneic CD4⁺ T cells into the BALB/c recipients, which was still detectable but greatly reduced after transplantation of 5 x 10⁵ allogeneic CD4⁺ T cells. Protection not only comprised reduced clinical signs of aGvHD but also an increase in survival.

Analysis of secondary lymphoid organs and liver demonstrated that in DMAG-treated mice CD4⁺Foxp3⁻ alloreactive T cells accumulated less than in control mice and that the frequencies of CD4⁺Foxp3⁻ regulatory T cells among the allogeneic T cells were elevated. The latter constitutes a potential mechanism for the long-term protection from aGvHD we observed despite the short treatment interval. Furthermore, we noticed that DMAG slightly decreased the proliferation of alloreactive CD4⁺Foxp3⁻ T cells (CFSE dilution) and increased the percentage of AnnexinV⁺ cells among CD4⁺Foxp3⁻ cells in mesenteric lymph nodes and spleen of allogeneic recipient mice.

Conclusion:
These results suggest that inhibition of Hsp90 by DMAG constitutes a novel immunotherapeutic strategy for the treatment of aGvHD after ASCT.

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Modulation of antibody responses to porcine xenoantigen by targeting the PD-1/PD-Ligand pathway

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Background:
Human T cells express several receptors (e.g. programmed death-1 receptor, PD-1) which deliver inhibitory signals and downregulate T cell activation. Thus, transgenic overexpression of corresponding ligands on porcine cells and tissues could be a strategy to prevent human immune responses to xenografts. We have previously shown that human in vitro T cell responses to porcine transfectants overexpressing human PD-Ligand 1 (PD-L1) are reduced as compared to responses triggered by control cells.

Question:
It was the aim of this study to assess the effects of PD-1/PD-L1 targeting on B cell activation and antibody responses to xenoantigen. We asked whether (a) PD-1 can be induced/up-regulated in B cells by stimulation with xenoantigen, and (b) B cell responses are diminished in the presence of PD-L1.

Methods:
In vitro activation of human B cells was induced by co-culturing with the porcine cell line L23 and L23 cells transfected with human PD-L1 (L23-PD-L1 cells). In vivo immune responses to L23 and L23-PD-L1 cells were characterized in a rat model by immunization experiments.

Results:
Stimulation of human peripheral blood mononuclear cells (PBMC) with L23 cells resulted in up-regulation of the early activation marker CD69 on CD19+ B cells. Furthermore, we observed proliferation of B cells as well as up-regulation of PD-1 on 8-10% of B cells, with a peak expression on day 5. After 7 days, anti-porcine IgM antibodies could be detected in the culture supernatant of human PBMC stimulated with xenoantigen and the IgM titer increased till day 12. To further characterize the antibody responses to L23 and L23-PD-L1 cells, we analyzed the level of anti-pig antibodies in rats immunized with the two cell types. Here we found lower levels of antibody responses in the serum of rats immunized with L23-PD-L1 cells. Flow cytometry analysis of the B cell population in draining lymph nodes revealed a lower frequency of activated B cells (Ig+CD44high) after immunization with L23-PD-L1 cells.

Conclusion:
These data suggest that antibody production to porcine xenoantigen is diminished in the presence of the inhibitory ligand PD-L1. Application of PD-L1 might be a strategy to prevent induction of anti-graft antibody responses, which are particularly strong in the pig-to-primate combination and represent a significant immunologic barrier for clinical xenotransplantation.

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Host B cell-derived IL-10 ameliorates murine Graft-versus-Host Disease

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Allogenic hematopoetic stem cell transplantation (HSCT) is the treatment of choice for a variety of hematologic malignancies. Graft-versus-Host disease (GvHD) is a key contributor to treatment related morbidity and mortality and consequently limits the efficacy of allogenic HSCT. Interleukin 10 (IL-10) is a well-known cytokine with immunoregulatory and anti-inflammatory properties, also important in context of GvHD. B cells have been described as potent IL-10 producers in various situations. Here we show how host derived B cells contribute to GvHD amelioration through IL-10 production.

We address the role of IL-10 in GvHD in an acute murine MHC mismatch model: Mice on a C57BL/6 background received bone marrow and CD90+ T cells from mice on a BALB/c background or vice versa. Transplantation experiments with IL-10 deficient recipient mice clearly show the importance of host derived IL-10 in general. To further dissect the cells contributing to IL-10 production in this situation we employed an IL-10 knock-in reporter mouse in which expression of eGFP is under control of the Il-10 locus. Lethal irradiation as used in the conditioning regimen before transplantation revealed B cells as major contributors of host derived IL-10. A phenotypical characterization of the eGFP+ B cells exhibited a CD1d+TIM-1+CD5+ phenotype. This is in line with the IL-10 producing B cells reported in the literature. To finally confirm our findings and to evaluate the relevance of host B cell derived IL-10 in GvHD, we transplanted lethally irradiated B6.B-Il-10−/− mice that have a B cell specific IL-10 knock-out with BALB/c cells. Here we found a reduced survival associated with the incapability of the host B cells to produce IL-10.

Taken together, our results provide new insights in the mechanisms and the variety of cells contributing to the course of GvHD. An improved understanding of this aspect might help to pave the way for new treatment options to overcome current limitations of allogenic HSCT.
Benign Inflammation Protects Liver Allografts in Late Subclinical Rejection Compared to Acute Cellular Rejection

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Background and Aims:
The subclinical rejection (SCR) of liver transplants constitutes a histological state of acute cellular rejection without any relevant biochemical abnormalities. It was shown that for SCR in kidney transplants a higher infiltration of regulatory T cells (Treg) and a higher Treg/CD3 ratio were associated with a better clinical outcome and renal graft function. Late SCR after liver transplantation, however, has not been studied yet. We therefore aim to define its clinical relevance and the pattern of T cell infiltration within the allograft.

Method:
In this prospective study we analysed the clinical course of 25 patients who presented with subclinical graft rejection late (i.e. more than three months) after transplantation during an average follow up period of 21 months. Furthermore protocol liver biopsies were stained for CD4, CD8, FOXP3 and DAPI with multicolour immunofluorescence.

Results:
Late SCR represents no risk factor for subsequent ACR, graft dysfunction, progressive liver fibrosis, graft loss or liver related death in our protocol biopsy program. This is despite the fact that liver biopsies of SCR and ACR are indistinguishable in terms of pathological diagnosis, size of portal infiltrates and the density of T lymphocytes (CD4 & CD8) within the infiltrates. Immunophenotyping of the infiltrated portal areas revealed a possible explanation.

CD4+ T cells increase significantly in both SCR and ACR with higher levels of inflammation (measured by the rejection activity index - RAI). CD8+ T cells, on the other hand, only correlate with RAI in ACR and do not increase in SCR. This causes a positive correlation of portal CD4+/CD8+ ratio with RAI in SCR and a negative correlation in ACR.

The portal infiltration of Treg (CD4+FOXP3+) increases with RAI in both SCR and ACR. Treg enrich with the severity of any inflammation and suppress in a dose dependent manner. Therefore, it is necessary to analyse the ratio of Treg to effector T cells (Teff). The overall ratio of Treg/Teff (CD4+ / CD8+) correlates with RAI in both SCR and ACR. The same is true for the Treg/CD4+ ratio. The ratio of Treg to cytotoxic CD8+ T cells, however, only increases with RAI in SCR and remains steady in ACR. This is an important finding since CD8+ cells correlate with higher transaminases in ACR. Compared to normal protocol biopsies without rejection Treg are significantly enriched in SCR and ratios of Treg to overall Teff, CD4+ and CD8+ cells are also significantly higher in SCR.

The lymphocyte infiltrates in SCR are therefore characterised by a lack of CD8+ enrichment with increasing severity of RAI, leading to a positive correlation of CD4+/CD8+ ratio with RAI compared to ACR where CD8+ cells increase significantly. Most striking is the increasing Treg/CD8+ ratio with RAI in SCR that is missing in ACR. All these changes in the T cell compartment are only detectable in the allograft itself and not in the peripheral blood.

Conclusion:
The clinical outcome of late SCR patients in our study was unimpaired. Late SCR thus appears to have the same benign course as the previously reported early SCR. The liver infiltrate in late SCR is indistinguishable from ACR with routine pathological reviewing. Immunophenotyping, however, shows the signs of successful regulation by Treg within the allograft. These patterns of T cell immunoregulation in portal infiltrates may contribute to the unimpaired clinical outcome of SCR after liver transplantation and may represent one aspect of the liver as an immunologically privileged organ.
Multiallelic amplification for haplotype separation of HLA alleles using PCR-SBT – unambiguous HLA high resolution typing by a new PCR based sequencing strategy

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Established methods for human leukocyte antigen (HLA) typing which have been considered routine laboratory tools in recent 5 years are now threatened with extinction by the rapid development of next generation sequencing HLA DNA technology. In comparison to the conventional molecular typing techniques for detection of high level of HLA polymorphisms as the PCR-SSO (Sequence Specific Oligonucleotides) as well as the PCR-SSP (Sequence Specific Primers), is the PCR based sequencing (PCR-SBT) the most reliable method for typing of HLA genes. The full complexity of HLA polymorphisms makes the sequencing method of choice for HLA low- and high resolution typing. The current sequencing methodologies utilizes combination of HLA locus or/and HLA group specific amplification strategies followed by cycle-sequencing the desired number of exons on one/both strands. In many laboratories is at time the DNA sequencing using the Sanger dideoxy chain termination reaction the preferred method for sure and reliable detection of HLA polymorphisms. A problem by an efficient analysis of the HLA allelic combinations after Sanger sequencing is the frequent occurrence of allelic ambiguities in the majority of samples. To solve this problem, a separation of haplotypes must be performed. The haplotype separation of HLA alleles will be achieved by PCR using multiple numbers of Group-Specific-Primers (GRPs), or during the sequencing approaches using Group-Specific-Sequencing-Primers (GSSP). Both strategies enable a high (4 - 8 digits) resolution, the differences lie in the practical implementation. For construction of the PCR as well as sequencing primers a further complication are HLA alleles with unknown sequences outside of clinical, or HLA typing relevant exons 2 and 3. This has the consequence that not for each allele a specific sequence motif is available. Sequence variations within the same populations or between several ethnic groups are also possible. In order to handle this problem, we have developed a new PCR system based on recognition of multi allelic sequence motives located in noncoding regions of HLA-A, B, C, DRB1 and DQB1 genes using the GRPs. The principle is based on construction of a variable number of GRPs of different HLA specificities whose specific sequence motifs are located in different noncoding areas outside the peptide binding sites of exons 2 and 3 (5´-UTR/ intron1/intron5/intron6). This new approach has been validated on example of selected samples encompassing all different allelic combination as available. For the haplotype separation of HLA-A* alleles a total number of 12 different GRPs, of the HLA-B* alleles 16, of the HLA-C* 16, of the HLA-DRB1* 17 and for the haplotype separation of the HLA-DQB1* alleles 6 different GRPs will be used. The disadvantage of this system is an incomplete cis/trans discrimination (HLA-A*1,9%, HLA-B* 3,1%, HLA-C*0,9%, HLA-DRB1*0,2%, HLA-DQB1*0, 8%) between all HLA currently described allele combination (IMGT-HLA Database, Release 3.12/April 2013). In conclusion, this method is capable of delivering nearly all unambiguous typing results. A flexible system is created which is independent of the sequence variations of only one HLA specific region. As a result, a creating of several specific PCR products of the same HLA specificity will be achieved. This approach allows an easy and cost efficient implementation in each diagnostic HLA laboratory and provides formats for each level of throughput in order to match the laboratory’s individual requirements.
Reconstitution of GPI-anchor negative lymphocytes with impaired function after Alemtuzumab-based T cell depletion

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Introduction:
The anti CD52-antibody Alemtuzumab is used for T cell depletion (TCD) to prevent graft versus host disease (GvHD) in the context of allogeneic hematopoietic stem cell transplantation (HSCT). We recently showed long-term persistence of CD52 negative T cells (TC) in patients following Alemtuzumab-mediated TCD. The lack of the GPI-anchored CD52 protein was due to the loss of glycosyl-phosphatidyl-inositol (GPI)-anchors themselves. Here, we provide additional functional analyses on these GPI-anchor negative cells.

Methods:
Using FACS-staining, we analysed the phenotype of immune cells of 58 patients at different time points (up to 4.5 years) after HSCT and looked for the presence of GPI-anchors via FLAER. The TC function ex vivo was evaluated by studying proliferation (CFSE), secretion of IFN-γ in Secretion-Assays, and intracellular cytokine-staining. For in vitro analysis, TC were sorted according to their GPI-anchor expression and tested for IFN-γ secretion in ELISPOT-Assays. The lytic function was assessed in Cr-Release-Assays. Antigen specific stimulation was accomplished either with peptide-loaded (for CMV and EBV) Dendritic cells (DC), CMV-infected fibroblasts or allogeneic DC. Treg were identified as CD3⁺CD4⁺CD25⁺CD127⁻ or CD3⁺CD4⁺CD25⁺FoxP3⁺ cells. Their function was evaluated when measuring their suppressive capacity on effector TC proliferation. GARP and CD45RA, HLA-DR identified the status of activation in Treg. We also correlated Treg subpopulations with the clinical course of patients who recovered from acute (a)GvHD and those with ongoing late aGvHD.

Results:
GPI-anchor negative T-lymphocytes and NK cells were observed in all patients. The majority of T-cells had a memory phenotypes. However, newly reconstituting naive TC-populations found later after transplantation, were exclusively GPI-anchor positive. GPI-anchor negative effector memory TC (CD45RO, CD62L, CCR7) were present in relevant numbers up to 4 years and showed an impaired antigen-specific function to EBV, CMV and allogeneic DC. The fraction of GPI-anchor negative cells among Treg was significantly elevated in patients with aGvHD: median 80.35% compared to 17.4% in patients with cGvHD or without GvHD. This Treg population did not exhibit the activated (GARP-positive) phenotype that was found among GPI-anchor positive Treg. The percentage of GPI-anchor negative Treg populations remained high in patients with ongoing aGvHD, whereas patients resolving from aGvHD reconstituted GPI-anchor positive Treg in the later course. In vitro, GPI-anchor negative Treg showed less suppression of TC proliferation.

Conclusion:
We confirmed that GPI-anchor negative TC reconstituting after HSCT with Alemtuzumab mediated TCD show an impaired function. This functionally impaired GPI-anchor negative effector TC might be partly responsible for some of the known viral complications in patients undergoing Alemtuzumab-based conditioning regimen. Furthermore, we demonstrate for the first time the reconstitution and persistence of GPI-anchor negative Treg with compromised suppressive capacity and additionally provide clinical evidence that a high proportion of GPI-anchor negative cells among Treg is associated with aGvHD.

GPI-anchors themselves or GPI-anchored proteins seem to be important for antigen-specific effector TC function as well as for immune-regulatory aspects mediated by Treg.
Age depending development of engraftment level and functionality of the lymphoid and myeloid lineage in humanized mice

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Humanized mouse models are a powerful tool in animal experiments to simulate the human immune system (Ishikawa et al., 2005; Shultz et al., 2012). Especially in infectious diseases like HIV or Dengue virus the help of humanized mouse models led to several important research results (Zhang et al., 2010). However, fundamental research of this model helps to get insights into different immunological aspects.

Our humanized mouse model is based on the transplantation of hematopoietic stem cells into radiated newborn mice. After 10 weeks and further time points, blood, spleen and bone marrow were analyzed for engraftment of human immune cells in these mice by flow cytometry. We developed different flow cytometric panels for several issues. We used a general panel with anti-mouse and anti-human CD45 as control for engraftment and markers for human monocytes, B and T lymphocytes as well as granulocytes and natural killer cells. After in vivo stimulation with LPS we also analyzed different kind of dendritic cells and activation markers like anti-human CD25, CD80, CD86 and HLA-DR. In dependence to graft versus host disease there was a panel for T-cell differentiation. To get a deeper insight on the functionality of the cells we measured human cytokines and immunoglobulins by cytometric bead array. Additionally, we did histological examination of several organs like spleen, kidney, small and large intestine. Also immunohistochemistry was done with an anti-human CD45 antibody.

The human immune cells in mice produce cytokines after stimulation, we could detect myeloid as well as plasmacytoid dendritic cells and there is a measurable amount of immunoglobulin. Over time the functionality and amount of human immune cells increase and there is a change from an almost only lymphoid cell population with mainly B-cells to more T-cells and also an increase in cells of the myeloid lineage. Especially in older animals human granulocytes are detectable. Histological examination with an anti-human CD45 antibody showed that the functional structure in the spleen of humanized animals is mainly build by human immune cells.

We found this mouse model as a functional and forward-looking tool in immunological research. In the future different disease models in humanized mice could be developed to replace rodent models of human diseases with these humanized disease models. This could lead to more translational research results than disease models in non-humanized mice do.

Ishikawa, Fumihiko; Yasukawa, Masaki; Lyons, Bonnie; Yoshida, Shuro; Miyamoto, Toshihiro; Yoshimoto, Goichi et al. (2005): Development of functional human blood and immune systems in NOD/SCID/IL2 receptor (gamma) chain(null) mice. In: Blood 106 (5), S. 1565-1573.


Targeting DEC-205 vs. TLR2/6 on dendritic cells: Which strategy is qualified for cellular immunotherapy against hepatitis C virus infection?

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Introduction:
Hepatitis C virus (HCV) represents an important global health-care burden, which is likely to even increase. Prevention and treatment of HCV infections are still challenging and should benefit from improvements in the induction of antigen-specific T cell immunity to the virus. Since dendritic cells (DCs) orchestrate innate and adaptive immune responses they are an exceptionally promising target for developing improved vaccines.

Objectives:
The major objective of this study is to develop a DC-based immunotherapy to enhance HCV-specific cellular immune responses for treatment of HCV infections. To follow this approach two different in vivo DC targeting strategies were compared, utilizing either the endocytosis receptor DEC-205 or the Toll-like receptor (TLR) 2/6 heterodimer, with respect to their applicability for inducing antiviral immunity in the liver. Based on these results analyses are extended towards the design of a DEC-205-based HCV vaccine.

Materials and methods:
C57Bl/6 mice (n=5) were s.c. immunized thrice (day 0, 14, 28) either with αDEC-205/OVA, with OVA protein and BPPcysMPEG or with BPPcysOVAMPEG (BPPcysMPEG directly linked to OVA MHC class I and II peptides) followed by infection with a recombinant adenovirus expressing OVA (AdOVA-GFP-luc) or control virus (Ad-GFP-luc). 4 days after infection, serum ALT-levels and luciferase activity in the liver were determined as well as FACS analyses have been performed. Recombinant HCV-proteins Core (aa1-191) and NS3 (aa1027-1218) containing N-terminally His-Tag are expressed using “expression-ready-clones” and purified by metal-affinity chromatography on Ni-NTA-agarose followed by conjugation to αDec205.

Results:
Targeting DEC-205 on DCs in vivo is considered to be superior to the TLR2/6 strategy since the immunization with αDEC-205/OVA, but not with the full-length OVA protein in addition to BPPcysMPEG, leads to the exceptionally potent induction of IFNγ secreting cytotoxic T lymphocytes capable of efficiently clearing virus-infected hepatocytes. Even though BPPcysOVAMPEG immunization is as efficient as αDEC-205/OVA vaccination, the limited number of antigenic determinants is of great disadvantage in the context of the highly mutating HCV. In order to exploit the successful induction of antigen-specific cellular immunity in the liver towards the design of an effective DEC-205-based HCV vaccine, protocols for recombinant expression of selected HCV candidate antigens NS3 and Core in E. coli, their purification and finally the chemical conjugation to αDEC-205 were established.

Conclusion:
In conclusion, αDEC-205/HCV-protein conjugates represent a promising tool to target HCV-antigens to immunogenic DCs in vivo and may represent a valid strategy to enhance HCV-specific immunity as therapeutic vaccine in patients suffering from chronic HCV infection.
Biodegradable calcium phosphate nanoparticles encapsulating retroviral T cell peptides and a TLR9 ligand are a powerful tool to protect against acute and treat chronic retroviral infection

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Unlike most bacterial infections, many viral infections do not result in pathogen clearance but rather in viral persistence and the development of a chronic state of infection. Therefore the prevention of viral infection is the major goal of antiviral therapy. However, retroviral infections (e.g. HIV) have been an unresolved challenge for vaccine designs where especially the induction of a virus specific T cell immune response seems to be crucial for the prevention of viral manifestations. Nanoparticles have been considered to be ideal vaccination vehicles to mimic invading pathogens and thereby enhance the immunogenicity. In this study we present biodegradable calcium phosphate (CaP) nanoparticles functionalized with TLR 9 ligand CpG and CD4 and CD8 T cell specific peptides from Env and Gag protein of Friend virus (FV) for the prevention of retroviral infection. In comparison to the application of soluble CpG and T cell peptides, immunization with functionalized CaP nanoparticles results in a higher number of virus specific CTLs and T<sub>H</sub>1 cells and therefore a significantly improved protection against acute FV infection in challenge experiments. However, due to mechanisms such as T cell exhaustion viral persistence is the outcome of many viral infections. Thus, reactivation of the adaptive immune system is one of the main goals during therapeutic vaccination. Here we demonstrate that a single shot of CpG/peptide functionalized CaP nanoparticles efficiently increases the expression of antiviral molecules, including cytotoxic granzyme B and IFN<sub>γ</sub> by CD4<sup>+</sup> and CD8<sup>+</sup> T cells in persistently FV infected mice which leads to a significant decrease of viral loads. The additional transient depletion of Foxp3<sup>+</sup> regulatory T cells (Treg) in transgenic mice can furthermore potentiate the antiviral effect of nanoparticle treatment, demonstrating the importance of Treg cells during therapeutic therapy of persistent viral infections.

Taken together, these results demonstrate that functionalized CaP nanoparticles successfully prime cellular immunity for prophylactic and therapeutic immunization and therefore represent a powerful vaccination tool for the treatment of both acute and chronic retroviral infection.
The precursors of long-lived and resting memory T helper cells

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Immunological memory provides a long-term protection against a variety of infectious pathogens and is a hallmark of adaptive immunity. Long-lived memory T helper (Th) lymphocytes play a crucial role in the generation and reactivation of other memory lymphocytes. We have so far shown that in an immune response some CD4⁺ T cells activated in secondary lymphoid organs relocate to the bone marrow (BM) and then reside and rest there as memory Th cells. However, it remains unclear how memory Th cells are generated during immune responses.

Following infection, antigen-specific CD4⁺ T cells clonally expand and differentiate into effector cells for the clearance of the pathogen. During the subsequent contraction phase, the majority of antigen-experienced cells die off, while a small population of the activated cells migrates into the BM and matures into long-lived memory Th cells. The precursors of memory Th cells migrate into the BM via sinusoids using integrin alpha2 (CD49b) and CD69. Loss or blockade of CD49b and CD69 expression on CD4⁺ T cells impairs the generation of resting memory Th cells in the BM. Thus, activated CD4⁺ T cells expressing both CD49b and CD69 during immune responses can be defined as the precursors of resting memory Th cells. Actually, on days 4 to 6 after immunization 10-20% of antigen-specific CD44⁺ CD4⁺ T cells express both CD49b and CD69. The cellular and molecular analyses of CD49b⁺CD69⁺ memory cell precursors contribute to the understanding of how memory Th cells are selected and which molecules induce their maturation. We here discuss how Th cell memory is initiated, determined and established.
CD8+ memory T cells are resting in murine bone marrow

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Question:
Memory T cell generation and persistence are still poorly understood and are therefore major targets of immunological research, aiming for the development of new vaccination procedures against infections and tumours. Previously, our group has shown that memory CD4+ T cells that are generated following protein immunization preferentially reside and rest in bone marrow (BM). Memory CD8+ T cells generated upon a specific immune response have also been shown to relocate to BM; however, their function and persistence have not been thoroughly investigated. We have now taken a closer look at memory CD8+ T cells in the BM in order to define how they are maintained over long time periods.

Materials and Methods:
In order to generate antigen (Ag)-specific CD8+ T cell memory, C57BL/6 mice were immunized twice at 4-week intervals with cationized OVA and LPS. SIINFEKL-reactive CD8+ T cells were tracked in spleen and BM in acute and late phases of the secondary response. Memory CD8+ T cell localization in BM was investigated by analyzing sections of femoral bones by laser scanning confocal microscopy.

Results:
Our results show that there are approximately equal numbers of Ag-specific memory CD8+ T cells in BM and spleens of the immunized mice indicating that BM is one of the major compartments for memory CD8+ T cell maintenance. Compared to spleen, higher frequencies of Ag-specific, as well as Ag-non-specific memory CD8+ T cells express CD69 in BM. However, according to Ki-67 staining, most Ag-specific memory CD8+ T cells in the BM are resting in the G0-phase of the cell cycle. Microarray analysis of memory phenotype CD8+ T cells from spleen and BM indicates that BM T cells are also resting in terms of gene expression. Thus, CD69 expression does not reflect recent activation, but suggests that CD69 is important for memory T cell maintenance in BM, as has been shown for CD4+ memory T cells. By confocal microscopy we could show that memory CD8+ T cells are contacting IL-7 producing BM stromal cells, which are providing survival signals to memory CD8+ T cells.

Conclusion:
Our findings demonstrate that BM is a major site where memory CD8+ T cells are maintained as resting cells in dedicated niches defined by IL-7 expressing stromal cells.
Human bone marrow hosts resting polyfunctional memory T cells

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Purpose/Objective:
Memory T cells are essential components of immunological memory, but little is known about their maintenance. Recirculating memory T cells of blood and secondary lymphoid organs slowly decay in numbers. In mice, we have recently identified professional resting memory CD4+ T cells located in the BM and stably maintained in dedicated survival niches. The cells are resting, despite expression of CD69. Here we analyze and compare human memory CD4+ and CD8+ T cells from peripheral blood (PB) and BM.

Material and methods:
Mononuclear cells were isolated from paired PB and BM samples from individuals undergoing hip replacement surgery. Phenotypic analysis and cytokine profile of distinct memory T-cell subsets were measured by flow cytometry. Proliferation and cell cycle status were analyzed using Ki-67 and propidium iodide staining, respectively. Global transcription of cells was assessed by microarray analysis.

Results:
Significantly higher numbers of memory T cells were found in BM than in PB. BM memory T cells displayed polyfunctionality, secreted a multitude of cytokines, and specificities for all recall antigens tested, i.e. CMV, Candida albicans, Tetanus Toxoid, and Measles. CD69-expressing memory CD8+ and CD4+ T cells were detected in BM but not in PB. CCR7 and CD62L expression were decreased on CD69+ memory CD4+ and CD8+ T-cells from BM. Ki-67- and propidium iodide-positive cells were significantly lower in CD69+ memory T cells from BM compared to PB. BM CD69+ memory T cells showed a resting global gene expression profile.

Conclusions:
In the steady state, human BM is home of polyfunctional memory T cells with recall antigen-specificities, ranging from persistent low-dose antigens to short- and long-lasting vaccines. BM memory T cells express CD69. In spite of CD69 expression, BM memory T cells rest in terms of proliferation and transcription. These results establish BM as a home of resting, professional memory CD4+ and CD8+ T cells, providing systemic, stable longterm memory.
PD-1 limits expansion of vaccine-induced CD8$^+$ T cells

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Programmed death 1 (PD-1; CD279 belongs to the CD28 family and interacts with its two ligands Programmed death ligand 1 (PD-L1, CD274) and Programmed death ligand 2 (PD-L2; B7-DC). PD-1 is expressed on activated T cells and is implicated in peripheral tolerance and exhaustion. The aim of this study was to analyse the impact of PD-1 on the resulting T cell response after vaccination. To this end purified PD-1-deficient OT-1 cells or their wildtype counterpart were transferred into C57BL/6 recipient mice, which were subsequently immunized using a *Bordetella pertussis* adenylate cyclase toxoid fused to ovalbumin. At various time points after immunization spleen and liver cells were analysed by flow cytometry for the presence of antigen-specific T cells using MHC-I multimers.

PD-1-deficient OT-1 T cells expand up to 10-fold more than wildtype OT-1 cells after immunization. Although the pool of PD-1-deficient T cells also contract, they persist in spleen and liver at much higher levels. They rapidly expand upon boost immunization one year after primary immunization. To verify their functionality, mice that received OT-1-PD-1-deficient T cells or OT-1 wildtype T cells were challenged 14 weeks after immunization with OVA-expressing *Listeria monocytogenes*. Mice that received PD-1 deficient T cells displayed much less bacteria in spleen and liver. To further decipher if this approach can be used to boost vaccine efficacy, we applied anti-PD-1 during immunization. Already this transient blockade of PD-1 was accompanied by an increased expansion of antigen-specific CD8$^+$ T cells. Interestingly a later application of this antibody was not accompanied by a persistence of increased numbers of T cells in liver or spleen.

In this study we demonstrated that the coinhibitory molecule PD-1 limits the expansion of CD8$^+$ T cells after vaccination rather than preventing their contraction. Therefore a transient blockade of the PD-1/PD-L1 pathway during vaccination might be a promising strategy to increase the number of responding CD8$^+$ T cells.
Vaccination against retroviral infection using adenoviral vectors encoding immunostimulatory cytokines as genetic adjuvants

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A safe and efficient vaccine against retrovirus infection remains an unmet challenge. We are working with Friend Virus (FV) infection as a mouse model of retroviral infections. FV is a murine retroviral complex consisting of the Friend murine leukemia virus F-MuLV which is replication-competent but apathogenic and the Spleen Focus Forming Virus which is pathogenic but replication-deficient. In susceptible mice, FV infection leads to splenomegaly and lethal erythroleukemia. Immunization of susceptible mice with an adenovirus-based expression/display vector Ad.pIXgp70, that encodes the transgene F-MuLV Env gp70 and displays it on the capsid because of its fusion to the adenovirus capsid protein pIX, was found to confer strong protection against FV infection with low viral loads in plasma and spleen, enhanced CD4+ T-cell responses and high binding antibody titers, but neutralizing antibodies were detectable only after FV challenge. To further improve the effectiveness of the vaccine with regard to neutralizing antibody induction, here the adjuvant effect of adenoviral vectors encoding different interleukins involved in the development of TH2 cells or in B cell maturation was analyzed.

Mice were immunized twice with Ad.pIXgp70 in combination with adenoviral vectors encoding the interleukins IL4, IL5, IL6, IL7 or IL23, using a heterologous combination of Ad5 and Ad5F35 vectors. Virus-specific CD4+ T-cell responses and antibody titers were analyzed, and protection from FV infection was assessed in a challenge experiment.

Lower viral loads in plasma and spleen were observed in mice which were coimmunized with adenoviral vectors encoding IL5, IL6 or IL23 in comparison to mice which only received Ad.pIXgp70. The improved protection mediated by Ad.IL23 correlated with increased neutralizing antibody titers 10 days after challenge infection as well as higher virus-specific CD4+ T-cell responses. The improved protection mediated by Ad.IL5 correlated with improved neutralizing antibody titers, and coimmunization with Ad.IL6 resulted in enhanced virus-specific CD4+ T-cell responses.

The coapplication of adenoviral vectors encoding IL5, IL6 or IL23 has an adjuvant effect and further improves the vaccination efficiency of Ad.pIXgp70 which correlates with increased neutralizing antibody titers and enhanced virus-specific CD4+ T-cell responses.

From needle prick to Th1 and Th17 immunity: deciphering mechanisms of glycolipid adjuvanticity.

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Recombinant subunit vaccines are a safe alternative to live whole cell vaccines, but only weakly immunogenic and thus require adjuvants to induce T cell immunity. The choice of adjuvant critically determines the type of memory response elicited, depending on the receptors and pathways triggered in antigen presenting cells (APC), thus directing T helper cell differentiation. The novel glycolipid adjuvant Trehalose-dibehenate (TDB), a synthetic analog of the mycobacterial cord factor, potently induces Th17 and Th1 immune responses. TDB triggers the C-type lectin receptor Mincle to activate Syk-Card9-dependent activation of APC. In addition, MyD88-dependent signaling through the IL-1 receptor is required for TDB adjuvanticity in vivo.

In order to provide new insight into the adjuvant’s mode of action, we followed the vaccine’s and APC’s fate in vivo in wildtype as well as Mincle−/− and MyD88−/− mice. Adjuvant was targeted to APC equally well in all mice. However, results indicate that the co-stimulatory capacity of APC is impaired if Mincle or MyD88-mediated signals are missing. Monocytes and neutrophils are recruited abundantly to the site of injection, but their functional role for generation of the T cell response is unknown. Apart from the TDB-mediated influx of neutrophils, kinetics in Mincle−/− were remarkably comparable to those of wildtype controls. In order to dissect the role of monocytes and neutrophils, we performed depletion and cell transfer experiments. Our results indicate that monocytes play an important role in development of antigen-specific adaptive immune responses upon immunisation with a glycolipid-containing adjuvant, whereas neutrophils, even though strongly recruited to the injection site, are dispensable.
Vaccination of tumor-burdened mice with a triple-functionalized nanoparticle engineered to address dendritic cells and to codeliver antigen and adjuvant induces potent therapeutic anti-tumor responses

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Dendritic cells (DC) are professional antigen-presenting cells with the potential to either activate or inhibit immune response. Exploitation of the immune-regulatory capacities of DC holds a great promise for the treatment of cancer. In this project the usability of functionalized, nano-sized solid core particles (NP) to induce potent anti tumor immunity was assessed. In this regard, three issues were considered in terms of functionalization: Due to the inherent ability of CD8⁺DEC205⁺ DC to efficiently cross-present antigens and thereby prime CD8⁺ T cells, NP were conjugated with a DEC205-specific antibody (aDEC205) to target this DC population in vivo. In addition, NP were coated with the model antigen ovalbumin (OVA), constitutively expressed by the B16 melanoma subline (B16/OVA) used for subcutaneous tumor inoculation. To ensure potent activation of targeted DC, NP were also coated with immunostimulatory CpG oligonucleotides (ODN) that serve as a TLR9-binding ligand.

In vivo studies revealed superior efficacy of this trifunctional NP formulation (NP[OVA+CpG+aDEC205]) to evoke antigen-specific T cell proliferation (CD4⁺, CD8⁺), and to induce cytotoxic T lymphocyte responses, as compared with other types of NP (NP[OVA], NP[OVA+CpG]). Accordingly, in a therapeutic B16/OVA melanoma model, only tumor-burdened mice vaccinated with trifunctional NP showed a pronounced anti-tumor response as reflected by an arrested tumor growth and a significantly higher survival rate as compared with groups of mice left untreated or vaccinated with either of the other NP formulations.

Interestingly, the frequency of MDSC was reduced in case of vaccination with either the non-targeted NP formulation that codelivered OVA and CpG or the aDEC205-targeted trifunctional NP formulation. Our preliminary data suggest that MDSC engulf NP as well, and are affected in their state of differentiation by immunostimulatory CpG ODN, which may contribute to the DC-dependent induction of an anti-tumor response.
Vaccination and Immune Memory

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From mouse to man: Mincle expression in human myeloid cells and recognition of mycobacterial cord factor

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Induction of a robust cellular immune response is essential if vaccinating against intracellular pathogens such as Mycobacterium tuberculosis. The glycolipid Trehalose-dibehenate (TDB), a synthetic analogue of the mycobacterial cord factor, induces a strong Th1/Th17 cell-mediated immune response in animal models and has entered Phase I clinical studies.

The macrophage-inducible C-type Lectin receptor Mincle has recently been identified as a FcRγ-dependent receptor for cord factor and TDB by us and others. Yet it is unknown whether human Mincle is able to recognize TDB and if the same signaling pathway via Syk/Card9 is induced in primary human antigen presenting cells (APC).

To address these questions we first analyzed the expression of Mincle and other related C-type lectins such as MCL, Dectin-1 and Dectin-2 in different human antigen presenting cells by qRT-PCR. Mincle is highly expressed in human APC. Highest basal levels of Mincle mRNA were detected in purified granulocytes followed by CD14+ monocytes.

Purified PBMC and monocytes obtained from healthy donors as well as monocyte-derived M1 and M2-like macrophages and dendritic cells were stimulated with glycolipids in vitro for 24-48h and analyzed for their proinflammatory cytokine production. Performing ELISA and CBA we measured a significant increase of several cytokines following TDB stimulation. Furthermore, retroviral transduction of murine Mincle-deficient DC with the human Mincle receptor restored the production of TNF and G-CSF in response to TDB and cord factor, emphasizing a similar role of Mincle for cord factor recognition in mouse and man.

Our data indicate that human APC are able to respond to TDB stimulation and it is likely that the C-type lectin Mincle mediates TDB recognition in a FcRγ- and Syk/Card9-dependent manner in mouse as well as in human APC.
Mouse models and protein candidates - finding an effective vaccine against *Chlamydia trachomatis*

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*Chlamydia trachomatis* caused genital infections are, with more than 90 million new cases occurring each year, one of the most frequent sexually transmitted diseases (STDs) worldwide. Typical disease sequelae include cervicitis, endometritis, salpingitis, infertility and abortions. Recurrent infections increase the likelihood of these complications. Although *Chlamydia* genital tract infections can be cured with antibiotics, a definitive control of *C. trachomatis* caused STDs is only possible through the development of a safe and efficacious vaccine. Therefore we are establishing an adequate mouse model using the human pathogenic *C. trachomatis* strain UW-3/Cx and the *C. muridarum* (MoPn), which is adapted to *Mus musculus*. To develop a genital infection model we used female mice of the C57BL/6 strain, which we infected with titrated amounts of *Chlamydia*. In both models we could observe changes in the tissues of the genital tract. While we only noticed mild redness and tumescence after infection with *C. trachomatis*, these symptoms were more pronounced when using the *C. muridarum* (MoPn) strain. Additionally we could detect Hydrosalpinx and an enlarged spleen size in the *C. muridarum* (MoPn) model. FACS-analysis showed a three-fold higher granulocytes level in infected mice compared to control mice. Furthermore we measured an increased systemic cytokine level in the blood serum post infection. In contrast we observed reduced cytokine levels in the uterus. Moreover, we monitored the process of the chlamydial infection by incubating HEp2-cells with vaginal swabs and digested uteri. We found that the chlamydial burden peaked on day 4 and subsequently gradually decreased until 23. These results were confirmed by performing qRT-PCR. The developed infection model systems will be used to test the effectiveness of our vaccine candidates.

To produce a potent vaccine, we clone, express and purify immune-dominant proteins. We decided to use the major outer membrane protein (MOMP) on the one hand and the polymorphic outer membrane proteins (Pmp’s) on the other hand. These surface exposed antigens presumably function as *chlamydial* adhesion molecules and are recognized by CD4+ T cells and B cells. We will test these antigens in combination with different adjuvants, in particular CpG.
Molecular basis of immune enhancing effects of an RNA-based adjuvant


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Adjuvants are used to enhance immune responses against weak antigens. Currently, only few adjuvants are approved to be used in the context of human vaccines. In this study, a new RNA-based adjuvant (RNAdjuvant®) was characterized. Upon RNAdjuvant® stimulation of bone marrow-derived myeloid dendritic cells (BM-mDC) or plasmacytoid dendritic cells (BM-pDC), Toll-like receptor (TLR)7-dependent induction of the activation markers CD69 and CD86 was found on both DC subsets. Similarly, after RNAdjuvant® treatment of mice, a TLR7-dependent activation of DC was detected in draining lymphoid organs. Type I interferon (IFN-I) produced by both, cDC and pDC, turned out to be one effector molecule. After i.m. administration of RNAdjuvant®, induction of IFN-I as well as the activation of adaptive immune cells was detected inside the draining lymphoid organ exclusively.

To investigate the immune enhancing effects of RNAdjuvant®, mice were immunized with non-adjuvanted H1N1 flu vaccine Celvapan® or with the trivalent subunit vaccine Influvac®. The adjuvanted vaccine induced increased flu-specific antibody responses when compared to the non-adjuvanted vaccine. Especially, T<sub>H</sub>1-dependent IgG2 responses were increased. In conclusion, the experiments performed in this study revealed that the adjuvant capacity of RNAdjuvant® is associated with an excellent safety profile.
An Artificial PAP Gene Breaks Self-tolerance and Promotes Tumor Regression in the TRAMP Model for Prostate Carcinoma

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Prostate cancer (PCa) is the most commonly diagnosed type of cancer in men in western industrialized countries. As a public health burden, the need for the invention of new cost-saving PCa immunotherapies is apparent. In this study, we present a DNA vaccine encoding for the prostate-specific antigen prostatic acid phosphatase (PAP) linked to the J-domain and the SV40 enhancer sequence. The PAP DNA vaccine induced a strong PAP-specific cellular immune response after electroporation (EP)-based delivery in C57BL/6 mice. Splenocytes from mice immunized with PAP recognized the naturally processed PAP epitopes, indicating that vaccination with the PAP-J gene broke its self-tolerance against PAP. Remarkably, DNA vaccination with PAP-J inhibited tumor growth in the Transgenic Adenocarcinoma of the Mouse Prostate (TRAMP) mouse model that closely resembled human PCa. Therefore, this study highlights a novel cancer immunotherapy approach with the potential to control PCa in clinical settings.

![Diagram of DNA vaccine coding for PAP-J, PAP-S, and PAP-J](image1)

**Figure 1**

![Graph showing immune response](image2)

**Figure 2**
Subviral dense bodies of human cytomegalovirus trigger DC maturation and induce a broad neutralizing antibody response

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Infection with the human cytomegalovirus (HCMV) may cause serious and life-threatening clinical conditions in individuals with an immature or compromised immune system. For disease prevention, an effective vaccine should target both cellular and humoral immune responses. To meet this goal, we focused on the use of subviral dense bodies (DB) of HCMV as the basis for vaccine development. These particles contain some of the most prominent antigens and glycoproteins in their natural conformation, rendering DB particularly interesting as a vaccine. Our current experiments demonstrate that DB have a unique capacity to efficiently trigger maturation of human monocyte-derived dendritic cells (DC). DB loading of DC resulted in up-regulation of co-stimulatory molecules as well as MHC complexes. Furthermore these DC were functionally active, as indicated by their release of cytokines and their capacity to stimulate T cell responses. In further analyses we tested if DB immunization induced neutralizing antibodies (NT-Abs), directed against a pentameric complex of viral envelope proteins, consisting of the polypeptides gH/gL/UL128-131A. This complex is exclusively found on clinical isolates of HCMV. For immunization studies we generated a variant of an HCMV laboratory strain that expressed the pentameric complex (Towne_rep). DB from Towne_rep led to the induction of a broad neutralizing antibody response. Taken together, these experiments demonstrate the distinct immunogenic properties of DB that render them interesting for further evaluation as a vaccine.
Delivery of functional DNA and messenger RNA to murine and human antigen-presenting cells by recombinant yeast

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The import of functional nucleic acids like DNA and messenger RNA into mammalian cells has proven to be a powerful tool in cell biology and several delivery systems have been described. However, as targeting of particular cell types is a major challenge and DNA/mRNA vaccination represents a promising means for the induction of cellular immune responses, there is a need for novel delivery systems that permit the introduction of functional nucleic acids to immune cells. Here, we describe a delivery system based on the yeast *Saccharomyces cerevisiae* that allows the delivery of functional DNA and mRNA to mammalian antigen-presenting cells such as human dendritic cells. Further, we prove antigen processing and presentation by stimulation of human autologous T lymphocytes after transfer of antigen-encoding nucleic acids. In conclusion, this new yeast-based system suggests itself as a promising approach for the development of a novel type of live vaccines [Walch et al. (2012), Gene Ther 19; 237].
Enhancing Tetanus Toxoid (TT) specific cellular immune responses through targeted delivery of TT-peptide containing Nanocapsules to human Dendritic Cells

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Question:
Targeted delivery of antigens to Dendritic Cells (DCs) is a promising approach to induce antigen specific cellular immune responses. Inducing protective immunity is of particular interest in neonates, since the evolving immune system often lacks the ability to respond to common vaccines. Aim of the present study was to evaluate the properties of nanocapsules (NCs) with encapsulated Tetanus Toxoid (TT) peptide and functionalized with anti-CD40, and MPLA and IFN-γ with respect to the overall uptake and the released cytokine profile by human DCs. In addition, the ability of DCs after phagocytosis of TT-NCs to induce recall immune responses in autologous T-cells from immunized humans and to induce immunity in T-cells from naïve Neonates was investigated.

Material & Methods:
NCs consisting of hydroxyethyl starch (HES) served as a carrier for TT-peptide, anti-CD40, MPLA and IFN-γ. Cord blood was acquired from caesarean sections of healthy full-term newborns with healthy mothers. Adult samples were obtained from healthy volunteer donors with documented Tetanus vaccination. PBMCs were isolated via Ficoll density centrifugation. DCs were generated from CD14+ cells cultured in the presence of IL-4 and GM-CSF for 4 days. Autologous CD4+ T cells were co-cultured with DCs after loading the DCs with various formulations of Nanocapsules. Uptake of NCs by DCs was investigated by flow cytometry, cytokine response of DCs to NCs by cytometric bead array, and the response of autologous T cells to TT loaded DCs a. o. by CFSE proliferation assay.

Results:
All formulations of NCs were tested with respect to their effect on the viability of DCs. Phagocytosis of NCs by DCs was evidenced by confocal microscopy. The release of NC content was documented through encapsulation of CellTracker. NC uptake by DCs was significantly enhanced by functionalizing of NCs with anti-CD40, anti-DEC205 and MPLA. Interestingly, combined coating of NCs with anti-CD40 and MPLA or anti-DEC205 and MPLA had the most pronounced effect on uptake by DCs. With respect to cytokine profile coating with MPLA and anti-CD40 evoked a TH1 type profile by DCs. Simultaneous administration of TT peptide along with anti-CD40, IFN-γ and MPLA induced enhanced TT specific immune responses in autologous T-cells.

Conclusion:
HES-NCs are a promising delivery system which can be exploited for targeting antigens to human DCs. Simultaneous delivery of antigen along with signals which promote TH1 type immune responses, offers the opportunity to overcome tolerance and to induce antigen specific cellular immunity.
A Modular Approach for Epitope Discovery and High-Resolution Profiling of Humoral Immune Responses

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Humoral immune responses can be displayed by circulating antibodies that recognize a vast repertoire of tumor specific antigens. The epitope pattern of relevant antigens recognized by antibodies is patient-specific and may change under disease progression, therapeutic intervention and other circumstances. This “epitope spreading” is not limited to cancers but is also observed in infectious and autoimmune diseases and allergies. Discovery of epitopes as well as deconvolution of epitope changes and spreading may lead to new biomarkers, individualized therapeutic vaccine strategies and enables correlation of humoral immune responses with clinical outcome and patient stratification.

High density peptide microarrays have been proven to be efficient tools to map the immune response at a single epitope resolution for large panels of antigens requiring only minute amount of serum or other patient samples (1 µl per assay) to perform a complete analysis [Barouch et al., 2013].

Herein we present a comprehensive but modular humoral immune response profiling platform allowing a systematic and high resolution analysis of the individual antibody repertoire comprising the following techniques:

1. Multiplexed Epitope Discovery: a limited number of samples (typically 20 to 200) are profiled on PepStar™-high content peptide microarrays displaying up to 6912 peptides in triplicates. This allows the coverage of a broad range of known and potentially relevant cancer antigens for multiple cancer types.

2. Selective Antigen Profiling: pre-selected antigens and peptides will be tested against a large number of samples (typically 200 to 1000). For this purpose PepStar™ Multiwell Microarrays will be used taking advantage of parallel and economic testing of 21 patient samples on a single microarray. The format is limited to 192 candidate peptides (in triplicate) per slide allowing systematic profiling of candidate peptides with large numbers of test samples.

3. Marker Validation: a robust peptide ELISA platform was developed based on highly purified peptides allowing both, the validation of the selected candidates as well as detailed analysis of the recognition. The platform enables transfer of results towards diagnostic assays and the selection of vaccine candidates.

The performance of this new platform is exemplarily demonstrated for selecting relevant epitopes from a large panel of antigens of the cancerogenic Epstein-Barr Virus.

Vaccination and Immune Memory

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Developing an enhanced transcutaneous immunization with Imiquimod to treat malignant diseases

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Vaccination is one of the most successful medical interventions within the past two centuries. Nevertheless, effective vaccines are overall only available for a limited number of infectious diseases, and infections remain the major cause of death worldwide. Hence there is an existing and even growing need for new immunization strategies to overcome the persistent difficulties related to classical vaccination approaches: one of the major problems being that most vaccines are only effective in prophylaxis, but not in the treatment of diseases. This may also be one of the key steps in the development of effective cancer vaccines.

Transcutaneous immunization (TCI) approaches are gaining increasing interest. Besides the advantage of self-medication, not requiring skilled medical personnel for application, such easy to use vaccines may be suitable for the use in 3rd world countries, mass vaccination programs and beyond this lack the need for injections.

In this context, we have developed a TCI method based on a synthetic TLR7 agonist Imiquimod which is the active component of the commercially available Aldara™ crème. Imiquimod is a synthetic imidazoquinoline derivative that activates DC in vitro by inducing DC maturation along with the release of inflammatory cytokines. Beyond this, imiquimod also has a proven efficacy in patients with human papillomavirus-associated or malignant skin diseases by topical administration. However, CTL responses induced by TCI with Imiquimod and a cognate peptide (50 mg Aldara/100 µg SIINFEKL, d0+d1) rapidly fade away, resulting in poor memory formation and only partial tumor protection. Concerning the mechanisms behind TCI-induced immune responses, we recently were able to identify bone marrow derived CD11c+ dermal DC as the essential APC population for CTL priming in this situation and that regulatory T cells and the presence of IL-10 have suppressive capacities in this situation.

Now the aim of our project is to optimize the pharmaceutical formulation of TCI incorporating our recent insights into the underlying mechanisms concerning cell participation and cytokine profile. To characterize our new Imiquimod based freeze-dried formulation we first used a Franz diffusion cell system to evaluate permeation through mouse skin in comparison to Aldara™. We found that imiquimod was readily released from Aldara™ and that our selfmade formulation shows markedly decreased drug release through mouse skin. In the next step we determined the vaccination potency by the analysis of peptide-specific T cells as well as in vivo cytolytic activity and detected a significant enhanced immune response after treatment with the new formulation. Analysing DCs, which migrated from the skin in the draining lymph nodes, revealed a more activated phenotype than after Aldara™ treatment.

Collectively, these results clearly support the notion that Aldara™ is not an ideal imiquimod preparation for vaccination purposes and demonstrate that we are able to generate superior formulations for topical application that overcome current limitations concerning for example cell-targeting for stimulation or circumvent suppressive processes.

All together the development of revised preparations and the deeper understanding of the underlying mechanisms may harbour the key for the rationale design of a next generation transcutaneous vaccination platform that can be used for the treatment of persistent infections and cancer.
Analysis, Isolation and *in vitro* Affinity Maturation of Target-Specific vNAR Molecules from Shark Antibody Repertoires

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Fish represent the oldest and most diverse group of vertebrates with over 29,000 species. Within this group, the cartilaginous fish represent the oldest extant jawed vertebrates, having evolved for more than the past 400 million years. This evolutionary ancient class already contains an adaptive immune system. Despite the fact that fundamental molecules of the immune system are similar in fish and mammals, chondrichthyans comprise a unique antibody-like molecule, named Novel Antigen Receptor (IgNAR), which is a homodimer of heavy chains devoid of light chains. Hence, the antigen-binding site is composed of only one variable domain, vNAR (fig. 1). Like camelid VHHs, vNAR domains have several structural and biophysical features that render them interesting molecules for biomolecular applications: vNARs are extraordinarily stable proteins and have remarkable folding properties. Furthermore, due to the loss of a conventional CDR2, the vNAR domain is the smallest naturally occurring antigen binding domain which leads to greater tissue mobility and to the possibility to target more cryptic and recessed epitopes otherwise accessible only to small molecules. [1, 2]

We analyzed the vNAR repertoire and the tissue-specific expression of this antibody-like molecule of the bamboo shark (*Chiloscyllium plagiosum*). Furthermore we developed a method for the generation and *in vitro* affinity maturation of antigen-specific vNAR molecules from semi-synthetic libraries. Binders against cell surface receptors were isolated using Yeast Surface Display[3] and further optimized via *in vitro* affinity maturation yielding shark vNAR antibodies with nanomolar affinities. This type of molecule may be a valuable tool for various diagnostic applications.


fig. 1: vNAR model structure. vNAR model was built based on a published crystal structure (PDB-ID: 1VES) through molecular replacement and energy minimization using YASARA structure and the AMBER03 force field. Images were rendered with POVRay.
Food-borne zoonotic infections of humans are still a major problem in Europe. Over 99000 people were infected with Salmonella in the year 2010 (EFSA), some with fatal outcome.

The control of infections in birds, which are asymptomatic carriers, is an important strategy to prevent human infections. Therefore, a comprehensive understanding of the immune response of chicken is crucial to develop new control strategies. In order to better understand the innate response of birds to infection, we performed global gene expression analysis using the micro-array technology, which led to the identification of candidate genes supposed to play an important role in the early immune defence of chicken against Salmonella enterica infections.

The CC-chemokine K203, which is supposed to be an ortholog of human CCL16, is amongst the most strongly induced genes in Salmonella infected primary macrophages and gut tissue. Even though this chemokine was identified more than 10 years ago, its function is still entirely unclear.

More detailed gene expression analysis of activated primary chicken macrophage cultures revealed that K203 is induced by a broad range of pathogen associated molecular patterns and IFN-γ.

In addition, K203 mRNA is rapidly induced upon Salmonella infection in ceecal tissue and remains strongly elevated for at least 14 days p.i. suggesting an important role in immune cell attraction and pathogen clearance. In order to identify K203 responsive cells, we cloned and expressed this chemokine as Fc-tagged version and performed binding studies with blood and spleen derived leucocytes. 100% of blood monocytes and subpopulations of CD4+ and CD8+ T-lymphocytes bound K203-Fc in a dose dependent manner. Two putative receptors were identified by chicken genome analysis (Kaiser et al.,2006). In order to identify the K203 receptor we cloned both molecules and expressed them in HEK293. Binding studies with K203-Fc showed that both CC chemokine receptors termed chCCRa and chCCRb bind K203.
Identification of Interferon-regulated genes in the chicken

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Type I interferons (IFNs) are an indispensable part of the early innate immune response against various infections. Receptor binding of IFNs induces IFN-regulated genes (IRGs), which mediate the main functions of IFNs. To get a better understanding of IRGs in chickens we (I) performed extensive comparative database analysis and (II) analysed the transcriptome of spleen and lung after intravenous injection of type I IFN and (III) performed transcriptome analysis in an lung infection model with NDV.

Database analysis revealed 1,400 “common IRGs”, which were identified in mammals and found in the chicken genome. Interestingly only 30% (IFN) and 50% (NDV) were confirmed in vivo after IFN and NDV stimulation, and only 20% of common IRGs were regulated in both experiments.

In addition to “common IRGs”, many “newly identified IRGs” and potentially chicken specific IRGs were found, which were regulated, but not in the mammalian databases.

IFN induced gene expression differed significantly between lung and spleen with distinct expression profiles of single genes and gene groups over time, which led the identification of tissue specific IRGs.

Gene Ontology analysis revealed less involvement in “apoptosis” of the “common IRGs” after NDV infection than after IFN injection, a possible result of a viral evasion strategy. Both “common” and “newly identified IRGs” were involved in many immune relevant pathways like “complement and coagulation cascades”, “Toll-like receptor signaling pathway” and “cytokine-cytokine receptor interaction”. Relevance of these genes is supported by the fact that many of the genes with highest changes in RNA-abundance are cytokines (e.g. Interleukin-6 and Interleukin-22) and chemokines (e.g. the lymphocyte attracting CCL19), and strong cell migration into the lung after NDV infection could be confirmed by histology and flow cytometry.
Milk haptoglobin - immunological biomarker for health monitoring of dairy cows

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Introduction:
After calving and in early lactation highly producing dairy cows show impaired immune functions. Additional physiological stress is caused by metabolic and hormonal transitions, which further increase the disease susceptibility. On-farm health monitoring routines are mostly still based on frequent controls by the farm staff or the veterinarian and monthly milk quality controls. Health monitoring has to be adapted to allow adequate control of an increasing number of animals and keep a high standard of herd health and welfare. For the automated and objective recording of health-related data immunological parameters such as acute-phase proteins (APPs) in milk are easily accessible and simple to measure. Haptoglobin (HP) is one of the major APPs in cattle. Increased concentrations in serum and milk have been associated with various infectious and inflammatory conditions but HP is mainly discussed as indicator for mammary gland infections. The post-partum extra-mammary infections and disorders, including uterus and respiratory tract infections, claw diseases or abomasal displacement, were focused in this study.

Objective:
Objective was to identify and validate a biomarker in milk which indicates the overall health status of the cow and further to develop an appropriate immunoassay that is suitable for routine analysis.

Animals and methods
Blood and milk samples of cows (healthy, mastitis, extra-mammary infections or disorders: uterus infections, enteritis, peritonitis, pneumonia, claw infections, sepsis) were collected in co-operation with local farms and animal clinics. HP in milk and plasma was quantified by ELISA. Statistical evaluation was performed by Mann-Whitney U-Test and ROC analysis. For validation purposes the suitability of milk HP measurement should be pre-assessed for usage in on-farm and clinical routines. Milk and plasma samples of selected animals were collected on-farm until 100 days after calving or during treatment of extra-mammary inflammations or disorders in the animal clinic. HP concentrations were subsequently determined by ELISA.

Results:
Confirming results of former studies, HP in milk of cows with clinical mastitis was highly increased (AUC= 1,00). Cows with mild extra-mammary diseases (early diagnosis on dairy farms) showed an elevated HP level in milk (AUC= 0,68). During severe extra-mammary inflammations or disorders (cows in animal clinics) HP concentration in milk was also significantly increased (AUC = 0,98). In early lactation several periods of increased milk HP revealed events of acute-phase response in individual cows to which no corresponding condition had been recorded under the current control system of the farm. Application of the test for therapy monitoring showed that plasma HP detection seems to be a more sensitive and faster endpoint for recognizing therapeutic effects following medication or surgical intervention than milk HP.

Conclusion:
Our results suggest milk HP as a biomarker of inflammation in cattle. Using a specially developed ELISA system milk HP measurement could significantly discriminate healthy cows and cows with clinical mammary or extra-mammary inflammations. Further validation is necessary to implement HP measurement for on-farm applications, it is a promising technique for advanced herd health and welfare monitoring programs to obtain objective, health-related data and show otherwise undetected cases of infection or disease.
Molecular characterization of pro-inflammatory cytokines interleukin-1β and interleukin-8 in Asian elephant (*Elephas maximus*)

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Introduction:
The Asian elephant is an endangered species listed in Appendix I of the Convention on International Trade in Endangered Species. Factors such as habitat loss, human-animal conflict and infectious disease contributes to the endangered status of the Asian elephant. With infectious disease, outcome is dependent on both direct pathogen-mediated damage to infected tissues and host immune system mediated effects. Host immune responses are modulated and enhanced by cytokines. Cytokines act on immune cells both locally and systemically to regulate cell proliferation, differentiation, and activation. Cell-mediated immune responses play a critical role in resistance against the virus and intracellular bacterial infections. Detection of cytokines in disease status may be useful in disease diagnosis as cytokines play important roles in T-cell differentiation. IL-1β and IL-8 are pro-inflammatory cytokines produced primarily by monocytes and macrophages in response to a variety of microbial and non-microbial agents.

Objectives:
As yet, no molecular data have been reported for IL-1β and IL-8 of the Asian elephant. The objective of this study was to characterize the IL-1β and IL-8 genes from Asian elephant at molecular level.

Materials and methods:
PBMCs were separated from elephant blood and cultured overnight in anaerobic environment. Concavalin A was applied during culture since stimulated PBMCs known to produce cytokines. Total RNA was isolated by NucleoSpin® RNA II Kit (Macherey-Nagel). The cDNA was synthesized using ProtoScript M-MuLV First Strand cDNA Synthesis Kit (New England Biolab). IL-1β and IL-8 genes were amplified from cDNA, using primers designed based on the published IL-1β and IL-8 sequences. The PCR products were purified using AxyPrep™ DNA Gel Extraction Kit (Axygen,U.S.A.). Further, both purified amplicon and the vector were ligated together by T4 DNA ligase and ligated mixture was transformed into DH5α. Positive clones were confirmed by restriction enzyme digestion using EcoRI and subsequently sequenced. The sequence data of both genes were submitted to GenBank, for which the assigned Accession number are KC633192 and KC633191, respectively. The nucleotide and deduced amino acid sequences were analyzed using the bioinformatics tools.

Result:
The open reading frame of Asian elephant IL-1β is 789 bp in length, encoded a propeptide of 263 amino acid polypeptide. The predicted protein revealed the presence of IL-1 family signature motif and an ICE cut site. Whereas, IL-8 contained 321 bp of open reading frame. Interestingly, the predicted protein sequence of 106 aa, contains an ELR motif immediately upstream of the CQC residues, common in all vertebrate IL-8 molecules. Identity levels of the nucleic acid and deduced amino acid sequences of Asian elephant IL-1β ranged from 68.48% (squirrel monkey) to 98.57% (asian elephant), and 57.78% (mouse) to 98.47% (sheep), respectively, whereas that of IL-8 ranged from 72.9% (Human) to 87.8% (African elephant), and 63.2% (primates) to 74.5% (African elephant, buffalo), respectively. The phylogenetic analysis based on deduced amino acid sequenced showed that the Asian elephant IL-1β and IL-8 were most closely related to African elephant. Characterization of these two cytokines in Asian elephant provides fundamental information necessary to progress the study of functional immune responses in this animal and gives the potential to use them to manipulate the immune response as recombinant proteins.
Fig. 1. Phylogenetic tree based on nucleotide sequences of Interleukin-1β from different animal species.

Fig. 2. Phylogenetic tree based on nucleotide sequences of Interleukin-8 from different animal species.
The gut micobiota is critical for the development of the mucosal immune system of chickens

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The development of the immune system critically depends on colonization of the intestinal mucosa by commensal microorganisms. While this has been studied intensively in mice over the last years, no data are available for avian species. In order to investigate the importance of bacterial colonization for the development of the mucosal immune system in chickens, we performed an experiment utilizing germ free chickens and compared the gut immune system with that of birds mono-reconstituted with E. coli (Nissle) and conventionally raised birds. The structural features of the gut immune system were analysed on day 28 after hatch by immunohistology on cryo-sections of caeca and caecal tonsills using chicken specific monoclonal antibodies reacting with B-cells, CD4⁺-, CD8⁺-, γ/δ- and α/β-Tcells, macrophages, NK-cells, IgM, IgY and IgA. Differences were observed in the caecum for all cells of the adaptive immune system, with lower numbers in germ free and mono-reconstituted birds, while the numbers and the distribution of innate immune cells such as macrophages and NK-cells did not seem to be affected. Importantly, caecal tonsills which represent the most prominent organized lymphoid structures in the chickens intestine were macroscopically absent in germ free birds, but visible in most of the mono-reconstituted animals and well developed in conventionally housed chickens. Thus, colonization of the gut by commensal bacteria is critical for the development of a fully functional mucosal immune system in birds. These morphological studies will be complemented by gene expression analysis to more precisely quantify this relation.
Autoantibodies recognizing citrullinated proteins (ACPA) are highly specific for rheumatoid arthritis (RA), precede the clinical onset of the disease by years and are the strongest known risk factor for bone loss. We have recently shown that ACPA specific for citrullinated vimentin directly interact with osteoclast precursors and induce bone loss. In patients with RA, ACPA-containing immune complexes can be detected in synovial fluid and tissue. We hypothesized that (I) immune complexes directly promote osteoclast maturation and, consecutively, bone loss and that (II) the type of IgG-glycan is important for the interaction with osteoclast precursors, since ACPA have been shown to be hyposialylated. We differentiated preosteoclasts from human monocytes and stimulated them with artificial immune complexes generated by heat aggregation from pooled human IgG (IVIG). Part of the IgG had been pretreated with neuraminidase or PNGase F to remove sialic acid or the whole Fc glycan, respectively. Stimulation of preosteoclasts with immune complexes resulted in their dramatically increased maturation to osteoclasts. This effect was even more pronounced with complexes formed from desialylated IgG. Monomeric IgG and fully deglycosylated immune complexes did not alter osteoclast maturation. qPCR and FACS analyses revealed that all Fcγ receptors (FcγR) are upregulated during osteoclastogenesis with FcγRI and FcγRIII being the most prominent ones. Desialylated immune complexes induced the activation of spleen tyrosine kinase (Syk) and phospholipase Cγ (PLCγ) as well as the upregulation of the transcription factor c-fos in preosteoclasts. Injection of murine immune complexes into the knee joints of C57-BL/6 mice caused accumulation of osteoclasts in the vicinity of the site of injection. Our data show that IgG immune complexes promote osteoclastogenesis. They upregulate the pro-osteoclastogenic transcription factor c-fos, after binding to activating FcγRs on preosteoclasts. This interaction is highly dependent on the absence of sialic acid in the Fc-glycan of the IgG. Altogether, we propose a novel mechanism by which ACPA promote bone loss independent of inflammation.
Targeting Toso enhances CD8+ T cell responses in murine malaria vaccination and disease

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The molecule Toso was currently identified as the receptor for IgM. It is highly expressed on murine B cells and plays a role in B cell survival and proliferation. Its ligand IgM has been shown to be an adjuvant in vaccine induced CD8+ T cell response. To elucidate whether directly targeting Toso can lead to an enhanced induction of antigen-specific CD8+ T cells, we used a monoclonal antibody against Toso in a malaria vaccination model. In this model, Plasmodium-specific CD8+ T cells mediate protection against liver stage parasites by killing infected hepatocytes. Upon vaccination and anti-Toso treatment, a higher amount of antigen-specific CD8+ T cells was observed. Furthermore, the parasite load in the liver upon challenge of vaccinated mice with Plasmodium berghei ANKA sporozoites was significantly lower in anti-Toso treated mice. To analyze the contribution of B cells to this effect, B cell deficient JHT mice were used. In JHT mice, the enhancing effect of anti-Toso on the CD8+ T cell response could not be observed. Furthermore, we show an enhanced expression of the costimulatory molecule CD86 on B cells upon anti-Toso treatment, providing enhanced B cell costimulation as a possible mechanism for the enhanced priming of the CD8+ T cell response. Similarly, in the model of cerebral malaria, where CD8+ T cells mediate pathology, an exacerbation of cerebral symptoms and an enhanced accumulation of CD8+ T cells in the brains was observed upon anti-Toso treatment. Our data show that targeting Toso enhances CD8+ T cell responses in malaria vaccination and disease and provide a new approach to enhance the efficiency of vaccines for the induction of CD8+ T cell responses.
IgG2a and IgG2c are unequal – distinct alleles, different serum abundance, disparate Fc-gamma-receptor (FcγR) activation capacities

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Immunoglobulin G (IgG) antibodies are a major effector of the adaptive immune response and immunological memory. IgGs are potent tools for the treatment of cancer, autoimmune diseases, bacterial, and viral infections. IgGs neutralise pathogens via antigen binding by their variable Fab-domains. Additionally, the constant Fc-domain triggers cellular responses upon activation of Fc-gamma-receptors (FcγR) like antibody dependent cellular cytotoxicity, enhanced phagocytosis, and cytokine secretion. In mice, four different FcγRs (activating: FcγRI, III, IV; inhibiting: FcγRII) and five IgG subclasses exist, whereas most mouse strains possess only four alleles coding for IgG1, IgG2b, IgG3 and IgG2a (e.g. BALB/c) or IgG2c (e.g. C57BL/6) [1]. Thus the potential analogy of IgG2a vs. IgG2c is a matter of controversial debate.

Mouse cytomegalovirus (MCMV) is a β-herpesvirus causing a persistent infection with phases of reactivation. Virus-specific IgGs are known to limit virus replication in the salivary glands after acute infection and to contribute to virus control during recurrent infection [2]. Binding and activation of FcγRs is known to differ between IgG subclasses. To functionally dissect distinct roles of IgG2a and IgG2c we compared the antibody response against MCMV in BALB/c and C57BL/6 mice. We found comparable titers of neutralizing IgG and total MCMV-immune IgG detected by ELISA in both strains. However, analysis of the IgG subclass composition revealed that the antiviral IgG response was dominated by IgG2a in BALB/c and by IgG2b followed by IgG2c in C57BL/6. Moreover, taking advantage of a newly developed cell-based assay measuring activation of defined FcγRs by MCMV-immune IgG [3], sera from BALB/c mice revealed a much stronger FcγRIII activation capacity contrasting with C57BL/6 sera. This indicated that IgG2a might be a more potent FcγRIII activator than IgG2b and IgG2c.

To systematically analyse the IgG subclass dependent activation of distinct FcγRs, monoclonal IgG antibodies recognizing the same epitope but possessing different IgG subclasses were generated. By FcγR activation assays we confirmed that IgG2a is more potent than IgG2c in activation of FcγRIII, but also FcγRI and FcγRIV. In addition, glycosylation of Asp297 of the IgG heavy chain plays a crucial role for the capabilities of a distinct subclass to activate FcγRs, because production of IgG isotypes in cells of different origin leads to altered FcγR activation.

Taken together, our data imply that although IgG2c genetically substitutes for IgG2a in C57BL/6 related mouse strains, the subclasses are functionally widely different. This finding is of obvious importance considering IgG-FcγR-mediated immune diseases and therapeutic approaches in the mouse model.

Complement and Fc Receptors

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Complement receptor usage of *Leishmania* parasites in different phenotypes of primary human macrophages

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*Leishmania* parasites can infect different human macrophage phenotypes by ligating different surface receptors and thereby triggering parasite uptake. Especially complement receptors are thought to play a critical role in activating or silencing host macrophages after infection. In a previous study we demonstrated a complement receptor-specific uptake of *Leishmania* parasites which was parasite stage-dependent. Now, we focus on the receptor usage of different *Leishmania* species, *L. major* and *L. aethiopica* as causative agents of cutaneous Leishmaniasis and *L. donovani* as origin of visceral Leishmaniasis.

First, we examined the surface expression of CR1 and CR3 in non-infected primary human inflammatory M1, as well as anti-inflammatory M2 macrophages using flow cytometry. Whereas CR3 was highly expressed on both cell types, CR1 was differentially expressed, with an intermediate expression on M1 cells, but low expression on anti-inflammatory M2 macrophages.

Second, we applied specific blocking antibodies against CR1 and CR3 to evaluate species-specific uptake of *Leishmania* parasites into M1 and M2 cells. We found *Leishmania* species-specific differences for the usage of CR1. When blocking CR1 only *L. major* promastigotes exhibited a reduced uptake in M1 cells in comparison to a reduced uptake of *L. aethiopica* and *L. donovani* promastigotes in M2 cells.

Third, siRNA knock-down experiments of CR1 and CR3 have been conducted in M1 and M2 macrophages. The levels of mRNA were successfully reduced more than 80% for both receptors. Preliminary experiments with these knock-down cells suggest a *Leishmania* species- and stage-specific uptake dependent on the different complement receptors.

These results indicate that inflammatory M1 macrophages and anti-inflammatory M2 macrophages differentially express complement receptors. This might have consequences on parasite development dependent on the specific parasite entry mechanism. Especially CR1 might be critical because it is highly expressed on M1 cells, in which *Leishmania* parasites are killed, whereas M2 cells promote parasite development and have a low CR1 expression. Nevertheless, further investigations are necessary to understand possible implications of complement receptors in the outcome of parasite development and its contribution to cause the wide spectrum of Leishmaniasis disease.
Characterization of C5aR expression in a novel floxed C5aR GFP reporter gene knockin mouse

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Introduction:
C5a and its G protein coupled receptor (C5aR) mediate many of the proinflammatory properties of complement. They play important roles in many diseases like sepsis, cancer, autoimmune arthritis and allergic asthma. By using C5aR-specific antibodies, it was shown that the C5aR is expressed on innate immune cells like neutrophils, eosinophils, basophils, monocytes, macrophages, dendritic cells (DC) and activated NK cells, among others. For cells of the adaptive immune system such as B and T cells the data are controversial. For example, it was shown that C5a via C5aR signaling directly regulates the CD4⁺ T cell compartment. The mechanisms underlying this regulation are ill-defined since conflicting data exist regarding the C5aR expression in naive and activated T cells.

Objectives: We aimed at determining the cell-specific expression of the C5aR under steady state and inflammatory conditions in innate and adaptive immune cells.

Material and Methods:
We floxed exon 2 of the C5ar1 coding region with two loxP sites. Further, we inserted a GFP-IRES cassette at the 5' prime end of exon 2. This strategy resulted in a C5ar1 GFP-knockin mouse that can also be used for conditional cell or tissue specific knockout studies. We determined the distribution of GFP-positive cells under steady state and inflamed conditions (12 and 24 hours after i.p. injection of LPS) in several organs and in CD3⁺CD28 stimulated CD4⁺ T cells. Neutrophils, macrophages, DC, NK, B and T cells were identified by lineage-specific markers using flow cytometry.

Results:
We found GFP⁺ cells in the spleen, bone marrow, peritoneum, blood, lung, bronchoalveolar lavage fluid and lamina propria (LP) cells of the small intestine under steady state conditions. In the bone marrow, 30-40% of the cells were GFP⁺, most of which were monocytes and neutrophils. Further, resident peritoneal, lung and LP macrophages and spleen-derived monocytes were GFP⁺. Lung DCs also stained GFP⁺. LPS administration resulted in mobilization of strong GFP⁺ monocytes and neutrophils into the bloodstream, which were also found in the BAL, lung, peritoneum and spleen. Further, GFP⁺ NK cells migrated into the lung, peritoneum and spleen. B and T cells were GFP negative under most tested conditions. CD3 stimulation resulted in a small GFP⁺ population of splenic CD4⁺ T cells.

Conclusion:
We have developed a novel floxed C5aR GFP reporter gene knockin mouse, which offers the opportunity for cell-specific tracking of C5aR expression independent of antibodies. The introduction of two lox P sites around the C5ar1 codon will allow us to conditionally delete the C5aR in immune cells in a cell- or tissue-specific manner. More specifically, we can create conditional knockouts helping us to define the role of C5aR for the induction, maintenance and contraction of B and T cell responses. This mouse will be a helpful tool to investigate the role of the C5aR in the pathogenesis of allergic asthma, autoimmune diseases and infections and its contribution to the regulation of adaptive immune responses.
C5a synergizes with IL10 and Toll-like receptors to control the peritoneal B1 cell compartment

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Introduction:
B1 cells are part of the innate immune system and mediate first line humoral immune responses and T cell-independent long-lasting immunity against encapsulated bacteria. In contrast to their B2 counterparts, B1 cells are characterized by surface expression of IgM, CD11b and low levels of B220. Body cavities like the peritoneum serve as reservoir for B1 cells, whereas their homing and egress are controlled by the chemokine CXCL13 released by resident peritoneal (PE) macrophages and stromal cells. B1 cells function as main source of natural and T-independent IgM antibodies in the body. The mechanisms regulating CXCL13 production from PE macrophages are ill-defined.

Objectives:
To evaluate the role of complement in the regulation of the B1 cell compartment.

Material & Methods:
To dissect the impact of complement and TLR signaling on CXCL13 production from PE macrophages, cells isolated from wt, TLR2, TLR4 and MyD88⁻/⁻ mice were stimulated with different TLR ligands +/- IL-10 or C5a in vitro and produced cytokines were determined by ELISA. To identify the IL-10 signaling pathways activated in PE macrophages, cells were incubated with Stat inhibitors before stimulation. To assess the importance of the complement system, we used C5⁻/⁻ and C5ar⁻/⁻ mice. Furthermore, we determined intracellular C5 expression in adherent macrophages by confocal microscopy. In search for the cellular source of IL-10, PE cells isolated from IL10 GFP reporter mice were stimulated with TLR ligands in vitro and subsequently analyzed via flow cytometry.

Results:
We found that the C5a/C5ar axis is critical for B1 cell functions, since C5ar⁻/⁻ mice have reduced PE B1 cell numbers, lower titers of protective natural and T-independent IgM antibodies. In search for mechanisms, we found that C5a stimulation upregulates CXCL13 production in PE macrophages when given together with IL10. Furthermore, TLR stimulation leads to C5 production in PE macrophages, which is increased by IL10. The TLR-driven CXCL13 and C5 production was abrogated in macrophages from MyD88⁻/⁻ mice. Specific blockade of Stat3 inhibited TLR + IL-10-induced CXCL13 production. Similar to C5ar⁻/⁻ mice, TLR2 and TLR4-deficient mice show reduced numbers of PE B1 cells. First experiments with C5⁻/⁻ mice suggest that C5a may also act directly on B1 cells modulating their IL10 release.

Conclusion:
We describe a novel role for C5a as an important regulator of the B1 cell compartment. Our data support a model in which C5a, TLR and IL10 are critical for the maintenance of the B1 cell pool in the peritoneal cavity by regulation of CXCL13 production from PE macrophages. Intracellular C5 from PE macrophages may serve as an autocrine source of C5a, acting as a positive feedback loop for CXCL13 production. Additionally, C5a may directly stimulate IL10 production of B1 cells. In a positive feedback loop, this cytokine then acts on PE macrophages driving Stat3-dependent pathways to promote CXCL13 release. Taken together, the C5a/TLR/IL10 axis is a crucial regulator of B1 cell-dependent innate immune responses.
A dual role for Fc receptor-mediated inflammation in an active model of experimental epidermolysis bullosa aquisita

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Introduction:
Epidermolysis bullosa aquisita (EBA) is an autoimmune blistering disease characterized by T cell-dependent production of antibodies specific for collagen type VII and immune complex (IC) formation. The subsequent activation of the complement-Fc gamma receptor (FcgR) axis results in the reduction of the activation threshold for activating FcgRs on macrophages and neutrophils thereby enhancing FcgR-mediated inflammation. Such inflammation leads to the separation of dermis and epidermis due to the release of reactive oxygen species and proteases. In contrast, the engagement of the inhibitory FcgRIIB suppresses activating FcgR-mediated inflammation. Previous work from our laboratory has shown that in passive transfer models of EBA, complement and FcgRs synergistically drive and control IC-mediated cutaneous inflammation. More specifically, FcgRIV and C5aR drive, whereas FcgRIIB and Dectin-1 control neutrophil-mediated tissue damage and blister formation. More recently, we developed an active model of EBA, in which cutaneous inflammation is induced by active immunization against the type A domain of the Willebrand factor (vWF). The role of FcgRs in the formation of anti-collagen VII antibodies and disease initiation after type VII immunization is unclear.

Objectives:
We aimed at evaluating the role of activating and inhibitory FcgRs in the production of anti-collagen type VII antibodies and the development of autoimmune blistering diseases in an active model of EBA.

Material and Methods:
To study the role of FcgRs in autoantibody production and disease onset, age- and sex-matched wild type (wt), Fcerg1−/− and Fcgr2b−/− mice were subcutaneously immunized with murine vWF and adjuvant in equal volumes. Antigen-specific serum titers were evaluated every 2 weeks via ELISA. Furthermore, the disease onset was determined by evaluating the affected body surface. Further, deposition of C3 and pathogenic IgG in the skin was determined by immunohistochemistry. The experiment was terminated 12 weeks after initial immunization.

Results:
Whereas wild type mice showed a continuous increase in disease onset resulting in 6.3% affected body surface at week 12, Fcgr2b−/− mice suffered from markedly increased phenotype involving 32.4% of the body surface already at week 6 so that the mice had to be sacrificed due to ethical reasons. In contrast, Fcerg1−/− mice were completely protected and did not develop clinical signs of EBA. However, the different clinical phenotypes did not match the anti-vWF antibody serum titers. In all mouse strains, we found high vWF-specific serum titers (1:40,000) already 2 weeks after immunization, which remained unchanged after 6 and 12 weeks.

Conclusion:
Engagement of activating Fcγ Rs is crucial for the development of clinical EBA in an active model of the disease as Fcerg1−/− mice, which lack functional Fcγ Rs, failed to develop a clinical phenotype. In line with this finding, the absence of the inhibitory receptor Fcγ RIIB strongly accelerates inflammation due to the lack of inhibiting signals on activating FcgRs. These data point towards an important protective role for Fcγ RIIB in active EBA. The strong induction of vWF-specific antibody titers in wt, Fcerg1−/− and Fcgr2b−/− mice suggest that the ligation of activating IgG Fc receptors has no impact on antigen sensitization but is crucial in the effector phase of the disease, which is in line with our data from the passive model of EBA.
Complement Inactivation by CspA Orthologs of *B. burgdorferi*, *B. afzelii*, and *B. spielmanii*

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Introduction:
The Lyme disease spirochete, *Borrelia burgdorferi*, is transmitted to the human host through the bite of infected ticks. Upon entry into the bloodstream the spirochetes are immediately confronted by the host’s innate immune response. The complement system is an integral part of innate immunity and in order to establish a persistent infection and survive in the host, borreliae have evolved a number of mechanisms to evade complement-mediated killing. The outer surface protein CspA of *B. burgdorferi* is the key molecule conferring resistance to complement-mediated killing. CspA contributes to complement resistance by binding host complement regulators such as factor H (CFH) and factor H-like protein-1 (CFHL-1). The CspA orthologs from *B. afzelii* and *B. spielmanii* have been reported to bind both these regulators as well, but unlike CspA of *B. burgdorferi*, these two orthologs have yet to be examined in more detail. In the present work, we compare the different binding capacities of all three CspA orthologs for CFH and plasminogen. We also investigate whether CspA of *B. afzelii* and *B. spielmanii* can directly inhibit complement, irrespective of their ability to bind CFH.

Material and methods:
Employing a gain of function approach, a serum-sensitive *B. garinii* strain was transformed with a vector containing the coding sequence for CspA orthologs of *B. burgdorferi*, *B. afzelii* and *B. spielmanii* under the control of their respective native promoters. Transformants ectopically producing CspA orthologs on the cell surface were then used to study the role of those molecules in serum resistance *in vivo*. Transformants were characterized with respect to their complement-resistance employing a colorimetric bactericidal assay. In addition, recombinant CspA orthologs were used to analyze their potential to inactivate C3b both by binding of CFH and plasminogen. Finally, hemolytic assays were employed to investigate whether CspA orthologs can directly interact with and inhibit complement, irrespective of their ability to bind fluid phase complement regulators.

Results:
Regardless of their origin, all three CspA orthologs impart resistance to complement-mediated killing when produced ectopically in a serum-sensitive *B. garinii* strain. While no difference was observed in CFH binding, CspA orthologs differ in regard to their capacity to inactivate C3b via bound plasmin(ogen) and inhibit formation of the membrane attack complex (MAC). The CspA ortholog of *B. afzelii* binds plasminogen more efficiently when compared to *B. burgdorferi* and *B. spielmanii*, resulting in more efficient degradation of C3b by bound plasmin(ogen). Furthermore, CspA of *B. afzelii* also inhibits the MAC more efficiently than the other CspA orthologs investigated.

Discussion:
Taken together, we found that CspA orthologs of serum-resistant Lyme disease spirochetes act as multifunctional immune evasion molecules: They acquire the fluid-phase complement regulator CFH, they bind plasmin(ogen), which can inactivate C3b and finally, CspA orthologs also directly interact with complement, inhibiting formation of the MAC.
Prognostic values of the soluble adhesion molecules E-selectin, P-selectin, ICAM-1, VCAM-1 and the chemoattractant MCP-1 during the acute and convalescent stages of Mediterranean spotted fever

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The aim of the study was to trace the quantitative changes in the dynamics of soluble cell adhesion molecules (sCAMs) sE-selectin, sP-selectin, Intercellular adhesion molecules-1 (sICAM-1), Vascular adhesion molecules-1 (sVCAM-1), and Monocyte chemoattractant protein-1 (MCP-1) in patients with Mediterranean spotted fever (MSF) in order to reveal their pathogenetic, diagnostic and prognostic values.

Materials and methods:
Indicative for MSF confirmation was the increased antibody titer to Rickettsia conorii by an indirect Immunofluorescence assay. The blood serum of 80 patients was studied during the acute period of the disease and of 60 of the same patients during convalescence. The control group comprised 20 healthy age-matched individuals. To study sCAMs a commercially available Enzyme-linked immunosorbent assay (Quantikine IVD colorimetric ELISA) of R&D Systems, MN (USA) was used.

Results:

sE-selectin as well as sP-selectin levels do not differ statistically between themselves during the acute and convalescent stages of MSF. The levels of sE-selectin are significantly higher in comparison with the controls during both stages (p<0.001), whereas that of sP-selectin are significantly lower than the control ones (p<0.05). There is no significant difference in the sE-selectin values among the mild, moderate and severe forms of MSF during both stages: in the three forms sE-selectin levels remain elevated till the end of the study period. The levels of sP-selectin do not differ statistically among themselves in the three forms of severity during the acute stage of MSF. During the convalescent stage sP-selectin levels distinctly tend to increase and the gradation is directed from the mild towards the severe forms, exceeding the control levels in the latter ones.

During the acute stage of MSF the levels of sICAM-1 and sVCAM-1 increase significantly. The increase of sICAM-1 values is parallel to the degree of severity of MSF and the gradation is directed from the mild towards the severe forms (p<0.001). During the acute stage of disease the levels of sVCAM-1 in the three forms of severity are significantly higher compared to the controls. The concentrations of sVCAM-1 in parallel to the degree of severity and their levels are most elevated in the severe forms of MSF as well (p<0.01). During the convalescent stage sICAM-1 and sVCAM-1 values return to the normal ranges.

The serum levels of MCP-1 are reliably higher than those of the controls (p<0.01) during the acute stage of MSF. The concentrations of MCP-1 increase in parallel to the degree of severity and their levels are most elevated in the severe forms of disease. In the mild and moderate forms the values of MCP-1 are significantly lower than the control ones (p<0.01). During convalescence the quantities of MCP-1 in the three forms of severity do not exceed the controls. Interestingly MCP-1 levels are lower for the mild form in the acute stage compared to the same form in convalescence (p<0.001); the same phenomenon is observed for the moderate form of disease severity (p<0.01). However, the comparison of the severe forms among themselves in both stages shows that the concentrations of MCP-1 are nearly double higher in the acute stage in comparison with the convalescence.

Conclusion:
sCAMs reflect the endothelial inflammatory potency. It is delineated that the endothelial activation is stronger in the severe forms of MSF and its estimation is an early sign to predict the disease outcomes and an orientation mark to choose a therapeutic approach.
Quantifying of adhesion forces of individual immune cells by Single Cell Force Spectroscopy

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The interaction of cells with their external environment plays a key role in multiple cellular processes, from tissue development and cohesion (1), to cell motility, cancer development and metastasis (2), and immunology (3-4). Using single cell force spectroscopy (SCFS) (5), cell adhesion can be quantified, and the contribution of different components e.g. from the extracellular matrix, can be assessed (6).

Using the atomic force microscopy early adhesion phenomena of single living cells can be investigated in pN-force range. We have developed an instrument, data analysis software and workflow to run such measurements on individual cells, in a temperature controlled environment in combination with light and fluorescence microscopy. The CellHesion® system has two different pulling ranges of 15µm/100µm to investigate the binding forces between cell and target surface, between two cells or between cell and cellular monolayer down to the single-molecule level. Based on new literature field of applications in immunology, used cell types and established cell attachment protocols will be discussed (7,8).

3) Flach et al., Nature Medicine 17, 479-487 (2011)
7) Hoffmann et al., J. Immunology 186(5): 2757-64 (2011)
Basophils control T cell Responses and limit Disease Activity in Experimental Murine Colitis

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Basophils have been recognized as important inducers of Th2 responses. Using the experimental colitis model induced by adoptive transfer of CD4+ CD62L+ T cells into lymphopenic hosts we characterized the potential of basophils to modulate T helper cell responses in vivo and regulate disease activity. Transferred T helper cells rapidly proliferate and produce large amounts of IL-3 increasing the number of basophils (FceR1+ CD49b+). Among these expanded cells a subpopulation of basophils became detectable expressing higher levels of FceR1 (FceR1++) with high potential to downregulate the development of colitogenic Th1 cells. Consequently depletion of basophils (using MAR1 or Ba103) substantially upregulated IFN-γ, IL-2 and TNF in proliferating T helper cells in week 1 and 2 after transfer. This proinflammatory profile of initial basophil depleted mice persisted until the end of the experiments (week 10 and 12) leading to exacerbation of colitis with more severe loss of weight, histological damage, colonic leukocyte infiltration and upregulation of Th1 cytokines in colon and plasma. Ex vivo we show that transferred T cells from wildtype but not from IL-3ko mice induce basophil derived IL-4 and IL-6 production in spleen and bone marrow, downregulating expression of IFN-γ, IL-2 and TNF in proliferating T helper cells. These data show a beneficial role of basophils in a T cell driven model of autoimmunity.

Figure legends:
Figure 1: Characterization of basophils after reconstitution of SCID mice with CD4+CD62L+ T cells.
(A-C) SCID mice (n = 6 / group) were reconstituted with 2 x 10^6 CD4+CD62L+ T cells from BALB/c mice (white bars) or received just PBS without T cells (grey bars) and analyzed 7 days later. (A) Plasma cytokine levels were quantified by cytometric bead array. (B) The plasma level of IL-3 was measured by in vivo cytokine capture assay. (C) By staining with antibodies against FcεR1 and CD49b the numbers of FcεR1+ and FcεR1++ basophils were quantified in the spleen of SCID mice 7, 14, 25 and 35 days after reconstitution with CD4+CD62L+ T cells from BALB/c mice. (D) SCID mice were adoptively transferred with 0.5 x 10^6 CD4+CD62L+ T cells from BALB/c mice (white bars) or IL-3 deficient mice (black bars) (9 mice per group). The control group received no T cells (grey bars; 10 mice per group). After 8, 16, 29 and 80 days basophils were quantified in the peripheral blood by flow cytometry with counting beads. Data are represented as mean +/- SEM.

Figure 5: Depletion of basophils with MAR1 aggravates colitis in SCID mice.
(A-D) Basophils were depleted in SCID mice by injections of the antibody MAR1 (black bars) or remained undepleted (white bars) (11 mice per group). 0.5 x 10^6 CD4+CD62L+ T cells from BALB/c mice were adoptively transferred 2 days later (day 0) to induce colitis and mice were monitored until day 84. A control group received no T cells (injection of PBS) (grey bars; 4 SCID mice).
(A) The weight of the mice was measured until day 84. One out of three representative experiments is shown. (B) Flow cytometric analysis of colon tissue and mesenteric lymph nodes of initially basophil-depleted mice (black bars; MAR1) and isotype-treated mice (white bars; Isotype). Leukocytes were identified by expression of CD45. The majority of infiltrating leukocytes consisted of CD4+ T cells. (C) Plasma cytokine levels measured with a cytometric bead array. (D) Histological analysis of colon tissue to quantify epithelial damage and inflammation. Scale bars indicate 100 µm. Data are represented as mean +/- SEM.
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The role of basophils during immune responses against helminths

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Introduction:
Basophils are associated with T helper 2 (Th2) cell-polarized immune responses found during allergic inflammation and parasitic infections. They are able to produce and release high amounts of IL-4, the hallmark cytokine for type 2 immune responses. But the role of basophils in the initiation of Th2 immune responses and their role during the effector phase is still highly debated. Nonetheless, they play a pivotal role during the protective immune response against N. brasiliensis.

Objectives:
We characterized the role of basophils in immune responses against infections with different helminths.

Material & methods:
We established a new mouse strain (Mcpt8Cre) where basophils are specifically and constitutively deleted. Additionally, the mice were crossed to IL-4-reporter mice (4get) to reveal IL-4-producing cells. These mice were infected with the gastrointestinal nematodes Nippostrongylus brasiliensis and Heligmosomoides polygyrus, and the trematode Schistosoma mansoni. The mice were then subjected to FACS analysis, restimulation assays, serum ELISAs and immunofluorescence microscopy.

Results:
Differentiation of Th2 cells during the initial immune response in draining lymph nodes and their accumulation in tissues appeared to be basophil-independent in all three infection models. In the S. mansoni-infection model we could not observe any differences between Mcpt8Cre and control mice in egg counts, number and size of granulomas, collagen expression, eosinophilia and Th2 cell cytokine production. Basophils were also not required for eosinophilia and worm expulsion during primary infections with N. brasiliensis or H. polygyrus. In the immune response during the second infection with these helminths, basophil-deficient mice showed normal IgE and IgG1 antibody production and normal Th2 polarization, albeit delayed during the immune response against H. polygyrus. However, in the absence of basophils the mice exhibited significantly higher worm burdens.

Conclusion:
These results demonstrate the importance of basophils during the memory type 2 immune response against certain helminths, while they appear dispensable for Th2 polarization and antibody production. Basophils therefore constitute a critical component for protective immunity against reinfection with helminths which is probably triggered by helminth-specific antibodies generated during the primary infection.
A complex network of trans diaphyseal blood vessels – highway for bone marrow-derived cells?

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The marrow of long bones is a major source of hematopoietic stem cells, which generate all myeloid and lymphoid blood cells. The mechanisms by which bone marrow derived hematopoietic cells circulate in the vascular system, adhere to the vessel walls and enter the surrounding tissue in case of physiologic stimuli or peripheral inflammation are well understood. However the processes and the structural conditions that permit the emigration from the marrow of long bones into the general circulation are still unclear. The currently available concept is not able to explain the extremely rapid mobilization of bone marrow derived cells to peripheral sites following acute stimuli. Furthermore the blood vessel system of long bones is critically affected by cartilage destructive diseases, which can cause structural changes of the compact bone and the associated blood vessel system. A precise knowledge on the blood vessel system of long bones would therefore be essential to quantify the damage associated with such diseases.

2-photon intravital microscopy of murine long bones demonstrated the presence of multiple pores in the bone surface of the diaphysis potentially filled with small blood vessels. Since bone marrow resident hematopoietic cells use the vascular system to reach the general circulation we hypothesized a functional role of these pores and the associated blood vessels as direct connections of the bone marrow with the surrounding tissue. Therefore we investigated the structural organization of the entire blood vessel system in the marrow and compact part of murine long bones. We also examined which vessels the hematopoietic cells use to enter the peripheral circulation outside of the bone and how the entry of these cells from the bone marrow into blood vessels is regulated.

For the quantification of the pores on the bone surface and the analysis of the enclosed vessels different transgenic mouse strains and multiple established antibodies for blood vessel staining were used. To identify the spatial arrangement and the function of these blood vessels we established variable clearing protocols for murine long bones and analyzed the samples using the advanced visualization techniques optical projection tomography (OPT), selective plane illumination microscopy (SPIM), scanning electron microscopy (SEM), ultramicroscopy and intravital 2-Photon microscopy.

Based on these analyses we could reveal structural differences in pore distribution with defined areas of increased or decreased amounts of pores on the bone surface. We could verify multiple direct blood vessel connections from the bone marrow through the compact bone into the peripheral tissue (trans shaft vessels, TSV) and interestingly we could demonstrate the transport of neutrophils through these blood vessels after systemic injection of G-CSF, a potent neutrophil mobilizer. Furthermore, osteoporotic TNFtg mice demonstrated an altered TSV structure of the compact bone and a reduced mobilization of neutrophils by G-CSF. These findings suggest a so far unknown way for rapid leukocyte mobilization from the bone marrow into the circulation via a complex network of TSV.

The detection of the blood vessel structures in the compact bone, the blood vessel system in the bone marrow and its connection to the TSV provides new insights into the question, how bone marrow derived, hematopoietic cells leave the bone marrow. The importance of the blood vessel network for hematopoietic physiology needs to be investigated further.
Mast cells expedite the control of pulmonary cytomegalovirus infection by enhancing the recruitment of protective CD8 T cells to the lungs

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The lungs are a noted predilection site of cytomegalovirus (CMV) infection, and interstitial CMV pneumonia is the most dreaded manifestation of CMV disease in the immunocompromised host, in particular in recipients of hematopoietic cell transplantation. In the immunocompetent host as well as after hematopoietic reconstitution, lung-infiltrating CD8 T cells confine the infection and prevent CMV pneumonia. By using murine CMV (mCMV) infection as a model, we provide here first evidence for a supportive role of mast cells (MC) in the recruitment of protective, antiviral CD8 T cells to the lungs. Notably, mCMV infection was found to induce a TLR3/TRIF-signaling-dependent activation of MC in vivo. Upon systemic infection, T cells - predominantly CD8 T cells - were recruited less efficiently into peripheral blood and lungs in MC-deficient KitW−sh/W−sh “sash” mice when compared with MC-sufficient congenic WT C57BL/6 or MC-reconstituted “sash” mice. Whilst MC did not notably alter the recruited epitope specificity repertoire, capacity to recognize infected cells, and antiviral effector function of pulmonary infiltrate CD8 T cells, percentages of activated, epitope-specific CD8+CD44+CD62L−KLRG1− short-lived effector cells (SLEC) were moderately increased but their absolute numbers significantly increased in the lungs by the recruitment-promoting function of MC. Most importantly, peak virus titers were found to be lowered and virus clearance accelerated by MC. These results thus reveal a novel cross-talk axis between innate and adaptive immune defense against CMV and identify MC as a hitherto unconsidered parameter in the immune surveillance of CMV infection with relevance for approaches to a cytoimmunotherapy of CMV pneumonia.
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Mast cell-deficient Kit<sup>W<sub>sh</sub></sup> "sash" mice display aberrant myelopoiesis leading to the accumulation of myeloid-derived suppressor cells

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Introduction:
Besides mast cells, the Kit<sup>W<sub>sh</sub></sup> mutation is known to affect other cells including melanocytes and interstitial cells of Cajal. Furthermore, it has been described that the Kit<sup>W<sub>sh</sub></sup> mutation causes splenomegaly and the expansion of Gr-<sup>1</sup>pos cells which has been interpreted as neutrophilia.

Results:
We investigated the latter phenomena in detail and demonstrate that naive sash mice develop extramedullary hematopoiesis in the spleen characterized by the expansion of immature lineage negative cells, common myeloid progenitors and granulocyte/ macrophage progenitors. A consistent feature shared by these cell types is the reduced expression of c-Kit in sash mice. The expansion of CD11b<sup>pos</sup>Gr-<sup>1</sup>pos cells in the spleens appeared mostly due to the accumulation of CD11b<sup>int</sup>/Ly6C<sup>int</sup>/Ly6G<sup>hi</sup> cells with mostly ring-shaped nuclei. These cells are able to suppress T cell proliferation in vitro and thus function as myeloid-derived suppressor cells (MDSC).

Based on our observation of accelerated tumor development in sash mice following transplantation of the tumor cell line L1C2, we investigated whether this effect is based on the absence of mast cells or due to the activity of MDSC. Transfer of bone marrow from sash mice into irradiated wild-type recipients leads to the expansion of MDSC derived from donor bone marrow in the presence of radio-resistant mast cells. Inoculation of these chimeric mice with L1C2 cells accelerates tumor growth to an extent equivalent to sash mice. This mast cell-independent effect on tumor growth was further corroborated by comparing mast cell-deficient Cpa3<sup>Cre/Cre</sup> "Cre-Master" mice and their wild-type littermates. Consequently, transfer of MDSC isolated from naive sash mice into wild-type recipients accelerated L1C2 tumor growth to an extent comparable to that seen in sash mice.

Conclusions:
Thus, it should be considered that the sash mutation causes aberrant myelopoiesis and the expansion of MDSC when sash mice are chosen to investigate the potential roles of mast cells.
Strain-specific effects of LPS and IL-1 on the production of IL-9 in IgE/Ag-activated bone marrow-derived mast cells

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Introduction:
Bone marrow-derived mast cells (BMMC) as part of the innate immune system express a variety of Pattern-Recognition Receptors (PRRs). The Toll-like receptor (TLR) family has emerged as an important family of PRRs. Mast cells express different TLR on their cell surface, e.g. the TLR4, which recognizes Lipopolysaccharide (LPS), a Gram-negative bacterial cell-wall component. Our previous work demonstrated that TLR4-induced signaling leads to the enhanced expression of inflammatory cytokines by IgE-activated mast cells, including IL-9, at least partly by boosting the activation of NF-κB. However, our knowledge about the regulation of IL-9 expression in mast cells is still scarce and deserves further investigation.

Objectives:
Based on our unpublished observation that BMMC derived from C57BL/6 mice are low responders to LPS compared to BALB/c regarding the production of IL-9, we performed comparative analyses of BMMC to get insight into the underlying mechanisms of IL-9 expression.

Materials & Methods:
We generated BMMC derived from C57BL/6 and BALB/c mice. The output of cytokine production in vitro was assayed by ELISA. In RT-PCR and Western Blotting the expression of transcription factors, cytokines and other members of the LPS downstream signaling pathway were analyzed. Luciferase reporter gene assays specific for NF-κB were performed.

Results:
In accord with our previously published work, LPS strongly promotes the production of IL-9 in IgE-activated BMMC derived from BALB/c mice. However, whereas the IL-9 production induced solely by IgE receptor signaling is comparable in BMMC derived from either BALB/c or C57BL/6, the latter only marginally respond to LPS as a cosignal for producing IL-9. Yet, the expression of IL-6, induced by IgE receptor signals in absence or presence of LPS, is comparable in BMMC derived from both strains. Production of IL-9 strongly depends on IL-1 and IL-1 mRNA expression induced by IgE and LPS is indeed lower in BMMC from C57BL/6, but IL-9 expression cannot be rescued by adding exogenous IL-1. Reporter gene assays revealed a stronger NF-κB activation in BALB/c BMMC along with higher c-Rel mRNA levels in response to IgE and LPS. Furthermore, under these conditions, the expression of the transcription factor IRF-4 in BALB/c-derived BMMC is also increased. Although the underlying mechanisms are still obscure, higher activity of NF-κB and expression of IRF-4 in BMMC from BALB/c likely explain stronger expression of IL-9.

Conclusion:
Profound strain-specific differences regarding the production of key cytokines may deeply influence the results of both in vitro and in vivo experiments. However, side-by-side analyses of high (BALB/c) and low responders (C57BL/6) to LPS- and IL-1-induced signals can help to further elucidate critical components of IL-9 expression in mast cells.
Mast cells determine the adaptive immune response in contact hypersensitivity

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Question:
Contact allergens trigger a rapid inflammatory response of skin resident innate immune cells. By these inherent adjuvant properties, sensitizing compounds elicit allergic-specific T cell responses. However, the mechanisms by which organic sensitizers trigger innate immunity are incompletely understood. Since mast cells are considered important sentinel cells of innate immunity at body surfaces we aimed to study the relevance of mast cells in innate and adaptive immune responses during contact hypersensitivity.

Method:
Conflicting results were published on the role of mast cells in contact allergy based on mast cell-deficient kit mutant mice. We used our novel Cre/loxP-based mouse models of mast cell deficiency and mast cell-specific gene inactivation to study mast cell functions in contact allergy.

Results:
We found that in the absence of mast cells, allergic responses to organic haptens were massively reduced. Mast cell-deficiency abrogated the early innate response to contact allergens as determined by quantification of vasodilatation, vessel permeability and neutrophil influx. Importantly, we demonstrated that DC migration from sensitized skin to lymph nodes and expansion of T lymphocytes was reduced in the absence of mast cells, and that the CD8+ T cell response to secondary allergen encounter was impaired in mast cell-depleted mice. To address mechanisms of the mast cell effect on contact hypersensitivity, we generated mice lacking TNF selectively in mast cells. Also these animals featured reduced CD8+ T cell-mediated inflammatory responses of allergen-challenged skin. However, we found that mast cell-derived TNF selectively supports migration and maturation of CD8+ DCs responsible for CD8+ T cell priming.

Conclusion:
Conclusively, we demonstrated that mast cell responses to contact allergens are essential for the induction of the pathogenic T cell response.
Granulocytes, Mast Cells, Immune Response in the Skin

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The PI3K isoforms p110γ and p110δ are non-redundantly involved in the development and survival of myeloid cells

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The class I phosphoinositide 3-kinase (PI3K) members PI3Kγ and PI3Kδ are hematopoietically expressed signalling enzymes that play important roles in function, proliferation, differentiation and survival of leukocytes. While the catalytic PI3K isoform p110δ is typically activated by receptor tyrosine kinases (RTKs) and/or Ras proteins, the catalytic isoform p110γ is mainly activated by GPCRs through direct interaction with G-protein βγ dimers and Ras proteins. Characterization of gene-deficient mice demonstrates that p110γ and p110δ are both required for the development and function of T lymphocytes. Moreover, using p110γ and p110δ double knock-out mice, we have previously shown that p110γ and p110δ also harbour specific non-redundant roles in development and peripheral maintenance of B lymphocytes. Here we studied the relative significance of the two PI3K isoforms in the generation and survival of various myeloid cell lineages. To this end we analysed major innate immune cell populations in p110γ and p110δ double knock-out mice. We found that, in contrast to single knock-out and WT controls, double knock-out mice had increased numbers of granulocytes and monocytes, whereas cell counts of dendritic cells and macrophages were reduced. Moreover, granulocytes from double knock-out mice exhibited an altered apoptosis rate. We now carry out a detailed analysis of the development and survival of neutrophils and eosinophils in these mice. Our results demonstrate that p110γ and p110δ are non-redundantly involved in the development and survival of myeloid cells and thus influence the homeostasis of granulocytes and other major innate immune cell populations.
The Effect of Irradiation on the Functions of Human Neutrophil Granulocytes using F-18 Fluoride

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Introduction:
During the initial phase of inflammation the polymorphonuclear neutrophils (PMN) are one of the first-responding cells which descend towards the site of inflammation. PMN are sensitive for radiation [2]. Many publications have shown that low dose radiotherapy (doses between 0.1 und 1 Gy per fraction) has anti-inflammatory effects, like the inhibition of the nitrogen oxide pathway of mouse monocytes [1]. Treatment of furuncles on the face by radiation therapy is established for doses between 0.2 and 0.5 Gy per fraction [3]. A biphasic phagocytic response was observed with an increased irradiation time. Following a first increase in phagocytosis, collapse of PMN was observed with longer irradiation time [2]. The reactive oxygen species (ROS)-concentration in PMN after fractioned irradiation with 6 Gy (3 x 2) - 18 Gy (9 x 2) decreased [4]. Irradiation of isolated PMN showed a biphasic curve of the oxidative Burst [5]. Decreased ROS synthesis by PMN with increased dosages between 0 and 32 Gy. could be observed [6]. However, there have been only a few publications regarding the effects of low dose irradiation on the functions of PMN (phagocytosis, oxidative burst and killing of pathogens) in vitro.

Objectives:
The aims of this project are the investigation of the changes in the main functions of PMN by low dose irradiation and the potential risks caused by radiation explorations in cases of problems with medical devices. Further questions are the correlation between dose and effect, the existence of a definite threshold for the expected effect and causes of therapeutic low dose irradiation effectiveness.

Material & Methods:
To investigate the underlying therapeutic effects of low dose radiation treatment on PMN and potential risks of radiation exposure on medical staff using 18Fluor with syringes, we designed a simple irradiation device. The blood is provided within thin cuvettes in order to maximize homogenous irradiation. They are arranged around the radiation source at variable distances in order to test different radiation dosages by a constant temperature (37°C) and radiation exposure (30 minutes). The irradiation of heparinised whole blood has been performed with doses between 0.1 and 0.2 Gy. After irradiation measurements of activation, phagocytosis, oxidative burst and killing of S. aureus by PMN were performed. Dosimetry was performed with TLD rods directly in the cuvettes.

Results:
The activation (CD11b and CD66b expression) of human neutrophils shows low decrease after irradiation with 18Fluor. Same effects were observed in the phagocytosis of S. aureus through PMN.

Conclusion:
The increase in CD 11b and CD66b observed following the radiation doses tested is considered to have influence on the functions of PMN further to phagocytosis. Further experiments concerning these functions will be performed.

References:
[6] De Vries et al.; Cancer 2001;92(9); 2444-2450
Human neutrophils aggregate via CD66b, which is independently expressed from CD11b

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Introduction:
Clearance of invaded bacteria belongs to the key mechanisms of neutrophils. The expression of cell surface receptors is crucial for their tasks as every step in the neutrophil recruitment is based on receptor interaction. The surface molecule CD66b has been shown to be involved in neutrophil aggregation. Its function is rather unknown but is mainly interpreted as co-expression with known surface receptors like CD11b/CD18, which recognizes complement-opsonized bacteria for phagocytosis.

Objective:
The aim of this study is to find out whether CD66b is specifically and independently expressed from other neutrophil receptors, and whether it could serve, e.g., as target for modulation of neutrophil function.

Materials & Methods:
Whole blood samples from healthy volunteers were incubated with S.aureus supernatant (SaS) obtained from two different strains. At defined time points (1.5 - 120 minutes) samples were taken and analysed by means of flow cytometry and fluorescently labelled antibodies for CD11b and CD66b expression and cell aggregation. The liquid growth medium IMDM was used as negative control (NC) and the bacterial peptide fMLP as positive control (PC) for CD11b expression.

Results:
CD11b expression following treatment with two different SaS (A and B) was rather unaltered, except that SaS A induces a slightly faster increase from 1.5 - 5 min.
The CD66b expression significantly increased after SaS A treatment, contrary to treatment with SaS B which markedly reduced CD66b expression comparable to fMLP. The NC shows that the procedure itself results into a slightly increased CD66b expression.
The aggregation of neutrophils 45min after SaS B and fMLP stimulation was at the NC level, whereas the sample stimulated by SaS A showed approx. 4-fold higher number of aggregated cells.

Conclusion:
The results show that the CD11b expression is increased in approx. the same level by SaS A and B, as well as by fMLP. In contrast, CD66b expression is only increased after stimulation by SaS A, but not by SaS B and not by fMLP. In conclusion, it was possible to induce a CD66b expression independent from CD11b expression on human neutrophils. Moreover, we showed again that cell aggregation seems to be dependent on CD66b.

The results indicate that the regulation of both cell surface receptors is different. Thus, further investigations on CD66b expression and its role in phagocytosis are planned.
Alternative non-coding transcript of the murine proteinase 3-orthologue and its probable immunological function

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Introduction:
Proteinase 3 (PR3) is the major autoantigen of the granulomatosis with polyangiitis (Wegener’s granulomatosis). We have investigated the murine PR3-orthologue and have discovered an intronic promoter, which is localized in intron 2. This promoter controls the expression of a so-called long non-coding mRNA (lncRNA). Investigations on mammalian genomes show that alternative promoters are associated with several diseases. They control the expression of protein variants and are apparently involved in the expression of the lncRNAs. As we could also detect an alternative PR3-transcript in humans, a species-overlapping function of these transcripts is most likely.

Methods:
To identify the elements which are essential for basal promoter activity, several promoter deletion constructs were generated, luciferase reporter assays performed and the proximal promoter sequence identified. FACS- and expression analyses as well as multiple alignments were used to analyze the currency of the transcript. The translation of the transcript was detected by immunoblots.

Results:
We could depict, that the alternative PR3-promoter is active in murine bone marrow, and in mouse embryos, as well as in leukemia- and other tumor cells. In addition, in the spleen of healthy mice the alternative transcript is exclusively expressed in immunosuppressive “myeloid-derived suppressor cells” (MDSCs). As we could not detect an appropriate polypeptide, the transcript apparently represents a so-called “long non-coding RNA” (lncRNA), a recently discovered RNA-species with regulatory functions. In humans the alternative mRNA-transcript is expressed in the bone marrow and in different tumor cell lines, so that a species-overlapping function can be assumed.

Conclusions:
The activity of PR3-promoters as well as the expression of alternative non-coding mRNA-transcripts (lncRNAs) in both humans and mouse myeloid or cancer cells suggests a regulatory function in the differentiation of myeloid cells as well the development of cancer cells. The potential role of these transcripts in autoimmune diseases, such as the granulomatosis with polyangiitis (Wegener’s granulomatosis), is currently being investigated.
The transcription factors IRF4 and NFATc2 contribute to the MyD88-dependent expression of IL-9 in mast cells

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Mast cells crucially contribute to anaphylaxis as well as to several Th2- or Th9-dominated diseases like asthma. Especially, mucosa-resident mast cell contribute to these pathophysiology by their ability to produce interleukin (IL)-4, IL-5, IL-13, and IL-9 upon activation via IgE/Ag. Recently, we were able to show that the development and function of IL-9-producing Th9 cells essentially depends on the transcription factor (TF) IRF4. Genetic ablation of the Irf4 gene renders mice resistant to allergic airway diseases. Initial in vitro analyses revealed that mast cells deficient in Irf4 also display a defective expression of the asthma promoting cytokine IL-9, which is believed to contribute to mucus production, tissue remodeling, and the influx of eosinophils. Further analyses revealed that in addition to IRF4, NFATc2 crucially contributes to the transcriptional regulation of the IL9 gene in mast cells. Detailed molecular analyses employing reporter gene studies, oligonucleotide precipitation and Chromatin Immunoprecipitation (ChIP) revealed that IRF4 and NFATc2 bind and transactivate the murine Il9 promoter. Additional analyses discovered that the expression of IRF4 is disturbed in Il1r1-deficient mast cells and Nfatc2-deficient mast cells leading to greatly impaired IL-9 production. In summary our data clearly demonstrate a central function of MyD88 signaling and the TFs IRF4 and NFATc2 in the production of IL-9 by mast cells.
Protection from poly(I:C)-induced liver damage requires type I IFN-dependent recruitment of myeloid suppressor cells and IL-1RA expression


Question:
The production of type I interferons (IFN) is one of the earliest immune responses upon detection of a danger signal. Acting at the interface of innate and adaptive immune responses, type I IFN can exert both pro- and anti-inflammatory functions.

Methods:
As type I IFNs are induced by so-called danger signals, we established a model of poly(I:C) (artificial ds RNA)-induced hepatitis in type I IFN-α/β-receptor chain 1 (IFNAR) deficient mice.

Results:
We observed that injection of artificial double-stranded RNA (poly(I:C)) caused severe liver damage and inflammation in IFN-α/β-receptor chain 1 (IFNAR)-deficient mice, but not in wild-type controls. These findings indicate an important anti-inflammatory function of type I IFN particularly in the liver, since all other analyzed organs showed no signs of inflammation. Studying mice with conditional IFNAR ablation revealed that IFNAR-triggering of myeloid cells is essential to protect mice from poly(I:C)-induced liver damage. Accordingly, in poly(I:C)-treated wild-type mice, monocytic myeloid-derived suppressor cells (MDSC) were recruited to the liver after poly(I:C) treatment whereas no liver-infiltrating MDSC were detectable in the liver of treated IFNAR−/− mice. Upon poly(I:C) injection IFNAR−/− mice showed an increased production of pro-inflammatory interleukin (IL)-1β and a severe lack of anti-inflammatory IL-1 receptor-antagonist (IL-1RA) production. Of note, IL-1RA production was type I IFN-dependent and could be assigned to liver-infiltrating myeloid cells. Depletion of IL-1β or treatment with IL-1RA both rescued IFNAR−/− mice from poly(I:C)-induced liver damage directly linking the deregulated balance of IL-1β/IL-1RA expression and the observed liver damage in IFNAR−/− mice. Thus, type I IFN-signaling protects from severe liver damage by recruitment of monocytic MDSC and maintaining a balance between IL-1β and IL-1RA production.

Conclusion:
Taken together, our data reveal new anti-inflammatory properties of type I IFN by inducing the production of IL-1RA by liver-infiltrating myeloid cells. Thus, data obtained could guide a way in new treatment opportunities for inflammatory liver diseases.
The alarmins S100A8 and S100A9 promote hyporesponsiveness of phagocytes in sepsis and polytrauma patients

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Question:
Phagocytes of polytrauma and burn patients develop a stage of hypoinflammation leading to secondary infections and high mortality. In Gram-negative sepsis hypoinflammation of phagocytes is a well-known phenomenon which is triggered by a process called endotoxin-tolerance. Endotoxin-tolerance is induced by stimulation of Toll-like-receptor-4 (TLR4) with low-dose endotoxin resulting in hypo-responsiveness of phagocytes to inflammatory re-stimulation. However, an initial microbial trigger is often missing in polytrauma and burn patients. Therefore we asked if this inflammatory process can additionally be modulated by endogenous activators of TLR4, like the Damage Associated Molecular Pattern (DAMP) proteins S100A8 and S100A9.

Methods:
Tolerance was induced in vitro by pretreatment of phagocytes with low doses of either LPS or S100A8/S100A9 prior to activation. Inflammatory response was quantified by determining cytokine release and validating corresponding mRNA-levels via RT-PCR. In vivo experiments were performed by determining survival rates of mice in a septic shock model. NF-κB activation was determined on nuclear fractions by western blot analysis. ChIP-assays were used to manifest epigenetic modifications of the TNFα promoter.

Results:
We observed highly elevated S100A8/S100A9 serum concentrations in the initial phase of inflammation in polytrauma and burn patients which predict lethal outcome in the latter. Pre-activation of TLR4 by S100A8/S100A9 induces tolerance, accompanied by diminished secretion of proinflammatory cytokines in vitro and enhanced survival rates of mice during septic shock. NF-κB transcription factor analysis and TNFα promoter studies revealed an increase of nuclear RelB and of methyltransferase G9a-dependent histone methylation in S100-tolerized phagocytes.

Conclusions:
S100A8/S100A9 complexes induce endotoxin-tolerance in phagocytes via activation of TLR4 signaling pathways and epigenetic modification of gene expression, relevant for development of hypoinflammation in burn and polytrauma patients.
Fibrocytes develop outside the kidney but contribute to renal fibrosis in a mouse model

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Introduction:
Renal fibrosis is associated with an exacerbated deposition of extracellular matrix proteins, e.g. collagen I. Collagen-producing bone marrow-derived cells, so called fibrocytes, have been detected in animal models and patients with fibrotic diseases. In vitro data suggest that they develop from monocytes with the help of accessory cells and profibrotic soluble factors. However, fibrocytes are also found in the peripheral blood or the spleen of healthy humans and mice.

Objectives:
Here, we analyzed whether fibrocytes develop locally from monocytes or migrate as already differentiated collagen-producing cells from the peripheral blood or the spleen into injured tissues.

Materials & Methods:
Monocytes and fibrocytes were differentially depleted with monoclonal antibodies and diphtheria toxin (DT) in bone marrow chimeric mice and the number of fibrocytes, the time course of their appearance, and the outcome of fibrosis were analyzed. We used the model of unilateral ureteral obstruction (UUO) that results in rapid infiltration of the obstructed kidney with inflammatory cells. Fibrocytes can readily be detected in the obstructed kidney and fibrosis develops within 5-7 days after UUO.

Results:
Before UUO (day 0), we detected only low numbers of fibrocytes in the obstructed kidney, whereas there was a strong increase after ureteral ligation. In the spleen, fibrocytes were clearly detectable on day 0 and increased on days 3 to 7. The phenotype of splenic and renal fibrocytes was very similar and distinct from classical monocytes as fibrocytes expressed no CD115, medium levels of CCR2, and high levels of CD11b and Ly-6G. Therefore, the number of circulating fibrocytes was not reduced when monocytes were depleted with a monoclonal antibody against CCR2 or when CCR2−/− mice with very low numbers of circulating or splenic monocytes were analyzed. However, the absence of CCR2 interfered with migration of fibrocytes into the kidney. Furthermore, we could efficiently deplete fibrocytes from the kidney using a depleting monoclonal antibody against Ly-6G or bone marrow chimeric mice expressing the DT receptor under the control of CD11b. Depletion of fibrocytes or reduced migration of fibrocytes into the kidney resulted in lower renal expression of collagen I. Although renal fibrocytes were almost completely depleted, there was still deposition of collagen I in the obstructed kidney, indicating that also other mechanisms contribute to renal fibrosis.

Conclusion:
In conclusion, these results demonstrate that fibrocytes develop outside the kidney independent of infiltrating monocytes and rely on CCR2 for migration into target organs. Further experiments are needed to determine the relative contribution of fibroblasts, fibrocytes, and epithelial cells to production of collagen I and other extracellular matrix proteins.
Characterization of the murine CD163 receptor

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Introduction:
CD163 is a scavenger receptor exclusively expressed on the surface of monocytes and macrophages. CD163 positive macrophages are found in the healing phase of acute inflammation as well as in wound healing tissue. This suggested an involvement of CD163 in the resolution of inflammation. Anti-inflammatory factors like glucocorticoids and IL-10 increase CD163 expression. The expression of CD163 has been the most excessively studied in man and rat. Nevertheless very little is known regarding the structure and regulation of CD163 expression, especially on the protein level, in mice. The aim of this study was to analyze the structure, expression and regulation of murine CD163 (mCD163).

Material and methods:
Extracellular domain of mCD163 was cloned in the pet28a expression vector, protein was expressed in BL21 E.coli cells and used to produce rabbit polyclonal antibody (LFR1-Ab). Regulation of CD163 expression in freshly isolated murine bone marrow cells, bone marrow-derived macrophages as well as the CD163 distribution in different organs were assessed using fluorescence microscopy, western blot and quantitative real time PCR.

Results:
In western blot LFR1-Ab specifically recognized a 170kDa protein in lysates of murine bone marrow cells. LFR1-Ab has also shown cross-reactivity with human CD163 (130kDa). The differences in molecular weight (MW) were due to significant amount of N-linked glycans in mCD163, since the pre-treatment with PNGaseF reduced its MW to 128kDa. High expression of CD163 was detected in freshly isolated bone marrow cells. However, the expression of this molecule was lost during in vitro culture. Subsequent stimulation of bone marrow cells with dexamethasone, but not IL-4, IL-6 or LPS and IFNγ, restored CD163 expression. Similar, but slightly weaker effect was observed in bone marrow-derived macrophages stimulated with DEX. In vivo analysis of CD163 distribution in mouse revealed the presence of CD163 positive cells in all tested organs (spleen, liver, lung and intestine). However i.p. injection of DEX enhanced expression of CD163 only in the lung, but not other organs. CD163 is known as a M2 macrophage marker; however we detected an increase of a specific CD163-positive subpopulation in several experimental diseases (S. aureus, L. major).

Conclusions:
Using our polyclonal antibody we could perform for the first time detailed analysis of the expression and regulation of murine CD163 on the protein level.
Glucocorticoids promote an anti-inflammatory feedback mechanism in activated monocytes.

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Introduction:
Synthetic glucocorticoids (GC) are still the most potent immunosuppressive agents used in the treatment of chronic inflammatory and autoimmune diseases. However patients treated for prolonged periods with GC suffer from deleterious side effects, which also limit their use in many clinical conditions. GC affect nearly all cells of the immune system, but there is growing evidence for cell-type specific mechanisms. Especially the precise mechanisms by which GC exert their anti-inflammatory effects on monocytes and macrophages are still not well defined. In our previous studies we have shown that treatment of monocytes with GC does not cause a global suppression of monocytic effector functions, but rather induces specific differentiation of these cells to an anti-inflammatory phenotype (regulatory monocytes, Mregs) (Ehrchen, Blood 109, 1265-1274 (2007); Barczyk, Blood 116, 446-455 (2010)). However, the impact of GC on inflammationally stimulated monocytes is still not well defined. Lipopolysaccharide (LPS) is a potent and well characterized trigger of monocyte activation. However, after long-term treatment (16-48 hours) LPS induces a negative feedback resulting in a subsequent induction of anti-inflammatory mechanisms.

Objectives:
The purpose of these studies was to investigate the interplay between GC-treatment and LPS-induced monocyte activation.

Material and methods:
Monocytic response to simultaneous stimulation with LPS and GC was analyzed by microarray analysis and compared with isolated GC- and LPS-treated monocytes. The LPS-GC-induced phenotype of monocytes was functionally characterized with special emphasis on migration, adhesion, phagocytosis, production of pro- and anti-inflammatory mediators as well as cell survival.

Results:
As expected simultaneous stimulation of monocytes with LPS and GC resulted in suppression of many LPS-induced pro-inflammatory factors. Some of them have not been described so far to be regulated by GC (IL-15, IL-31 and chemokines CCL3, CCL4, CCL8). Surprisingly we also found, that combined treatment with LPS-GC led to synergistical up-regulation of many genes, which have been affected neither by stimulation with LPS nor GC alone, playing a role during macrophage polarization toward M2 phenotype, resolution of inflammation and which are important for cell differentiation, tissue remodeling and wound healing. We have also observed additive effects of GC and LPS on expression of many anti-inflammatory genes, which have not been reported to be mutually regulated by LPS and GC until now. Functional clustering analysis identified specific functions of monocytes which were modulated by GC-treatment of LPS-activated monocytes. GC specifically inhibited adherence but enhanced spontaneous migration and chemotaxis, phagocytosis (latex beads, opsonized bacteria, apoptotic cells) as well as the ability to produce pro-resolving lipid mediators. All these aspects could be confirmed at protein level and by functional assays.

Conclusion:
Taken together, our results clearly indicate that GC do not simply suppress LPS-mediated activation of monocytes but rather induce their reprogramming toward a specific anti-inflammatory phenotype involved in resolution of inflammation.
Activation-induced cell death of human monocytes: a novel mechanism fine-tuning inflammation and autoimmunity

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Introduction:
Monocytes are circulating cells with high plasticity. They respond to various stimuli with distinct activation and differentiation patterns, are able to secrete several humoral factors and they contribute to inflammation in the immune system, either by governing host defense response to invading pathogens or driving reactions to self-molecules in conditions of tissue-damage. Control of these mechanisms is necessary to ensure the self-limitation of inflammatory reactions and avoid perpetuated autoinflammation or autoimmunity. This aspect of immunoregulation is crucial and has been mainly associated with adaptive immunity. To date it is unclear how activated monocytes can regulate early cytokine signals promoting their survival or cell death.

Objective:
The goal of the study was to explore GM-CSF- and IFNγ-stimulated mechanisms leading to activation-induced cell death (AICD) in human monocytes.

Methods:
Primary human monocytes were isolated and subjected to stimulation with GM-CSF and IFNγ. Cell death was measured using Annexin V and propidium-iodide staining and analyzed by FACS. To explore the mechanism behind AICD of monocytes signaling pathways were analyzed by Western blot using the respective antibodies against phosphorylated and non-phosphorylated proteins. TNF-blockers were used to analyze the role of TNF in the process of AICD.

Results:
In the present study we demonstrate in vitro, that simultaneous treatment with GM-CSF and IFNγ promotes AICD of human monocytes. Analyzing the signaling pathways that lead to cell death revealed that pyronecrosis is induced by GM-CSF and IFNγ. Pyronecrosis has morphological characteristics of necrosis, is caspase- and RIP kinase1-independent but cathepsin-B-dependent. GM-CSF/IFNγ-induced cell death of monocytes involved IL-1β and TNFα-hypersecretion. Furthermore, pyronecrosis was found to be dependent on TNFα and could specifically be inhibited by TNF-blockers such as etanercept.

Conclusion:
Taken together, we identified AICD of monocytes as a novel mechanism, which could regulate inflammatory processes that may be altered in the context of autoinflammation. The involvement of different mediators and pathways in this process could have consequences on therapeutic strategies, e.g. for combination therapies involving TNF-blockers.
Lymphotoxin-beta receptor signalling regulates cytokine expression via TRIM30α in a TRAF3-dependent manner

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So far most studies have focused on the critical role of the Lymphotoxin-beta-receptor (LTβR) in the development of secondary lymphoid organs and the maintenance of lymphoid structures. Our previous work has shown that activation of the LTβR by T cell derived LTα1β2 regulates inflammatory cytokine expression in an experimental model of intestinal inflammation. Furthermore, we were able to demonstrate that LTβR activation is involved in the induction of TLR cross-tolerance as the result of the LTβR-dependent induction of TRIM30α. So far, TRIM30α has been reported as a negative regulator of TLR-mediated NFκB activation inducing a negative feedback mechanism to control prolonged and excessive inflammatory reactions. However, up to now, the molecular mechanisms underlying the protective role of LTβR activation have not been elucidated in detail.

In this study we demonstrate that LTβR activation on mouse macrophage cell line J774 results in a strongly reduced TLR-mediated induction of cytokine (TNF, IL-6) and NO production demonstrating that LTβR activation on mouse macrophages dampens pro-inflammatory cytokine and mediator expression. Thus, LTβR signalling renders macrophages hypo-responsive to subsequent re-stimulation with TLR ligands. These results are associated with the induction of TRIM30α expression, which negatively regulates the TLR-induced NFκB activation. The observation of an LTβR-mediated TLR-tolerance in the human monocyte cell line THP-1 suggests that similar signalling mechanisms seem to exist in human cells. Furthermore, the signalling pathway leading to LTβR-mediated TRIM30α induction was characterized. J774 cells retrovirally transduced with specific inhibitors of NFκB proteins, which block either the canonical pathway (IκB alpha superrepressor) or the non-canonical pathway (dominant-negative mutant of NIK), were analyzed for TRIM30α expression. Hence, we showed that LTβR-induced TRIM30α expression seems to be mediated by the canonical NFκB signalling pathway. To investigate the role of the signalling proteins called TNFR-associated factor proteins (TRAF) in LTβR-induced TRIM30α expression, we made use of J774 cells over expressing a dominant negative form of TRAF2, 3, 5 or 6. Our findings demonstrate a crucial role for TRAF3 in LTβR-mediated TRIM30α induction. Further signalling pathway analysis revealed an increased activation of RelB (non-canonical NFκB pathway) in J774 cells expressing a dominant negative mutant of TRAF3. In contrast an activation of RelA (canonical NFκB signalling pathway) seems not to be modulated by a dominant negative mutant of TRAF3 after LTβR activation. These data indicate that TRIM30α expression which is induced by LTβR activation on macrophages seems to be mediated by an IκBα-dependent signalling pathway in a TRAF3-dependent manner.

Collectively, our study suggest that LTβR activation on mouse macrophages is involved in the regulation of pro-inflammatory cytokine and mediator expression by activation of a signalling pathway that controls exacerbating inflammatory cytokine production.
Beta-defensins activate macrophages and synergize in pro-inflammatory cytokine expression induced by TLR ligands

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Our previous studies indicated that mouse beta defensin 14 (mBD14, Defb14), a newly identified member of the beta-defensin super family, interacts with the chemokine receptors CCR2 and CCR6. In this study we report that pre-stimulation of primary mouse macrophages with mBD14 results in a synergistic, enhanced expression of pro-inflammatory cytokines and chemokines induced by TLR ligand re-stimulation. Experiments using specific inhibitors of Gi-protein-coupled receptor signalling provide evidence that this effect seems to be mediated by a Gi-protein-coupled receptor expressed on bone marrow derived macrophages. However, using primary macrophages derived from CCR6- and CCR2-deficient mice clearly demonstrated that the enhanced pro-inflammatory cytokine and chemokine expression is independent of the chemokine receptors CCR6 and CCR2. Additionally, signalling pathway analysis indicated that mBD14 is capable of inducing MAPK ERK1/2 phosphorylation and the induction of CD86 and F4/80 expression in bone marrow-derived macrophages after mBD14 stimulation. Collectively, our data indicate that b-defensins activate primary macrophages and enhance pro-inflammatory responses by using GiPCRs in order to support inflammatory reactions induced by TLR ligands.
CD44-dependent induction of myeloid derived suppressor cells by activated human hepatic stellate cells induce from peripheral blood monocytes


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Myeloid derived suppressor cells (MDSCs) are a heterogeneous population of cells associated with the suppression of immunity. However, how MDSC are induced and where is unclear. The liver is known for its immune regulatory functions. Here, we investigated the capacity of human hepatic stellate cells (HSCs) to transform peripheral blood monocytes into MDSCs. Mature peripheral blood monocytes co-cultured with HSCs down-regulated HLA-DR and developed a phenotypic profile similar to CD14+HLA-DR-/low MDSCs. Only activated but not freshly isolated HSCs were capable of inducing CD14+HLA-DR-/low cells. Such CD14+HLA-DR-/low monocyte-derived MDSCs suppressed T-cell proliferation in an arginase-dependent fashion. HSC-induced development of CD14+HLA-DR-/low monocyte-derived MDSCs was not mediated by soluble factors, but depended on physical interaction and was mediated by HSC-expressed CD44. Thus, activated human HSCs can convert mature peripheral monocytes into MDSCs. As HSCs are activated during chronic inflammation the subsequent local induction of MDSCs may prevent excessive liver injury due to inflammation. HSC-induced MDSCs functionally and phenotypically resemble those isolated from liver cancer patients. Thus, our data suggest that local generation of MDSCs by liver-resident HSCs may contribute to immune suppression during inflammation and cancer in the liver.
Induction of human anti-inflammatory monocytes by glucocorticoid application \textit{in vivo}

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Introduction:
Glucocorticoids (GC) are still the most widely used drugs in the treatment of chronic and autoimmune diseases. However, prolonged continuous GC-therapy leads to deleterious side effects, which frequently limit their clinical use. Thus, other therapeutic regimes like interval therapy with very high doses have been developed. The cellular and molecular mechanisms of different GC therapeutic regimes however are currently unclear. We previously showed that GC induce specific differentiation of long-lived monocytes with an anti-inflammatory phenotype \textit{in vitro} (Ehrchen, Blood 109 (2007); Barczyk, Blood 116, 446-455 (2010)). We now wondered whether induction of long-lived anti-inflammatory cells could be relevant for the clinical efficacy of GC therapies.

Objectives:
The aim of the studies was to determine, whether administration of GC in humans \textit{in vivo} leads to generation of an anti-inflammatory population of monocytes similar to that induced by GC-treatment \textit{in vitro}. We analyzed, if GC-induced regulatory monocytes occur under \textit{in vivo} conditions in peripheral blood of healthy donors treated with oral steroids in therapeutic doses as well as patients with localized scleroderma treated with high dose GC pulse therapy.

Material and methods:
For \textit{in vitro} studies human monocytes were isolated from buffy coats. Healthy volunteers were treated with orally administered steroids (prednisolone) in therapeutic doses (1.5 mg/kg body weight) for 2 consecutive days. Peripheral blood monocytes were isolated from fresh EDTA-blood pre- and 8h, 24h and 48h post-administration of GC. Patients suffering from localized scleroderma were treated with dexamethasone pulse therapy (100 mg i.v. for 3 consecutive days). Peripheral blood monocytes were isolated from fresh blood obtained before first injection of dexamethasone and 24h after termination of therapy. The effect of GC on mRNA expression (qRT-PCR), protein expression (flow cytometry and western blot), apoptosis (AnnexinV-staining, Nicoletti assay) and phagocytosis was analyzed.

Results:
We could demonstrate that \textit{in vivo} administration of GC induces population of monocytes with characteristic features of \textit{in vitro} generated anti-inflammatory monocytes: enhanced expression of scavenger receptor CD163, IL1 receptor type II (CD121b) and down-regulated expression of T-cell-attracting chemokines CXCL9 and CXCL10, increased capacity to phagocyte pro-inflammatory agents as well as protection from cell death. Already moderate doses of glucocorticoids administered orally to healthy probands induced in peripheral blood population of monocyte showing characteristic features of \textit{in vitro} generated GC-induced anti-inflammatory monocytes. Moreover, GC-effects were even more pronounced in autoimmune patients after very high dose interval GC-therapy.

Conclusion:
Using different glucocorticoids and routes of administration we could demonstrate that peripheral blood monocytes from healthy donors and autoimmune patients change their phenotype toward a population with anti-inflammatory properties after application of GC. Moreover, induction of long-lived anti-inflammatory monocytes was even more pronounced after pulse therapy with very high concentration of GC and this could be especially relevant for the strong and long-term efficacy of interval GC-therapy.
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12/15-lipoxygenase mediates GC-induced anti-inflammatory mechanisms in monocytes

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**Introduction:**
Glucocorticoids (GC) are drugs of choice for the treatment of many chronic and allergic inflammatory diseases. Our previous studies have shown that GC treatment does not suppress monocyte functions but induces a distinct anti-inflammatory phenotype in these cells. Similarly, the treatment of inflammatory monocytes with GC leads to re-programming of the cells towards a specific population involved in resolution of inflammation. Gene expression analysis has shown upregulated expression of 12/15-lipoxygenase (12/15-LOX) in GC- and LPS/GC-treated monocytes. Lipoxygenases react with fatty acids generating lipid mediators that contribute to the resolution of inflammation.

**Objectives:**
The aim of our studies was to determine lipoxygenase-dependent effects in GC-treated monocytes.

**Materials and Methods:**
Bone marrow-derived monocytes were isolated from wild type (wt) C57Bl/6 and 12/15-LOX-/- mice and stimulated with GC and/or LPS. Gene expression was analyzed using quantitative RT-PCR. Protein expression was examined by Western Blot, Flow Cytometry and CBA technology. Functional assays were performed to analyze monocyte migration, chemotaxis, phagocytosis, apoptosis and oxidative burst.

**Results:**
GC treatment induced a much more pronounced reduction of adhesion to plastic surface in wt monocytes as compared to 12/15-LOX-/- cells. Conversely migration was enhanced in GC- and LPS/GC-treated monocytes isolated either from wt or 12/15-LOX-/- mice.

12/15-LOX-/- monocytes exhibited a remarkable higher ROS level which could not be inhibited by GC treatment as compared to wt cells. Gene expression analysis has also revealed up-regulated expression of several components of NADPH-Oxidase Complex in 12/15-LOX-/- monocytes. In contrast, anti-inflammatory genes like IL-1R2 were down-regulated in these cells in response to GC-treatment.

Phagocytosis of carboxylated-modified latex-beads (mimicking apoptotic cells) was increased in GC-treated monocytes. However, this effect was much weaker in GC-treated monocytes isolated from 12/15-LOX-/- mice. In contrast, no significant differences between wt and 12/15-LOX-/- monocytes were observed in phagocytosis of latex beads (mimicking foreign particles).

Treatment with GC led to marked protection from both spontaneous and drug-induced apoptosis in wt but not 12/15-LOX-/- monocytes. Moreover, 12/15-LOX-/- monocytes seemed to be more prone to necrotic cell death as assessed by annexinV-staining and analysis of caspase activation.

**Conclusion:**
GC- and LPS/GC-treatment induced anti-inflammatory and pro-resolving phenotype of monocytes, which show increased expression of 12/15-LOX. Our results indicate that inhibition of ROS production, protection from cell death as well as migration and adhesion of GC-treated monocytes are at least partially mediated by 12/15-LOX. Specific targeting of the 12/15-LOX-pathway may be a promising strategy to block undesirable inflammation with fewer side effects.

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**Figure 1**

[Graph showing anti-inflammatory IL1-R2 expression following treatments.]
Leishmania major (L. major) is an obligate intracellular parasite with a promastigote flagellated stage dividing in the insect vector and an amastigote aflagellated stage multiplying in mammalian hosts. Both, disease inducing promastigotes as well as disease propagating amastigotes use macrophages (MF) as host cells. In the human body there are different phenotypes of MF, such as pro-inflammatory type I (MF I) and anti-inflammatory type II (MF II) and it is still not known which phenotype of MF is involved in parasite propagation or killing. In murine MF Leishmania parasites can be killed in an iNOS dependent manner. However, for human MF it is unclear which anti-parasitic mechanisms are used to control Leishmania.

In this study we search for MF phenotype specific killing mechanisms for both life stages of L. major parasites. We found that pro-inflammatory MF I are more resistant to L. major infection as compared to anti-inflammatory MF II. Interestingly, we found that the antimicrobial peptide cathelicidin (LL-37) was specifically up regulated in more resistant MF I as compared to MF II. LL-37 is located in lysosomal compartments and is demonstrated to be involved in the innate host defence against a broad range of pathogens. We could show that recombinant human LL-37 kills extracellular L. major parasites. We established a siRNA knockdown for the LL-37 peptide in primary human MF and could show LL-37 to be involved in the intracellular degradation of L. major promastigotes in human MF I. Both parasite load as well as parasite survival were significantly higher in the knockdown MF I as compared to control MF I, demonstrating LL-37 to be involved in the intracellular degradation of L. major promastigotes but not amastigotes. Interestingly, the lack of anti-leishmania LL-37 activity in MF II suggests this MF phenotype to be more suitable host cells for Leishmania parasites.

In conclusion, this study suggests that pro-inflammatory MF I can eliminate L. major parasites via LL-37.
SWAP-70: A regulator of myeloid/lymphoid lineage determination

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SWAP-70, identified and investigated by us, controls cell migration and adhesion of hematopoietic cells by modulating processes such as integrin activity and F-actin rearrangement. Swap-70−/ mice are osteopetrotic, their osteoclasts although present in the bone in increased numbers fail in efficient bone resorption. In addition to the increase of mature TRAP+ osteoclasts, we observed enrichment of ckit+cfms−CD11b+ and ckit+cfms−CD11b+ early-stage and potential late-stage osteoclast precursor cells. Furthermore, an unusual population that expresses the myeloid (CD11b) and B-lymphoid (B220) surface markers, is strongly expanded in the bone marrow (BM) of Swap-70−/ mice. However, the B220+CD11b+ population does not express Gr-1, which is expressed on mature granulocytes, but also transiently on monocytes during their differentiation within the BM. Deep RNA sequencing revealed a significant up-regulation of Siglec5, which has been implicated in the differentiation and activation of monocytes, while some Tripartite Motif (TRIM) family gene members, which function in specific ubiquitin-mediated protein degradation pathways, and are involved in several of biological processes, including differentiation of cells, are dramatically down-regulated. The impact of these and other potential candidate genes on the accumulation of B220+CD11b+ cells is currently studied by gain and loss of function experiments. During ontogeny, B220+CD11b+ cells are found in the fetal liver and in the BM of newborn Swap-70−/ and WT mice, but thereafter only in Swap-70−/ mice, suggesting impaired differentiation of Swap-70−/B220+CD11b+ cells. To determine, whether these cells represent an enriched population of myeloid-lymphoid bipotential precursors, FACS-purified B220+CD11b+ cells were cultured under different cell culture conditions, showing that B220+CD11b+ cells can either adapt a B cell (B220+CD11b+) or a myeloid (B220+CD11b−) lineage fate. Since Osteoclasts and macrophages share a common progenitor, our data implicate SWAP-70 as a regulator essential for fate decision of lymphoid/macrophage/osteoclast lineage progenitors.
Activation of ID3 expression by TGFβ in primary human macrophage reveals involvement of Smad1/5 mediated signaling

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Introduction:
Macrophages encounter TGFβ in various normal and pathological situations. The effect of this growth factor on macrophages is, however, poorly studied. Recently we demonstrated that glucocorticoids are necessary to maintain the ability of macrophages to respond to TGFβ stimulation. Expression profiling of TGFβ-treated macrophages revealed activation of the expression of ID3 a members of the ID family of helix-loop-helix (HLH) proteins.

Objectives:
The objective of the study was investigation of the mechanism of TGFβ-mediated regulation of ID3 expression.

Results:
To understand mechanism of ID3 expression regulation the promoter of ID3 was cloned and analysed using luciferase reporter assay. Interestingly stimulation of cells transfected with the reporter constructs, containing ID3 promoter, with TGFβ did not induce an increase of luciferase activity. Also addition of enhancers identified using ECR Browser to the reporter constructs did not provide TGFβ reactivity. Since ID3 mRNA expression was shown to be BMP dependent we tested whether BMP signalling is activated in TGFβ-treated macrophages. In contrast to TGFβ signalling that is initiated by TGFβ receptor I ALK5 and involves phosphorylation of Smad2 and Smad3, BMP signalling is activated by ALK1/2/3/6 type I receptors and involves phosphorylation of Smad1 and Smad5. We established that stimulation of macrophages by TGFβ leads to rapid phosphorylation of Smad1/5. Inhibition of ALK5 receptor by SB431542 inhibitor efficiently blocked this phosphorylation. Analysis of concentration dependency of observed effect revealed, that while 5 µM of SB431542 are sufficient to block Smad1/5 phosphorylation, 25µM are needed to block Smad2/3 phosphorylation. Since it was suggested that Smad1/5 phosphorylation may be mediated via MAPK/ERK pathway we tested whether treatment of macrophages with PD98059 - a Ras/MAPK inhibitor will block Smad1/5 activation. PD98059 used in concentrations from 5 to 50 µM did not have any effect on Smad1/5 or Smad2/3 phosphorylation.

Conclusions:
Taken together we established that in primary human macrophages TGFβ activates both TGFβ and BMP signalling pathways. In contrast to published data in macrophages TGFβ-mediated phosphorylation of Smad1/5 is not mediated by MAPK/ERK pathway.
Role of FoxQ1 in the transmigration of monocytes in inflammatory conditions

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Introduction:
Monocyte polarization in the circulation is a result of the interplay between circulating and tissue-derived molecules. However, the molecular mechanism of the specific activation of monocytes in blood stream is still poorly understood. Recent studies showed that the levels of cytokine IL-4 are increased in the circulation during Th2-associated inflammation. Using microarray assay we found that monocytes respond to IL-4 by strong overexpression of FOXQ1 and observed its upregulation in monocytes of patients with acute atopic dermatitis. However function of FOXQ1 in monocytes was unknown. In the presented study we investigated the expression regulation of FOXQ1 in human monocyte-derived macrophages and analyzed effect of FOXQ1 on monocyte transmigration.

Methods and Results:
Analysis of FOXQ1 expression in human monocyte-derived macrophages by qRT-PCR confirmed that cytokine IL-4 induces FOXQ1. We showed that TGF-β1 in combination with dexamethasone amplifies the effect of IL-4. In order to identify FOXQ1-induced genes and analyse function of FOXQ1 murine macrophage-like RAW264.7 cells were stably transfected with mFOXQ1 or empty vector. A microarray analysis revealed that FOXQ1 target genes can be involved in the monocytes motility. In fact, the macrophage-like cells showed increased migration activity during FOXQ1 overexpression. The stimulatory effect of FOXQ1 on monocyte migration correlated with its ability to suppress expression of receptor PLXNC1 known to inhibit migration of monocytes and dendritic cells. In parallel with FOXQ1, PLXNC1 was downregulated in human monocytes upon IL-4 stimulation and in monocytes of patients with acute atopic dermatitis.

Conclusions:
Our data indicate that FOXQ1 is upregulated by IL-4 and TGF-β1 and stimulates monocyte migration in response to inflammatory stimuli by suppression of PLXNC1. We hypothesise that FOXQ1 supports increased monocytes extravasation through the activated endothelium during chronic inflammation.
Complement Component C5a is a selective modulator of IL-27-dependent innate immune responses

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Activation of the complement system serves as a first line of defense against bacteria, fungi and viruses. In addition to lysis and opsonization of pathogens, complement cleavage products orchestrate the later stages of innate and adaptive immune responses. Here, we have investigated the effects of the potent anaphylatoxin, C5a, on gene expression and release of Interleukin-27 (IL-27) in macrophages during acute inflammation in vivo. The signature subunit, p28, of IL-27 was strongly induced by ligation of the TLR3-receptor (Poly I:C) or the TLR4-receptor (LPS) in cultures of mouse macrophages. LPS was unable to induce IL-27p28 in macrophages genetically deficient of TLR4 or LPS-binding protein (LBP-/-). Recombinant mouse C5a suppressed the release of IL-27p28 from LPS-activated macrophages as detected by ELISA or RT-PCR in a dose-dependent and time-dependent manner. After treatment with LPS plus C5a, lower frequencies of F4/80+/CD11b+/IL-27p28+ macrophages were present as compared to LPS-controls. The degradation product, C5a-desArg, displayed less activity than C5a to silence IL-27p28. The effects of C5a, required ligation with its G-protein coupled receptor, C5aR (CD88), but were independent of the second receptor C5L5 (GPR77), Figure 1. During endotoxic shock, C5aR-deficient mice displayed higher concentrations of circulating IL-27p28 in plasma as compared to wild type mice. Interestingly, when IL-27p28 was induced by TLR3-activation (viral stimulus), the co-presence of C5a, did not interfere with IL-27p28 appearance, Figure 2. These observations were reflected by a selective role of C5a-induced phosphorylation of the Akt signaling pathway in situations of TLR4- but not TLR3-mediated activation of macrophages. Blockade of Akt by the irreversible small molecule inhibitor wortmannin, resulted in a 2.5-fold increase in concentrations of IL-27p28 during endotoxic shock. A second mechanism of C5a mediating suppression of IL-27p28 may relate to the ability of C5a to up-regulate IL-10 in LPS-activated macrophages, with IL-10 recruiting STAT3 for further suppression of IL-27p28. Collectively, these results elucidate new molecular mechanisms how C5a may selectively modulate immune responses against bacteria (TLR4-dependent), while having little effects during immune responses related to viral infections (TLR3-dependent).

Figure 1: Relative suppression of IL-27p28 in LPS-activated peritoneal macrophages from wild type (C57BL/6J), C5aR-/- or C5L2-/- mice when incubated with C5a (1 mcg/ml). Values of LPS alone (1 mcg/ml) were used as 100 % value for each strain, ELISA, 10h. Data from 3 independent experiments with absolute concentrations of IL-27p28 (2000-4000 pg/ml) after LPS alone.

Figure 2: Relative suppression of IL-27p28 by C5a (1 mcg/ml) in macrophages (C57BL/6J) when incubated with LPS (1 mcg/ml) or Poly I:C (1 mcg/ml) for 20 h. Data from 3 independent experiments.
Macrophages (Mφ) are main targets during human cytomegalovirus (HCMV) infection and even though they are professional antigen presenting cells their contribution to the anti-viral T-cell function is not well known. In view of the different pro- and anti-inflammatory abilities of M1- and M2-Mφ, we investigated how HCMV alters the antigen presenting properties of both types of Mφ. HCMV established a productive and persistent infection in M1- and M2-Mφ and skewed both cell types towards classical activation by inducing high release of pro-inflammatory cytokines and chemokines. Further experiments revealed that HLA-A,B,C was selectively reduced in HCMV-infected M1- and M2-Mφ but up-regulated in uninfected bystander cells, thus suggesting that a paracrine mechanism lead to the pro-inflammatory Mφ activation.

HCMV-infected M1- and M2- Mφ were able to induce autologous T-cell proliferation in HCMV-seropositive donors, thus suggesting that HCMV-Mφ efficiently stimulate memory T cells. Comparable percentages of CD4+ and CD8+ T cells were induced to proliferate, expressed the activation marker CD69 and contained IFN-γ. The degranulation potential of stimulated CD8+ T cells was confirmed by a CD107a (LAMP-1) translocation assay.

In summary, our findings indicate that Mφ preserve proper antigen presentation capacity upon HCMV infection while enhancing inflammation.
TLR driven ROS production upon direct TLR-NOX interaction

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Introduction:
Toll-like receptors (TLRs) are pattern recognition receptors (PRRs) that specifically recognize pathogen associated molecular patterns (PAMPs). Upon binding of PAMPs intracellular signalling is initiated by the recruitment of TIR-domain-containing adapter molecules such as TIRAP, TRAM, MyD88 or TRIF to their cytoplasmic domain and the activation of interleukin-1 receptor-associated kinases (IRAKs). NF-kB activation and subsequent release of proinflammatory mediators such as cytokines are typical effects of TLR activation. TLRs are also known to induce the release of gases like NO and reactive oxygen species (ROS) to kill invading microorganisms or induce signaling events. The time period between pattern recognition and ROS release is short, which implies direct protein-protein interactions rather than gene activation and consequent protein synthesis in the signaling process.

Objectives:
TLRs not only play a role in host defense but also can have impact on the development of specific diseases like rheumatoid arthritis or atherosclerosis. Aim of this study is to identify new binding partner of TLRs and their associated signaling molecules to find possible targets for new therapies.

Methods:
For identification of new direct interaction partner of TLRs we screened a leukocyte cDNA expression library in Yeast. Further interaction studies were done upon overexpression of both binding partners in Human Embryonic Kidney (HEK) 293 cells. Phosphorylation was analysed by western blot analysis with specific antibodies. ROS was measured by lucigenin chemiluminescence assay.

Results:
We identified one phagocyte NADPH oxidase (NOX) 2 subunit as direct interaction partner of the intracellular domain of TLR7 in Yeast. This finding was corroborated by time and stimulation dependent co-immunoprecipitation of TLR2 or -4 with the NOX2 subunit. Upon challenge with heat inactivated *Staphylococcus aureus* the NOX2 component was phosphorylated indicating synchronous TLR2 and -13 activations as a requirement for activation of the responsible kinase. However, single TLR ligands induced ROS production by NOX2 depended on the respective TLRs in primary macrophages and a neutrophil cell line. Respiratory burst upon stimulation with whole bacterial and single TLR agonist was also dependent on the kinases IRAK4 and ERK.

Conclusion:
Our results suggest a direct signal transduction pathway from TLRs via IRAK4 and ERK to the active NADPH oxidase.
Role of Gr1+ inflammatory macrophages in pyelonephritis-induced fibrosis

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Urinary tract infections (UTI) such as cystitis, urethritis and pyelonephritis are one of the most common bacterial infections worldwide. They are caused by Uropathogenic strain of E. Coli (UPEC). When UTI’s ascends and reaches the pyelum or pelvis of the kidney it is called as Pyelonephritis (PN). Chronic pyelonephritis results in inflammation, acute fever, nausea and diarrhea and in long-term and inevitably renal fibrosis and furthermore kidney failure.

Macrophages have been known to play an important role in fibrosis of other organs but their role in pyelonephritis-induced fibrosis is still unclear. Here we have developed a mouse model for pyelonephritis-induced fibrosis. Using this model, we could show that Gr1+ inflammatory macrophages accumulate 1-day post infection and decrease until day 6. In contrast, Gr1− macrophages do not accumulate on day 1, but increase until day 6 post infection. CCR2 KO animals, which lack Gr1+ macrophages, showed a reduced accumulation of Gr1− macrophages within kidney. Such reduction was not due to reduced proliferation indicating that differentiation of Gr1+ macrophages into Gr1− macrophages takes place. Furthermore, CCR2 KO mice showed increased bacterial load, reduced fibrosis and improved kidney function indicating a substantial role of Gr1+ macrophages in the defense against UPECs and in the development of fibrosis. These data indicate the important role of Gr1+ inflammatory macrophages in pyelonephritis and pyelonephritis-induced renal fibrosis.

Colonic ileus after intestinal surgery depends on CCR2

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Purpose/Objective:
The most severe complication after abdominal operation, the post operative ileus (POI), is characterized by a lack of bowel motility within the jejunum and colon. Such pathology depends on macrophages (MPS), which are initially activated by IFN-γ. However, the specific POI-promoting MP-subset and its origin remains unclear.

Materials and Methods:
After opening the peritoneal cavity POI was induced in CCR2⁻/⁻, Clodronate Liposome (Clo-Lip) depleted and C57BL6 mice by manipulating the small bowel with moist cotton applicators once from the oral to aboral direction. After 24h jejunal POI was assessed by oral FITC-Dextran application and measuring its bowel progression after 1,5h. Colonic POI was examined by inoculation of a bead into the colon and measurement of excretion time. Cell numbers were determined using flow cytometry.

Results:
We found radiosensitive, resident Ly6C⁺CX3CR¹Bright MPs as the main MP population within the unmanipulated muscularis. After manipulation we observed an influx of Ly6C⁺CX3CR¹Dim MPs, which was dependent on CCR2 and derived from blood. However, jejunal POI was still present in CCR2⁻/⁻ and Clo-Lip depleted mice which either lack Ly6C⁺CX3CR¹Dim MPs in blood and muscularis. Nevertheless we found that the colonic POI was improved in CCR2⁻/⁻ and also in Clo-Lip treated mice.

Conclusions:
These findings indicate that blood-derived Ly6C⁺CX3CR¹Dim MPs are dispensable for jejunal POI, but contribute to colonic POI. Further experiments are needed to investigate the mechanism of jejunal POI, which will include Ly6C⁺CX3CR¹Bright MPs.
Network engineering on transcriptional level reveals a continuum of human macrophage differentiation

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Introduction:
Macrophages are a major component of the innate immune system with a diverse set of functions in their respective environment. Activation and function of these cells is triggered by the integration and computation of environmental signals leading to specific transcriptional regulation as the bases for activation and function. Particularly studies in murine model systems have clearly demonstrated that macrophages are among the cell types with the most versatile transcriptional programs.

Objectives:
Based on these current concepts, we were interested to answer the following questions. First, is there an underlying, stable and lineage-specific transcriptional program in human macrophages clearly distinguishing these cells from closely related cell types, e.g. dendritic cells (DC), second, is macrophage polarization into classical M1- and alternative M2-macrophages the only outcome of activation, third, is there a central activation signature of macrophages independent of differentiation and fourth, can we link findings obtained in the murine system to human macrophages?

Materials and Methods:
To address these questions, we used monocyte-derived macrophages as a human model system. We applied microarray-technology to generate 384 transcriptome expression profiles including a large variety of activation signals from 299 human macrophages as well as other immune cell types (T-, B-, NK-cells, DC). Bioinformatics analyses such as coregulation networks, reverse engineering of cellular networks (e.g. ARACNe and TINGe), Weighted Gene Correlation Network Analysis (WGCNA) on different conditions were performed in this study. Furthermore, we linked the findings to histone modification and PU.1 binding information by ChIP-Seq experiments. Using Gene Set Enrichment Analysis (GSEA) we overlaid our 28 in vitro differentiation signatures on human alveolar macrophages. Core signatures of murine macrophages derived from ImmGen were related to human macrophage activation and differentiation.

Results:
Based on comprehensive comparative analysis of a diverse set of activation signals from human macrophage transcriptomes we unequivocally extend the current M1- and M2-like polarization model into a continuum model of macrophage differentiation. Reverse network engineering revealed a core of transcriptional regulators associated with macrophage activation complemented by sets of regulators leading to specific differentiation programs. Overlaying this continuum of macrophage activation onto in vivo data lead to new models of human alveolar macrophage activation, e.g. a strong IL4 signature in alveolar macrophages of smokers. Lastly, linking this information resource of human macrophage activation to the ImmGen data helped to define an activation and differentiation-independent human and murine macrophage core signature.

Conclusion:
Taken together, large enough transcriptional datasets of immune cell activation as provided here for human macrophages are valuable resources not only to study immunological aspects of macrophage biology but also to better understand general mechanisms of transcription control and to develop novel mathematical models for signal integration in these transcriptionally highly active human cells.
Comparative transcriptomics reveals gain of proliferative capacity, loss of phagocytic capacity and a distinct phenotype of tumor-associated macrophages in murine chronic lymphatic leukemia

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Introduction:
Tumor-associated macrophages (TAM) are a key component of the tumor microenvironment linked to reduced survival or impaired treatment response in various tumor entities. In a murine spontaneous model of chronic lymphocytic leukemia (EµTCL1 transgenic, CLL) we recently identified a functional role of MIF and macrophages in the progression of CLL. It remains to be elucidated how macrophage polarity evolves during disease progression and functionally affects the tumor microenvironment, tumorigenesis and progression.

Methods:
We applied comparative transcriptomics of primary macrophages derived from spleen and bone marrow from EµTCL1 mice in the pre-leukemic versus the leukemic state. Macrophages were isolated via F4/80 fluorescent immuno-staining and subsequent sorting. Gene expression profiles were analyzed bioinformatically by hierarchical clustering, ANOVA, pathway analysis, gene ontology enrichment analysis and gene set enrichment analysis. Experimental validation of bioinformatically modelled transcriptional and functional changes in TAM were performed by flow cytometry assessing cell proliferation using BrDU assays, fluorescently labelled latex beads to assess ex vivo phagocytosis and dextrane particles for in vivo assessment of phagocytosis. Primary human monocyte-derived macrophages were used in co-culture systems with primary patient CLL cells prior assessment of phagocytic activity.

Results:
Comparative bioinformatical analysis of macrophages from pre-leukemic mice of two organ sites (spleen, bone marrow) with tumor-associated macrophages (TAM) derived from the same organs in leukemic mice revealed an unexpected magnitude of transcriptional reprogramming. Interestingly, the gene expression signatures of TAM in CLL showed little overlap with M1- or M2-like macrophage polarization patterns previously described based on in vitro polarization of macrophages. Instead, mathematical modeling of major transcriptional changes indicated a gain of proliferative capacity with cell cycle regulators being induced and loss of phagocytosis-associated genes. Indeed, increased proliferation of F4/80+ TAM was experimentally demonstrated in leukemic animals at local tumor sites. Moreover, the transcriptional signature related to reduced phagocytic capacity of macrophages was confirmed ex vivo by reduced incorporation of latex beads by TAM derived from leukemic mice. Phagocytosis by TAM was also impaired in vivo indicated by reduced incorporation of fluorescent dextrane particles. These functional findings could be recapitulated in human monocyte-derived macrophages co-cultured with primary CLL cells or healthy donor B-cells showing reduced phagocytosis induced by leukemia cells.

Discussion:
Taken together, an extensive reprogramming of macrophages takes place during leukemogenesis of CLL, causing the expansion of non-phagocytic macrophages proliferating within the tumor microenvironment with a distinct, non-M1/M2 polarization. These findings form the basis for a better understanding of the tumor microenvironment in this disease allowing the development of novel therapeutic strategies targeting CLL-nurturing cells in the tumor microenvironment.
Macrophages

Unique characteristics of murine neonatal macrophages

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Introduction:
Neonates, preterms in particular, are vulnerable to infections because they rely primarily on innate immunity. Macrophages (MΦ) are key players as innate immune cells. The aim of our study was to characterize neonatal tissue macrophages and to compare them to their adult counterparts.

Methods:
MΦ were isolated by peritoneal lavage from neonatal (<24h) and adult (42d) C57BL6/J mice. Transcriptomes were analyzed by microarrays, phenotypes were characterized by chipcytometry using a panel of 22 extra- and intracellular markers. Functional assays included cytokine release upon LPS stimulation (multiplexed bead assay) and T cell proliferation assays. In some experiments MΦ isolated for fetal mice were included as additional controls.

Results:
We observed a distinct neonatal phenotype with low expression of classical MΦ markers (F4/80, CD14, CD11b), Toll-like receptors (TLR 2, 4, 9) and antigen presentation markers (MHCII, CD80, CD86). MΦ isolated from fetal mice showed a similar phenotype. Furthermore, transcriptome analysis revealed significant differences between neonatal and adult peritoneal MΦ (PCA). Systemic transcriptome analyses revealed IFNγ as one of the most significant upstream regulators (p<0.05). Cytokine (IL6, IL1) and chemokine (ccl2, ccl3) release of neonatal MΦ upon LPS stimulation differed significantly compared to adults. Moreover, neonatal MΦ were unable to induce T cell proliferation.

Conclusions:
Neonatal MΦ express a distinct phenotype which appears not to be related to birth stress. The differences in gene expression, cytokine release and their lacking ability to induce T cell proliferation could help to explain neonatal immune reactions.
Macrophages

Macrophages (MØ) are an important part of the innate immune system [1]. They are plastic cells able to adapt to microenvironmental changes acquiring distinct polarization phenotypes termed M1 and M2 [2]. During normal inflammation, M1 prevail in the early phase orchestrating the first defense and the recruitment of effector cells, whereas M2 are more abundant at the end resolving inflammation and mediating tissue repair [3,4]. In chronic inflammatory diseases, the balance between M1 and M2 is disturbed leading to the predominance of M1 cells that arrest the inflammatory response in the initial phase and prevent the entry into the next finalizing phase [5,6]. At present, there are no tools to therapeutically correct this imbalance. Recently, we found that CD64-directed immunotoxins can be used to selectively target and kill M1 cells.

In contrast to the specific elimination of M1 macrophages by immunotoxins, modulation of the cytokine production using immunomodulatory proteins (IMP) may offer an alternative approach for therapeutic intervention in chronic inflammatory diseases. IMP are thought to skew M1 cells to an M2-like phenotype, preventing the production of pro-inflammatory signals (e.g., IL-12, TNF-α) and blocking the systemic amplification loop via activation of T cells. We tested two potential IMP candidates, one of which was non-targeted, whereas the other was specifically directed to human CD64 (hCD64). Both IMP were cloned and expressed in Escherichia coli followed by purification using standard chromatographic methods. Their immunomodulatory activity was successfully demonstrated by the down-regulation of the pro-inflammatory mediator IL-12 in M1 MØ. In addition, the hCD64-targeted IMP increased the level of the anti-inflammatory IL-10 in non-polarized (M0) and M1-polarized MØ. Our preliminary in vitro data demonstrate hCD64-mediated immunomodulation of inflammatory M1 MØ resulting in an anti-inflammatory M2-like phenotype. The in vivo performance of these IMP will be investigated in future experiments.

Concluding, our results may present a promising therapeutic strategy for the treatment of chronic inflammatory diseases.

References:
Khaya grandifoliola compared with Baicalin inhibits inducible nitric oxide synthase, p38MAPK Kinase and pro-inflammatory cytokines gene expressions in LPS- treated RAW 264.7 macrophages

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Khaya grandifoliola is a species of plant in the Meliaceae family. In this study, we prepared CH₂Cl₂/MeOH 1:1 V/V crude extract, fractions and sub-fractions from this plant, and find out its effects on RAW macrophages cells activities. K. grandifoliola sub-fraction 78 was shown to be a potent inhibitor of lipopolysaccharide (LPS)-induced expression of inducible nitric oxide synthase (iNOS) and pro-inflammatory cytokines mRNA expression (TNFα, IL6 and IL1β) in mouse macrophage cells. K. grandifoliola sub-fraction 78 effects were compared to Baicalin, a flavonoid isolated from Scutellaria baicalensis Georgi, for their effects on LPS-induced nitric oxide (NO) production and iNOS and the expression of pro-inflammatory cytokines gene expressions. K. grandifoliola crude extract, fractions and sub fractions, as well as Baicalin, inhibited LPS-induced NO production in a concentration-dependent manner without cytotoxicity. The decrease in NO production was in parallel with the inhibition of LPS-induced iNOS gene expression by K. grandifoliola sub-fraction 78 and Baicalin. In addition, the suppression of pro-inflammatory cytokine mRNA expression was very significant after treatment with K. grandifoliola SF-78 and Baicalin. Furthermore, K. grandifoliola SF-78 inhibited the activity of p38MAPK kinase (95% inhibition), suggesting that the inhibition of this kinase is one of the multiple signaling pathways supporting K. grandifoliola SF-78 effects on these cells. From these results, it was concluded that K. grandifoliola sub-fraction 78 contains prominent compounds with promising effects against inflammatory diseases.
Loss of the RNA-binding protein SYNCRIP is crucial for Nox2 destabilization and impaired ROS production during the hypoinflammatory phase of sepsis

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Question:
In the hypoinflammatory phase of sepsis macrophages are alternatively activated by contact to apoptotic cells or their secretion products. One hallmark of this phenotype shift is attenuated ROS-formation by the NADPH-oxidase [1], contributing to immune paralysis. Because mechanistic insights of this inhibition remain largely elusive, we focused on the catalytic subunit of the NADPH-oxidase [2], Nox2, assuming that sepsis reduces its expression, consequently diminishing ROS formation.

Methods:
To alternatively activate macrophages in vitro, J774 cells were stimulated with conditioned medium of apoptotic T cells (CM). Luciferase-assays were used to analyze the role of the 3’UTR in Nox2 mRNA stability. Assuming that a protein might be involved in mRNA destabilization, we performed a mRNA pulldown with biotinylated Nox2-3’UTR constructs followed by mass spectrometry. We verified the role of the identified factor by overexpression and knockdown approach. Additionally, we overexpressed Nox2 in J774 cells and analyzed ROS formation (w/wo CM treatment) with the hydroethidine- assay by FACS analysis. For in vivo experiments we induced polymicrobial sepsis in mice by cecal ligation and puncture (CLP). The ability of peritoneal macrophages (PMs) to produce ROS was determined by FACS using the hydroethidine-assay. Nox2 expression of primary PMs was determined at protein and mRNA level.

Results:
We noticed an impaired mRNA and protein expression of Nox2 along with decreased ROS-production after the induction of sepsis in mice as well as in vitro by stimulating J774 macrophages with CM of apoptotic T cells. As Nox2 overexpression restored ROS-production of CM-treated J774 cells, we assumed that expression of the Nox2 subunit is primarily crucial for maintaining NADPH oxidase activity. Using 5,6-dichloro-β-D ribofuranosyl benzimidazole (DRB) to block transcription we could showed the decrease in Nox2 is due to a reduced half-life of Nox2 mRNA following CM treatment. mRNA pulldown experiments followed by MS analysis identified SYNCRIP as a RNA-binding protein, which stabilizes Nox2 mRNA through binding to its 3’ UTR under control conditions. CM-dependent degradation of SYNCRIP prevents its stabilizing function, which is crucial for maintaining ROS-production during the hypoinflammatory phase of sepsis. In line, knockdown of SYNCRIP decreased Nox2 mRNA expression, whereas SYNCRIP overexpression rescued downregulated Nox2 expression following CM treatment and concomitant ROS-production.

Conclusion:
Our data suggest that during sepsis progression in macrophages Nox2 mRNA stability is affected by SYNCRIP. In the hypoinflammatory phase of sepsis SYNCRIP expression is suppressed, and Nox2 gets degraded. Consequently the assembly of a functional NADPH-oxidase complex, able to produce suitable amounts of ROS, is no longer possible. Only the binding of SYNCRIP to the Nox2 3’UTR, ensures its stability. Therefore, understanding the mechanisms of SYNCRIP regulation influencing NADPH-oxidase activity may indicate options to reconstitute NADPH-oxidase function, finally improving immune reactions in sepsis patients.

Acknowledgements:
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C/EBPβ is a key transcriptional regulator of IL-36α - a novel member of the IL-1 family

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The transcription factor C/EBPβ is a member of the CCAAT/enhancer binding protein family and is expressed in a variety of cell types where it plays a role in tissue-specific gene expression, proliferation, and differentiation.

In order to identify new target genes regulated by C/EBPβ in macrophages we generated C/EBPβ knock-down (shC/EBPβ) RAW 264.7 cell lines using a short hairpin interfering RNA construct. Analysis of knock-down efficiency by semi-quantitative RT-PCR revealed a comparable reduction of cebpβ mRNA in three analysed clones resulting in more than 90% reduction of C/EBPβ at the protein level. To identify genes regulated by C/EBPβ in response to inflammatory stimuli we treated wild-type and shC/EBPβ RAW 264.7 macrophages with LPS and performed microarray analysis. In addition to genes which are known to be regulated by C/EBPβ we also identified IL-36α as a potentially C/EBPβ-regulated gene. Quantitative RT-PCR confirmed the reduced expression level of IL-36α and rescue experiments restored IL-36α gene expression in shC/EBPβ cells.

IL-36α belongs to the IL-1 family of cytokines and was recently shown to be a potent regulator of dendritic and T cells. Since the transcriptional regulation of IL-36α is unknown, we first determined the transcriptional start site by 5′-RACE-PCR. In silico analysis of the IL-36α promoter identified five potential C/EBP-binding sites, one half-CRE site and two putative NF-κB binding sites. Reporter assays using serial deletion constructs of the promoter revealed a declined luciferase induction with a complete loss of promoter activity when deleting the proximal half-CRE and NF-κB site. Site directed mutational analysis of the IL-36α promoter showed that the half-CRE site is essential for promoter activation whereas the classical C/EBP binding sites were of minor importance. Reporter assays performed in shC/EBPβ cells further underline the importance of the half-CRE site as C/EBPβ responsive element. Furthermore, bisulfite sequencing analysis suggests a methylation-independent binding of C/EBPβ to the half-CRE element. Binding of C/EBPβ to this element is currently analyzed by electrophoretic mobility shift assays and chromatin immunoprecipitation.

Together, these findings suggest a distinguished role for C/EBPβ in the regulation of IL-36α via the proximal half-CRE element in response to inflammatory stimuli.
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The role of Nrf2 in Bone Marrow Derived Macrophages (BMDMϕ) regarding CD8+ T cell activation

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Question:
A major mechanism in defending reactive oxygen species (ROS) and maintaining cellular redox balance is Nrf2 (Nuclear factor erythroid-derived 2-related factor 2)-mediated transcriptional upregulation of antioxidant genes. Since myeloid derived suppressor cells (MDSCs) generate ROS to attenuate T-cell activation through modification of the T-cell receptor (TCR)/MHC complex [1], Nrf2 activation in MDSCs is necessary to maintain MDSC survival [2]. Disruption of Nrf2 in the bone marrow of cancer bearing mice correlates with elevated ROS levels in Nrf2−/− MDSCs, thus showing an increased susceptibility to metastasis by lung cancer cells [3]. To elucidate the role of Nrf2−/− macrophages in shaping T cell immunity, we established an ex vivo co-culture system with OT-1+ CD8+ T cells, encoding a TCR that specifically recognizes OVA(257-264) peptide presented by bone marrow derived macrophages (BMDMϕ) via MHCI H-2kβ.

Materials & methods:
Co-culture of Nrf2−/− or WT BMDMϕ and OT-1+ CD8+ T cells. WT and Nrf2−/− BMDMϕ were generated from bone marrow of 8-12 week old mice using a standard protocol. BMDMϕ were left untreated or pulsed with OVA(257-264) peptide and control peptide OVA(323-339) for 4h, before adding freshly purified CFSE labeled CD8+ OT-1+ T cells from spleens of OT-1 transgenic mice in a ratio of 1:2 for 72 h. T cell activation was characterized after 72h by labeling with anti-CD25 and anti-CD69 antibody as well as intracellular staining of granzyme B and perforin and gating on CFSE/FITC+ CD8+ T cells using flow cytometry. In parallel, BMDMϕ were washed, detached with accutase and labeled simultaneously with F4/80, CD80, CD86, B7-H1, B7-DC antibodies for flow cytometric analysis. Cytotoxic assay. CD8+ OT-1+ CFSE labeled T cells were purified from 72h co-culture supernatants and incubated with eFluor670 stained, stable OVA(257-264) presenting target cells EG.7 in a ratio of 1:2 for 24h. Cytotoxic activity, as well as cytokine expression, were measured by flow cytometry.

Results:
We could prove by flow cytometry, that WT and Nrf2−/− BMDMϕ are able to take up and cross-present the specific OVA(257-264) peptide via MHCI by staining the BMDMϕ with a specific anti-MHCI H-2kβ OVA(257-264) Ab. Moreover, expression of the costimulatory molecule B7-H1 was strongly upregulated after 72h of co-culture in WT and Nrf2−/− BMDMϕ presenting the MHCI restricted peptide OVA(257-264) compared to the control peptide OVA(323-339). Interestingly, CD8+ OT-1+ T cells co-cultured for 72h with OVA(257-264) presenting Nrf2 deficient BMDMϕ, show a decreased expression of the surface activation marker CD25 and reduced synthesis of the cytotoxic factors perforin and granzyme B compared to T cells incubated with WT BMDMϕ. In line, those T cells were less cytotoxic and produced lower amounts of soluble IFN-γ compared to T cells incubated with WT BMDMϕ.

Conclusion:
Our results indicate, that the loss of Nrf2 in BMDMϕ delayed or inhibited CD8+ T cell activation in an ex vivo OT-1 coculture system. Since MDSCs are responsible for tumor associated T cell tolerance by inducing a ROS-dependent modification of TCR/MHCI complex, we assume that Nrf2−/− BMDMϕ, also known to produce elevated ROS levels, act in the same way to induce non-responsive CD8+ T cells.

References:

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The role of nicotinamide as an immunmodulator of two human macrophage subsets

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Nicotinamide (NAM) a component of vitamin B3 is produced by several enzymes (sirtuins, ARTs, PARPs, CD38) that use NAD as a substrate. As the endproduct of these reactions it may act as a specific inhibitor. To sustain enzyme activity, NAM is recycled back to NAD by a salvage pathway. [1] Here we asked in how far these nicotinamide mediated processes have an impact on the differentiation and immunological properties on the 2 monocyte derived macrophage subsets termed M1 and M2.

Monocytes were differentiated into M1 and M2 macrophages in the presence and absence of NAM. After 7 days in culture the cells were incubated in the presence and absence of LPS. After 16h following parameters were measured: intracellular NAD/NADH concentrations, expressions of cell surface markers and NAD-dependent enzymes as well as the cytokine production.

We found that some surface markers differentiating between M1 and M2 macrophages were downregulated in response to NAM treatment whereas other surface antigens, in part of unknown function, showed an increased expression. Furthermore we identified nicotinamide as a compound capable of inhibiting LPS-induced cytokine production of M1 but not M2 macrophages. Changes of intracellular NAD concentrations observed under different experimental conditions may affect the activity of NAD-dependent enzymes. Among these enzymes we concentrated on sirtuins as they regulate transcription factors known to modulate some of the here described immune responses.

Taken together vitamin B3 has the potential to interfere with important immunological properties of M1 and M2 macrophages.

Macrophages

Hypoxia triggers inflammasome activation in human LPS-primed monocytes, but not in monocyte-derived macrophages

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Monocytes are part of the innate immune system and are recruited to sites of inflammation, where they differentiate into macrophages. The monocytes encounter varying environmental conditions on their way from the blood vessel to the inflamed tissue. One changing environmental factor is the concentration of oxygen.

This decreased oxygen level could be a triggering factor for the activation and survival of monocytes or macrophages. Aim of the study was to analyze the influence of hypoxia on lipopolysaccharide (LPS)-induced cytokine production in primary human monocytes and monocyte-derived macrophages.

Monocytes were immunomagnetically separated from the blood of healthy donors. Macrophages were differentiated out of primary monocytes for 7 days in the presence of human serum. For hypoxia experiments, cells were stimulated 16h with 100ng/ml LPS under hypoxic conditions (1% oxygen). Analysis of cytokines in the supernatant was done with ELISA. The concentration of Interleukin-1β in the supernatant of LPS-stimulated monocytes was significantly increased under hypoxic conditions (0.9ng/ml vs. 4.35ng/ml, p=0.0019). Similarly, the release of Interleukin-6 was elevated (63.8ng/ml vs. 125.9ng/ml, p=0.019), but not the concentration of TNF. Monocyte-derived macrophages showed no increase in either of these cytokines under the same hypoxic conditions. Therefore further analysing experiments were done with monocytes.

Cleavage of the IL-1β proform to its active form is dependent on the assembly of the inflammasome and the recruitment of caspase-1 followed by their activation. When inflammasome assembly was blocked with high extracellular K+-buffer or by inhibiting intracellular Ca-signalling with the Ca2+-chelator BAPTA-AM, hypoxia-induced IL-1β release was abrogated. Hypoxia-induced IL-1β cleavage is dependent on the NLRP3 inflammasome and the adaptor protein ASC, which was shown with genetically deficient THP1 cells.

Reactive oxygen species (ROS) are one already known activating signal for the inflammasome. Here we show that hypoxia leads to the release of mitochondrial ROS, and the induction of mitochondrial ROS through decoupling of the electron transport chain with Rotenone triggered an increase of IL-1β release under normoxic conditions.

This study shows that hypoxia leads to the activation of the inflammasome, the recruitment of caspase-1 and the subsequent cleavage and release of Interleukin-1β in human primary monocytes, but not in monocyte-derived macrophages. Intracellular Calcium mobilization and mitochondrial ROS production were shown to be essential mechanisms triggering inflammasome assembly.
The cAMP response element modulator (CREM) is a regulator of phagocytosis

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Defects in phagocytosis are of hallmark importance in the pathogenesis of systemic lupus erythematosus (SLE) resulting in persistently circulating apoptotic waste, which may encounter inflammatory removal pathways and serve as immunogen for the induction of autoreactive lymphocytes and as antigen for immune complex formation. We found that the transcriptional repressor CREM\textsubscript{α} is heavily expressed in monocytic cells from patients with SLE. CREM\textsubscript{α} binds to promoters of genes that contain cAMP response elements (CRE) and negatively regulates their transcription in a chromatin-dependent mechanism.

By ChIP on Chip promoter analysis we identified CREM binding to 1807 genes in LPS treated human monocyteic cells including an overrepresented binding to regulators of phagocytosis. We confirmed enhanced expression of phagocytosis-relevant genes like gulp, coronin and itgax in CREM\textsuperscript{−/−} macrophages compared to wildtype macrophages. Additionally macrophages of CREM\textsuperscript{−/−} mice show enhanced levels of phagocytosis, while mice with an overexpression of CREM\textsubscript{α} in all hematopoietic cells display decreased levels of phagocytosis \textit{in vitro} and \textit{in vivo}. We thus propose that the expression of CREM\textsubscript{α} influences phagocytic functions in monocyctic cells.
Sphingosine-1 phosphate (S1P) favors macrophage activation towards a M1 phenotype and has an impact on macrophage cytokine production and migration

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Introduction:
Macrophages play an essential role as phagocytic and cytokine producing cells in complex processes like wound healing and tumor development. In response to environmental signals like cytokines and other immunomodulators, macrophages can adapt various phenotypic activation states, favoring (M1 activation) or inhibiting (M2 activation) inflammation. S1P is a bioactive lysophospholipid that acts as intra- and extracellular signaling molecule in order to influence a broad spectrum of cell types and biological processes. S1P antagonists are increasingly used to modulate the immune response in various autoimmune diseases and after transplantation.

Objectives:
In this study the impact of the bioactive lysosphospholipid S1P on both macrophage activation as well as the biology of activated M1 and M2 macrophages was assessed.

Methods:
Bone marrow derived macrophages (BMDM) from C57 BL/6 mice were cultured under either M1 or M2 favoring conditions. S1P-receptor expression profiles were determined by quantitative RT-PCR, analyzing all known S1P-receptor subtypes (S1P\(_1\)–S1P\(_5\)). Influence of S1P on macrophage activation, migration, phagocytosis and cytokine secretion were assessed in vitro.

Results:
M1 macrophages significantly downregulate the expression of the S1P\(_4\)-receptor compared to unactivated or M2 macrophages whereas the expression levels of the remaining four S1P receptors were not significantly altered. Under M1 polarizing conditions, increasing S1P concentrations favor M1 activation. M1 macrophages showed a chemotactic response to increasing S1P concentrations. Increasing S1P concentrations increased TNF and IL-6 secretion of M2 activated and unactivated macrophages, while the production of other cytokines (MCP-1, IL-12, IL-10) showed no S1P dependence in vitro. Finally, phagocytotic activity of M1 as well M2 was independent from the presence or absence of S1P in vitro.

Conclusion:
Here we show that S1P influences both macrophage activation and biological activity. M1 and M2 macrophages show a divergent S1P-receptor expression profile. The role of S1P receptors and especially S1P\(_4\), in macrophage activation and function should be further assessed in order determine their potential as targets to influence macrophage activation and function in inflammatory and malignant diseases.
Chronic psychosocial stress promotes tumor growth by accumulation of MDSC in peripheral organs

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Stress is considered as a condition, which negatively affects health. Specifically, stress does not only induce behavioral and psychiatric manifestations but also physiological changes. An acute physiological stress response comprises release of epinephrine (Epi) and glucocorticoids (Cort) from the adrenals, which prepares the body for a “fight or flight” reaction. In contrast to a short duration of stress, which has beneficial effects, chronic stress and chronically elevated levels of Epi and Cort make the body more susceptible to infectious diseases and promote tumor growth. Cort is classically considered as an immune suppressive factor suppressing T and B lymphocyte proliferation. However, recent studies revealed that Cort supports CD11b\textsuperscript{+} myeloid cell survival. CD11b\textsuperscript{+} cells are important for the resolution of infections and antigen presentation, thereby initiating the adaptive immune response. In contrast to the beneficial function of these cells, a subpopulation of CD11b\textsuperscript{+} cells that promote tumor growth was identified in patients bearing tumors. Today such cells are termed myeloid derived suppressor cells (MDSC) and characterized by the expression of CD11b, Ly6G and Ly6C.

The aim of our study was to evaluate the effect of chronic psychosocial stress on tumor growth and potential mechanisms involved. We applied chronic subordinate colony housing (CSC), a previously established model for chronic psychosocial stress. We analyzed phenotype and function of CD11b\textsuperscript{+} myeloid cells in stressed mice and compared them to those appearing in tumor-bearing mice. After 19 days of CSC the fraction of myeloid cells was increased in blood and secondary lymphoid organs. Detailed analysis of such cells revealed their MDSC-like phenotype while they showed only weak suppressive activity. However, enhanced tumor-growth was measured in stressed mice. Previous exposure to CSC enhanced the suppressive activity of MDSC in tumor-bearing mice. In conclusion, our data indicate that exposure to chronic psychosocial stress induces the development of MDSC, that gain functional activity in tumor-bearing mice and promote tumor growth.
Interactive influence of macrophages and hepatocytes in co-culture

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Introduction:
The liver is the central organ for regulation of metabolism and plays an important role for the control of the systemic acute phase response as well as for innate and adaptive immunity. Moreover it has important function for inductions of immunological tolerance. It receives 80% of its blood supply from the gut and the spleen enriched in gut-derived bacterial products, environmental toxins, food antigens and spleen-derived immunological signals. These different functions are warranted by a complex assembly of highly specialized cell types which are organized in the sinusoidal unit, including hepatocytes, sinusoidal endothelial cells, hepatic stellate cells and liver macrophages (also termed Kupffer cells). Under homeostatic conditions liver macrophages represent about 15% of total liver cell population and 80 to 90% of all resident tissue macrophages in the body. They are present in the microvessels of the sinusoids, a strategic position for screening of pathogens, which enter the liver via the portal-vein. Here macrophages are responsible for removal of cellular debris and clearance of exogenous material. Furthermore they are central to innate immunity with key functions in host defence against invading pathogens, induction of the hepatic acute phase response and are critical regulators of liver damage and regeneration. Thereby, macrophages have a remarkable plasticity, enabling them to efficiently respond to the various environmental and endogenous signals.

Objectives:
Using different co-culture models the present study analyses the impact of intercellular communication between hepatocytes and macrophages on macrophage differentiation and cellular response of hepatocytes and macrophages towards stimulation with inflammatory stimuli or growth factors.

Materials & Methods:
Experiments were performed with bone marrow derived macrophages and primary hepatocytes of 8-12 week old C57/BL6j male mice. Differentiation markers were determined by FACS. Expression of chemokines, cytokines and acute phase proteins were measured by quantitative real time PCR and ELISA.

Results:
The data indicate that in bone marrow derived macrophages (BMDM) co-culture with hepatocytes induces substantial changes of the expression profile of differentiation markers, with an up-regulation of markers specific for sessile macrophages. These changes of the differentiation marker profile is accompanied by changes of the inflammatory response towards LPS with a sustained reduction of pro-inflammatory cytokine expression and a suppression of chemokine expression in hepatocytes in response to growth factors and cytokines such as TNF-alpha.

Conclusion:
Evidence is provided that hepatocytes and macrophages maintain a complex and reciprocal intercellular communication which modifies cellular response of both cell types towards distinct stimuli and results in changes of macrophage differentiation with an up-regulation of differentiation markers indicating a sessile macrophage phenotype.
Glucocorticoids and IL-4 synergize in M2 polarization of macrophages

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Macrophages play an important although ambivalent role in many inflammatory conditions. This is due to the fact that they can polarize into at least two different subtypes. Classically activated M1 macrophages are induced upon IFNγ and LPS stimulation, pro-inflammatory in nature and responsible for pathogen removal. In contrast, M2 macrophages are induced by Th2 cytokines such as IL-4 and IL-13, glucocorticoids (GCs) or TGFβ, have anti-inflammatory properties and play a role during parasite infections, cancer, tolerance and tissue repair. The balance between both subtypes is believed to strongly impact the outcome of inflammatory responses. In this study we set out to characterize M2 polarization induced in murine bone marrow derived macrophages (BMDMs) upon treatment with a combination of IL-4 and the synthetic GC dexamethasone (Dex). Each reagent caused distinct alterations in macrophage morphology and adherence. Flow cytometry and expression analysis by quantitative RT-PCR revealed that IL-4 and Dex acted synergistically in the induction M2 markers such as CD163 and CD206 while M1 markers including TNFalpha and CD86 were down regulated. The phagocytotic activity of macrophages was enhanced by Dex but IL-4 failed to further increase this property. Collectively, our study suggests that the combined treatment of macrophages with IL-4 and Dex is a highly efficient strategy to achieve alternative macrophage polarization which could become instrumental in treating inflammatory diseases in the future.
Maturation of Murine Monocytes and their Role in the Homeostasis of Tissue Macrophages

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Introduction:
Murine monocytes and macrophages have been well documented as important effector cells in immunological processes. However, there is still some controversy regarding the relation of classical Ly6C+ monocytes and their non-classical Ly6C- relatives. In addition, the origin of tissue macrophages after ablation vs. their steady-state homeostasis has not yet been entirely resolved.

Objectives:
Our aims were
i) to identify the source of macrophages repopulating their respective organ after experimental depletion and
ii) to disclose Ly6C- monocytes as either progeny of Ly6C+ monocytes or independently developing family members.

Materials & Methods:
We performed syngeneic transplantation of CX3CR1+/gfp CD45.1 bone marrow into irradiated CD45.2-expressing C57BL/6 mice and characterized tissue resident macrophages in untreated vs. clodronate treated mice. The developmental relationship of monocyte subsets in peripheral blood was investigated by differential in vivo staining.

Results:
Whereas tissue macrophage populations like hepatic Kupffer cells in - and originating from - recipient mice sustained themselves autonomously, their re-establishment after macrophage depletion involved bone-marrow derived donor cells. The fate-mapping approach of differential in vivo staining revealed that the initial staining pattern of Ly6C+ monocytes was progressively found among Ly6C- monocytes. In parallel, an increasing percentage of Ly6C+ monocytes showed a lack of staining which may correspond to the appearance of new monocytes from bone marrow.

Conclusions:
Our results support the hypothesis that Ly6C- monocytes substantially arise from Ly6C+ classical monocytes. The modulation of tissue macrophage populations with respect to their origin from donor bone marrow or resident cells of the recipient may be an important tool to investigate the role of distinct myeloid cell populations in immunological processes.
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