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ISNI 2012 Abstracts

B cells and antibodies

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Endogenous myelin-specific antibodies mediate demyelination and trigger spontaneous CNS autoimmune disease in the absence of myelin-specific B cells

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Objective: To dissect the relative pathogenic relevance of myelin-specific antibodies (ab) from myelin-specific B cells in the MS model experimental autoimmune encephalomyelitis (EAE).
Background: B cells and B cell-derived ab may both play a pathogenic role in CNS autoimmune disease. Transgenic mice in which a majority of B cells recognizes myelin oligodendrocyte glycoprotein (MOG) and plasma cells secrete high titres of MOG-specific ab (MOG BCR knockin mice) experience a fulminant course of actively-induced EAE. Double-transgenic mice which further contain myelin-reactive T cells (MOG BCR knockin × 2D2) spontaneously develop EAE. Utilizing both models in combination with anti (α)-CD20, which depletes B cells but spares CD20 negative plasma cells, we dissected the relative pathogenic contribution of myelin-reactive B cells from endogenously produced myelin-reactive ab.
Methods: MOG BCR knockin mice received 0.2 mg α-CD20 i.p./week starting 3 weeks prior to immunization with mouse MOG 1–117 or after EAE was established. MOG BCR knockin × 2D2 mice were injected with 0.2 mg α-CD20/week starting at the age of 4 weeks. T cell phenotype was determined by FACS for IFN-γ and IL-17. Serum from MOG BCR knockin mice immunized with MOG 1–117 was transferred into recipient wild-type (WT) mice with established EAE or naïve 2D2 mice. Serum from WT mice immunized with MOG p35–55 served as control.
Results: MOG BCR knockin mice developed fulminant EAE compared to WT mice, which was not affected by depletion of B cells. In double-transgenic MOG BCR knockin × 2D2 mice, α-CD20 treatment did not interfere with development of encephalitogenic T cells or incidence/severity of spontaneous EAE. While all peripheral compartments were efficiently depleted of B cells, α-CD20 did not affect constitutive secretion of α-MOG ab in either model. Further corroborating the deduced pathogenic role of myelin-reactive ab, serum from BCR MOG-knockin mice containing high titres of pathogenic α-MOG ab exacerbated established EAE in WT recipients and triggered spontaneous EAE in naïve 2D2 recipients.
Conclusion: Taken together, these data indicate that endogenously produced self-reactive ab contribute to CNS autoimmune disease independent of myelin-reactive B cells. Ongoing mechanistic experiments suggest that besides promoting CNS demyelination α-MOG ab may enhance myelin-recognition of antigen-presenting cells resulting in accentuated activation of myelin-reactive T cells.

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Clonally expanded and autoantibody producing B cells are present both in peripheral blood and cerebrospinal fluid of multiple sclerosis patients

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Objective: B cells and oligoclonal antibodies are present in the cerebrospinal fluid (CSF) of multiple sclerosis (MS) and clinically isolated syndrome (CIS) patients but their target antigens remain unknown. The focus of this study was to characterize B cells from the peripheral blood (PB) and CSF of MS and CIS patients following B cell immortalization.
Methods: Peripheral blood mononuclear cells (PBMC), peripheral immunoglobulin G^+ (IgG^+) B cells or CSF cells were infected with Epstein–Barr virus (EBV) to obtain continually dividing B cell lines. Molecular analysis of the Ig heavy chain variable region (V_H) genes was used to analyze clonality and diversity of the immortalized B cells. Binding of the produced antibodies to human oligodendroglioma (HOG), astrocytoma (U251), control epithelial alveolar carcinoma (A549) cell lines and PBMC was analyzed by flow cytometry and immunocytochemistry.

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Results: We generated 301 immortalized B cell lines from 18 MS patients, 45 from 4 CIS patients, 7 from 4 patients with a non-inflammatory neurological disease (NIND) and 40 from 2 healthy subjects (HC). Most B cell lines were obtained from the PB although 7 B cell lines were isolated from the CSF of 1 MS patient and 2 from 1 NIND patient. A monoclonality rate of more than 82% was obtained for these B cell lines, eliminating the need for subcloning. Antigen-stimulated clonal expansion and affinity maturation were evidenced both in PB and CSF of MS and CIS patients by the occurrence of B cell lines expressing identical CDR3 sequences and increased V_H mutation frequencies when compared to B cells from HC. Moreover, a proportion of peripheral B cells from MS and CIS patients displayed autoreactivity, as demonstrated by analysis of antibody binding to HOG cells, U251 cells, PBMC and A549 cells. Intracellular specificity to HOG cells was demonstrated for antibodies from 4 B cell lines of 3 MS patients. General intracellular binding to 2 or more tested cell lines was demonstrated for 75 other B cell lines from both MS and CIS patients. Interestingly, the major myelin lipid phosphatidyl choline was identified as specific target for 1 CSF B cell line of a MS patient.
Conclusions: In conclusion, clonal expansion and affinity maturation were demonstrated for the first time in peripheral B cells of MS and CIS patients. As several B cell lines showed reactivity to human brain cell lines, the antigen reactivity of the generated monoclonal antibodies is now further examined to identify the specific target antigens.

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Somatic hypermutation of intrathecal IgM-producing B cells in multiple sclerosis patients

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Objective: The vast majority of MS patients have oligoclonal bands (OCBs) of the IgG isotype in their CSF that can be identified by isoelectric focusing. Interestingly, about 40% of MS patients have OCBs of the IgM isotype (OCMB) and many of them bind lipids. Nearly 95% of OCMBs persist for many years. Therefore we hypothesize that OCMB antibodies presumably might not originate from the innate immune system but rather derive from an antigen-driven immune response.
Methods: We collected cells from 3–5 ml CSF from 10 MS patients. We amplified heavy chains by two independent rounds of nested PCR. Reverse inner primers were linked to multiplex identifier (MID), a combinatory tag to discriminate between equal sequences. Each MID was assigned to a particular patient. PCR products obtained from each sample were purified and sequenced by pyrosequencing.
Results: We analyzed the Ig transcriptomes of intrathecal B cells from 10 MS patients highly positive for OCMB. As controls we sequenced peripheral blood B cells from MS patients and a healthy volunteer. We analyzed V_H sequences derived from the different B cells subpopulations, compared them with their corresponding V_H germline sequences, and analyzed the rates of somatic hypermutations in the complementarity determining regions (CDRs) 1 and 2 to the framework regions (FR) of the IgM variable region genes of intrathecal B cells. We found significantly higher rates of somatic hypermutations in the CDR as compared to FR: 6.9% versus 3% nucleotide exchanges in intrathecal IgM-producing B cells; 10.2% versus 3.8% in intrathecal IgG-producing B cells; and 9.8% versus 4.7% in peripheral blood IgG-producing B cells, whereas the sequence analysis from the peripheral blood IgM-producing B cells showed the same rate of substitution between CDR and FR: 2.8% vs. 2.7%.
Conclusions: Our study demonstrates for the first time that somatic hypermutation occurs in IgM antibodies in an autoimmune disease that affects the central nervous system like MS. This strongly supports the view that IgM antibodies are generated by an antigen-driven immune response. In a prospective view our findings might give reason to focus on IgM antibodies as new potential therapeutic targets in MS therapy.

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B-cells as obligatory APC in the marmoset EAE model

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Objective: CD20+ B-cell depletion is a highly promising treatment for multiple sclerosis (MS), but the mechanisms underlying the therapeutic effect are poorly understood. Classically, B-cells are thought to contribute to the MS pathogenesis by production of autoantibodies that amplify demyelination via opsonization of myelin sheaths. However, this does not explain the almost immediate and long-lasting clinical effect.

The aim of this research was to assess antibody non-dependent contributions of B-cells in the experimental autoimmune encephalomyelitis (EAE) model in the common marmoset (*Callithrix jacchus*), a small-bodied Neotropical primate. The common marmoset is used as a valid preclinical model for MS that can be used for translational research into pathogenic mechanisms and therapy development.
Methods: EAE was induced in five marmoset twins with a single synthetic peptide derived from the human myelin oligodendrocyte glycoprotein corresponding with the amino acid sequences 34 to 56 ($MOG_{34–56}$) emulsified in incomplete Freund's adjuvant (IFA). MS-like pathology and disease in this model is induced via an uncommon autoantibody-dependent mechanism involving MOG_{34-56} specific cytotoxic T-cells producing high IL-17A. One sibling of a twin was treated 21 after immunization with HuMab7D8, a human IgG1κ monoclonal antibody against human CD20, and the fraternal sibling served as control.
Results: All control animals developed neurological deficit characterized by balance disturbance and paralysis of the limbs. Furthermore, demyelinated lesions in the white and grey matter were detected. White matter lesions contained infiltrated T-cells and activated macrophages/microglia. Grey matter lesions comprised only few granzyme B+T-cells. Treatment with anti-CD20 resulted in total inhibition or significant reduction of these clinical and neuropathological features.
Conclusions: Taken together, this experiment warrants the conclusion that a critical pathogenic role of CD20+ B-cells is the activation of MOG34-56 specific encephalitogenic T-cells in the marmoset MOG34-56/IFA model, since B-cell depletion impaired the mechanism of action

of the pathogenic T-cells in the autoantibody non-dependent EAE model.

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Differential roles of CXCR3 ligands in trafficking of antibody secreting cells during viral encephalomyelitis

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Objective: Viral infections of the CNS are often characterized by accumulation of antibody secreting cells (ASC) within the nervous tissue. These ASC play a critical role in clearance or controlling viral recrudescence by providing a local source of antibody (Ab). The signals fostering ASC recruitment into the inflamed CNS are poorly understood. Accumulation of ASC within the CNS during gliatropic coronavirus induced encephalomyelitis is CXCR3 dependent and associated with the sustained expression of CXCL9 and CXCL10 mRNA. The present study determined if either CXCR3 ligand was equally capable of recruiting ASC into the CNS.
Methods: To investigate the potential role(s) of CXCR3 ligand(s) on ASC recruitment during virus-induced encephalomyelitis ASC accumulation in the CNS of wild-type, CXCL9 deficient ($CXCL9^{-/-}$), $CXCL10^{-/-}$ and $CXCR3^{-/-}$ mice were compared. Mice were infected intracranially with the gliatropic JHM variant of mouse hepatitis virus (JHMV). Real-time quantitative PCR was used to measure parameters of CNS inflammation, while anatomical distribution of $CD138^{+}$ ASC, CXCL9 and CXCL10 in the CNS were examined by immunohistochemical analysis.
Results: Parenchymal $CD138^{+}$ ASC in the CNS were specifically reduced in $CXCL10^{-/-}$, but not in $CXCL9^{-/-}$ mice. Impaired ASC recruitment in $CXCL10^{-/-}$ mice coincided with reduced CNS levels of virus specific IgG relative to wt mice, but elevated levels relative to $CXCR3^{-/-}$ mice. Neutralizing serum Ab levels were not affected by CXCL9 or CXCL10 deficiency. Moreover transcript expression of BCMA and TACI, B cell receptors that function to support ASC survival and differentiation, remained near naïve levels throughout infection in $CXCL10^{-/-}$ mice. Consistent with similar T cell accumulation and effector function, control of infectious virus was not affected in either $CXCL9^{-/-}$ or $CXCL10^{-/-}$ mice. Immunohistochemical analysis revealed CXCL10 expression in parenchymal cells consistent with astrocyte morphology. On the other hand, CXCL9 expression was primarily confined to the perivascular spaces surrounding blood vessels. Increased magnitude and distinct focal expression of CXCL10 compared to CXCL9 may thus underlie preferential in vivo migration of ASC by CXCL10.
Conclusions: These results highlight the distinct localization of CXCR3 ligands in the CNS during JHMV infection and demonstrate CXCL10 is indispensable for driving ASC migration into the inflamed CNS parenchyma.

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Astrocytic autoantibody of neuromyelitis optica (NMO-IgG) binds to aquaporin-4 extracellular loops, monomers, tetramers and high order arrays

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Objective: To investigate the nature and molecular location of aquaporin-4 (AQP4) epitopes recognized by the pathogenic autoantibody of neuromyelitis optica (NMO-IgG) and the influence of AQP4 supramolecular structure. AQP4, the principal central nervous system (CNS) water channel, is confined to astrocytic and ependymal membranes. Two AQP4 isoforms, M1 and M23, exist as homotetrameric and heterotetrameric intramembranous particles (IMPs). IMPs organized in orthogonal arrays of particles (OAPs) are an ultrastructural characteristic of astrocytic membranes. NMO-IgG-seropositivity unifies a spectrum of relapsing CNS autoimmune inflammatory disorders (NMOSD) of which NMO exemplifies the classic phenotype.
Methods: We used freeze-fracture-electronmicroscopy to evaluate the supramolecular organization of AQP4 M1 and M23 isoforms in transfected HEK293 cells. We used these cells as substrate to test high-titered AQP4-IgG-positive sera from 32 individual NMOSD patients, and 85 control sera, by indirect immunofluorescence (live-cell binding assay), Blue-Native PAGE and SDS PAGE. We investigated by ELISA the binding of NMO-IgG to synthetic peptides corresponding to AQP4 extracellular and intracellular loops.
Results: NMO-IgG binds to denatured AQP4 monomers (68% of cases), to native tetramers and high order arrays (90% of cases), and to AQP4 in live cell membranes (100% of cases). Disease-specific epitopes reside in extracellular loop C more than in loops A or E. IgG binding to intracellular epitopes lacks disease specificity.
Conclusions: Our observations predict greater disease specificity and sensitivity for tissue-based and cell-based serological assays employing "native" AQP4 than assays employing denatured AQP4 and fragments. NMO-IgG binds most avidly to plasma membrane surface epitopes formed by loop interactions within tetramers and by intermolecular interactions within high order structures. The relative abundance and localization of AQP4 high order arrays in distinct CNS regions may explain the variable clinical phenotype of NMO spectrum disorders.
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Blood brain barrier

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Endothelial miR-155 promotes blood–brain barrier dysfunction in neuroinflammation

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Objective: Blood–brain barrier (BBB) dysfunction is thought to contribute to disease progression of many neurological conditions with an inflammatory component such as multiple sclerosis (MS). However, the molecular mechanisms leading to breakdown of the brain endothelial barrier

are largely unknown. Here, we have investigated the role of brain endothelial microRNAs (miRNAs), known post-transcriptional regulators of gene expression, in BBB dysfunction during neuroinflammation.
Methods: Initially, we used the immortalized human cerebral microvascular endothelial cell line, hCMEC/D3, to determine altered miRNAs after stimuli with cytokines at different time points using microarray analysis. We confirmed brain endothelial deregulated miRNAs at the neurovascular unit in MS brain tissue, using laser capture microdissection (LCM), and in spinal cords of the experimental autoimmune encephalomyelitis (EAE), by in situ hybridization (ISH). RT-qPCR was carried out to validate altered miRNAs in vitro and in vivo. To identify the molecular mechanisms mediating miR-155-induced brain endothelial breakdown we used transcriptome analysis and novel targets were validated using 3′ UTR analysis.
Results: Pro-inflammatory cytokines induced altered levels of many miRNAs in cultured human cerebral microvascular endothelial cells. Amongst the deregulated miRNAs, miR-155 showed the highest and earliest increases in brain endothelium during inflammation both in vitro and in vivo. Changes in the levels of selected brain-endothelial deregulated miRNAs, including miR-155, were confirmed in spinal cord of mice at different stages of EAE and in human MS brain tissue. MiR-155-deficient mice were partially resistant to develop EAE, an effect associated with a reduction in BBB leakage to paracellular tracers at the inflammatory plaque. In addition, miR-155 induced increases in brain endothelial paracellular permeability by targeting not only set of genes that maintain brain endothelial cell-cell junctions but also those that regulate focal adhesion contacts.
Conclusions: We propose miR-155 as a novel negative regulator of BBB function in inflammation-mediated neurodegenerative diseases. Thus investigating the roles of miR-155 at the BBB have a number of implications including uncovering new regulators that mediate BBB integrity and identifying potential therapeutic targets to ameliorate the progression of CNS inflammatory disorders.

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ABC transporters: Novel regulators of neuroinflammation

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Objective: The blood–brain barrier (BBB) is the regulated interface between the peripheral circulation and the central nervous system (CNS). One of the key protective features of the BBB is that it strictly regulates the efflux of various toxic compounds through specialized ATP-binding cassette (ABC) transporters, thereby maintaining brain homeostasis. ABC transporters can drive cellular exclusion of a wide variety of exogenous compound and drugs, but recent data indicate that endogenous substrates may include inflammatory mediators like cytokines, chemokines and bioactive lipids, suggesting a potential role in (neuro)inflammatory diseases like multiple sclerosis (MS), topic of study in the current project.
Methods: To study the role of ABC transporters in MS pathology in more detail, we investigated their expression pattern in well-characterized human post-mortem MS tissue and their expression/function in relevant CNS cells. Moreover, proof of principal was obtained *in vivo* by inducing experimental autoimmune encephalomyelitis (EAE).in mice that lack one of the ABC transporters P-glycoprotein.
Results: Here we demonstrate that in active and chronic inactive MS lesions ABC transporter expression is severely altered not only at the level of the blood–brain barrier (BBB) but also at reactive astrocytes, in which they play a role in regulating immune cell migration across *in vitro* BBB models. Moreover, we have provided conclusive *in vivo* evidence indicating that animals that lack P-glycoprotein experience significantly reduced clinical symptoms of experimental autoimmune encephalomyelitis (EAE).
Conclusions: Together, these data highlight a novel immunomodulatory capacity of ABC transporters during neuroinflammation and indicate their therapeutic potential to prevent MS lesion formation.

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microRNAs control brain endothelial cell barrier function and immune quiescence, implications for MS

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Objective: In normal physiology the blood–brain barrier (BBB) tightly regulates brain homeostasis. Perturbations of BBB function, including the loss of brain endothelial cell barrier integrity and immune activation are hallmarks of multiple sclerosis. Therefore, understanding of the BBB in health and disease may lead to novel approaches for MS treatment.
Methods: Using a combined genetic and bioinformatics approach, we uncovered a novel mechanism which regulates different aspects of the BBB, including barrier formation and immune quiescence, i.e. through microRNAs. microRNAs are recently discovered endogenous, small, noncoding RNAs which regulate the production of about 30% of human proteins and have been shown to be involved in cell biology and pathology
Results: Using a genomics approach, we have identified a microRNA (miR-125a-5p) which targets the activity of the transcription factor myc-associated zinc finger protein and plays a major role in the formation of a tight brain endothelial cell barrier and the paracellular trafficking of immune cells. Interestingly, lower levels of miR-125a-5p were associated with the inflamed BBB in vitro and in brain endothelial cells obtained from MS patients by laser capture. Most importantly, our recent analyses in brain capillaries which were isolated from MS patients have revealed that a large panel of BBB stabilizing microRNAs is significantly reduced in MS lesions.
Conclusions: We conclude that therapeutic application of microRNAs (such as miR-125a-5p) potentially could re-establish normal functioning of the BBB to prevent inflammation in multiple sclerosis.

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Sphingosine 1-phosphate receptor 5 mediates the immune quiescence of the human brain endothelial barrier

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Objective: The sphingosine 1-phosphate (S1P) receptor modulator FTY720P (Gilenya®) potently reduces relapse rate and lesion activity in the neuro-inflammatory disorder multiple sclerosis (MS). Although most of its efficacy has been shown to be related to immunosuppression through the induction of lymphopenia, it has been suggested

that a number of its beneficial effects are related to altered endothelial and blood–brain barrier functionality. However, to date it remains unknown whether brain endothelial S1P receptors are involved in the maintenance of the function of the blood–brain barrier thereby mediating immune quiescence of the brain. Here we demonstrate that the brain endothelial receptor S1P5 largely contributes to the maintenance of brain endothelial barrier function.
Methods: We analysed the expression of S1P5 in human post-mortem tissues using immunohistochemistry. The function of S1P5 at the blood–brain barrier was assessed in cultured human brain endothelial cells using agonists and lentivirus-mediated knockdown of S1P5. Subsequent analyses of different aspects of the brain endothelial cell barrier included the formation of a tight barrier, the expression of blood–brain barrier proteins and markers of inflammation and monocyte transmigration.
Results: We show that activation of S1P5 on cultured human brain endothelial cells by a selective agonist elicits enhanced barrier integrity and reduced transendothelial migration of monocytes in vitro. These results were corroborated by genetically silencing of S1P5 in brain endothelial cells. Interestingly, functional studies with these cells revealed that S1P5 strongly contributes to brain endothelial cell barrier function and underlies the expression of specific blood–brain barrier endothelial characteristics such as tight junctions and permeability. In addition, S1P5 maintains the immunoquiescent state of brain endothelial cells with low expression levels of leukocyte adhesion molecules and inflammatory chemokines and cytokines through lowering the activation of the transcription factor NFκB.

Conclusions: Our findings demonstrate that S1P5 in brain endothelial cells contributes for optimal barrier formation and maintenance of immune quiescence of the barrier endothelium.

Blood brain barrier

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Junctional Adhesion Molecule (JAM)-A contributes to monocyte but not T cell migration across the blood-brain barrier influencing clinical severity of EAE

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The migration of circulating leukocytes into the central nervous system (CNS) has been recognized as a key-event in the pathogenesis of multiple sclerosis (MS) and its animal model experimental autoimmune encephalomyelitis (EAE). To enter the CNS, immune cells have to cross either the highly specialized endothelium forming the blood-brain barrier (BBB) or the epithelial cells that establish the blood-cerebrospinal fluid barrier (BCSFB). Junctional adhesion molecule (JAM)-A is expressed in tight junctions of both barriers as well as by different leukocyte subsets and has been implicated in leukocyte adhesion and diapedesis across endothelia of peripheral vascular beds. During inflammatory conditions of $MOG_{aa35-55}$-induced EAE we detected an up-regulation of JAM-A in the inflamed BBB vasculature, BCSFB epithelium, and on microglial cells. At sites of leukocyte infiltration JAM-A was exposed on the luminal side of BBB endothelial cells, indicating an active involvement in leukocyte extravasation. In comparison to wildtype littermates, $MOG_{aa35-55}$-induced EAE in JAM-$A^{-/-}$ mice was milder, delayed and accompanied by reduced leukocyte infiltration into the spinal cord, while MOG-specific T cell priming was not affected. *In vitro* studies with primary mouse brain microvascular endothelial cells (pMBMEC) revealed maintenance of monolayer integrity in the absence of JAM-A and showed a role for endothelial JAM-A in diapedesis of $CD115^{+}CX3CR1^{+}$ monocytes but not of encephalitogenic T cells across pMBMECs under both static conditions and physiological shear flow. In summary, our study demonstrates a role for JAM-A in EAE pathogenesis by specifically regulating monocyte but not T cell recruitment across the inflamed BBB.

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Lesion formation in multiple slerosis and spontaneous experimental autoimmune encephalomyelitis is associated with early disturbances in the blood brain barrier

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Magnetic resonance imaging studies suggest that early changes in the normal-appearing white matter of multiple sclerosis (MS) patients precede appearance of gadolinium-enhancing lesions. Although these findings suggest the breakdown of the blood–brain barrier (BBB) as a central characteristic in MS pathogenesis, limited data is available regarding the changes to the CNS vasculature during lesion formation.
Objective: To study the differential changes of BBB components, particularly during the early stages of lesion formation in MS and in spontaneous experimental autoimmune encephalomyelitis (EAE).
Methods: CNS material from MS patients and from the novel T cell receptor (TCR)-1640 transgenic animals which develop spontaneous relapsing-remitting (RR) EAE were classified according to cellular infiltration, demyelination, astrogliosis and microglial activation. BBB disruption was determined at different levels. We first evaluated the extravasation of endogenous tracers to then examine the expression of the junctional protein molecules. These findings were correlated with changes in the basement membrane and the activation of endothelial cells was determined according to the expression of cell adhesion molecules (CAMs).
Results: Preactive MS lesions displayed considerable microglia and astrocyte activation, minimal leukocyte infiltration, a relative absence of demyelination and discrete basement membrane abnormalities. However, we detected considerable redistribution of junctional proteins and increased expression of all CAMs, suggesting that barrier breach occurs at early stages of lesion formation, before significant immune cell infiltration and demyelination. The pattern of BBB disruption in RR mice was established at various stages before clinical signs and the breakdown of the barrier in these mice was nearly identical to the changes detected in preactive lesions from MS brains.
Conclusion: Our findings indicate early disturbances in the expression of junctional proteins, CAMs and basement membrane proteins at the level of the BBB in non-demyelinating MS and EAE specimens. These early vascular changes coincide with perivascular immune cell infiltration and bring pathological support for important BBB disruption prior to demyelination, during the first stages of lesion formation. In addition, the involvement of peripheral immune responses in this process will be discussed.

Innate immune system

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IL-27 signaling in microglia

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Objective: It has been recently suggested that antigen-presenting cells are not only the source, but also the target of IL-27 - a novel pleiotropic cytokine with both pro- and anti-inflammatory properties. In contrast to the well established diverse effects of IL-27 on T cells, very little is known about how this cytokine might effect the antigen presenting cells themselves.

The aim of this study was to investigate the effect of IL-27 signaling on the phenotype and function of microglia, the resident immune cells of the central nervous system in vitro and under neuroinflammatory conditions in vivo.

Methods: The effect of IL-27 on the function and phenotype of microglia cells was investigated in vitro in primary murine microglia cells. The contribution of the IL-27 signaling to the establishment of the microglia phenotype involved in neuroinflammatory processes was investigated in murine experimental allergic encephalomyelitis (EAE), a model of multiple sclerosis.

Results: Upon IL-27 stimulation in vitro a complex microglia phenotype emerged. On the one hand IL-27 significantly enhanced the phagocytic capacity of microglia cells and induced a characteristic cytokine/chemokine profile (CCL5, CCL3, CCL2 and CXCL1). On the other hand IL-27 upregulated the surface expression of positive (eg. CD40) and negative (eg. CD274) co-stimulatory molecules. Further supporting the bivalent nature of IL-27, it exerted both synergistic (eg. iNOS induction) and antagonistic effect (eg. induction of the antigen presenting machinery) with IFN-gamma. Furthermore, we established that in microglia, unlike in other antigen presenting cells, IL-27 utilizes primarily the MAPK/Erk signaling pathway. In vivo, IL-27 receptor was upregulated in microglia predominantly during the chronic phase of EAE.

Conclusions: Our results demonstrate that IL-27 induces a characteristic microglia phenotype with both pro- and anti-inflammatory aspects. Furthermore, our data suggest that the effect of IL-27 on microglia depends on their state of activation. Nonetheless, the expression pattern of IL-27, IL-27 receptor and the related signaling molecules in the course of EAE, point to the possible involvement of this novel cytokine in the regulation of microglia phenotype and function during inflammatory demyelinating diseases.

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The human neuroinflammasome is activated by HIV-1 infection: Microglia contain requisite inflammasome components

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Objective: HIV-1 enters the brain early in infection and causes chronic neuroinflammation together with the eventual loss of neuronal viability, manifesting clinically as HIV-associated neurocognitive disorders. Several inflammasome complexes have been reported to mediate immune responses, most evident as interleukin (IL)-1β release by myeloid cells, in response to different microbial challenges. However, the expression and impact of the inflammasome in individual neural cell types and ensuing human neurologic disease remain unknown. Herein, we hypothesized the human brain contained an inflammasome and its activation contributed to HIV neuropathogenesis.

Methods: Cerebral white matter from HIV/AIDS (HIV+, n = 5) or other disease control (HIV-, n = 5) autopsied patients was investigated by deep sequencing and semi-quantitative RT-PCR of brain-derived RNA. Primary human microglia, astrocytes and neurons were exposed to prototype stimuli to induce inflammasome activation including ELISA and immunoblot analyses of IL-1β production and release.

Results: In primary human neural cells (neurons, astrocytes and microglia), only microglia constitutively expressed components of the inflammasome's machinery (caspase-1, NLRP1, NLRC4 and AIM2). Exposure of primary human microglia and astrocytes to activators of the inflammasome (LPS, ATP or MDP) induced IL-1ß maturation and release, but only in microglia. The absence of detectable IL-1ß protein in astrocytes was discordant with astrocyte IL-1ß transcript levels, which were induced by multiple stimuli. Conversely, astrocytes exposed to IL-1ß showed activation of TNFα, RANTES and iNOS expression. Human microglia exposed to HIV-1 gp120 or HIV-1 infected showed induction and release of IL-1ß, which was blocked by caspase inhibitors. Deep sequencing and network analyses revealed that NLRP1 transcripts were increased in cerebral white matter from HIV + patients, compared to the HIV- group ($p<0.05$) in conjunction with activation of innate immune pathways. Moreover, caspase-1 as well as the inflammasome targets, IL-1ß and IL-18, were also induced in the HIV + brains ($p<0.05$). IL-1β immunoreactivity was co-localized with MHC Class II in activated microglia within HIV + brains.

Conclusions: These observations highlighted the expression and activation of the inflammasome in human microglia, pointing to a role for these cells as the principal effectors of neuroinflammation, which underlies the development of HIV neuropathogenesis.

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Orchestrated monocyte trafficking via the blood-cerebrospinal fluid-barrier following spinal cord injury

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Objective: Monocyte infiltration following spinal cord injury (SCI) has been traditionally assumed to reflect an unregulated, and thus detrimental, invasion as a result of the mechanical disruption of parenchymal blood–brain-barrier (BBB). The delayed and insufficient infiltration of some beneficial monocytes, considering their abundance in the blood, is thus puzzling. Moreover, given the recently recognized macrophage heterogeneity (pro-inflammatory(M1)/anti-inflammatory(M2)) at the lesion site, the underlying mechanism that regulates the functional fate of the infiltrating monocytes is currently enigmatic.

Methods: A series of experiments that specifically follow the entry route of blood monocytes and their functional fate at the contused spinal cord parenchyma were done. Blocking antibodies, knockout mice and pharmacological inhibitors were used to reveal the underline mechanism utilized in each entry gate. flow cytometry, fluorescent microscopy, Luminex, adoptive transfer experiments as well as chimeras were used throughout the study.

Results: Here, we show that rather than leaking through the disrupted parenchymal BBB, monocytes are in fact actively recruited to the injured spinal cord in a well-orchestrated manner, via the blood-cerebrospinal fluid- barrier (B-CSF-B). Two novel entry zones, with differential recruitment capacities, were identified; the adjacent spinal cord leptomeninges and the anatomically remote brain ventricular choroid plexus. Each entry point was found to be selective towards specific monocyte phenotype and to utilize distinct immunological guiding molecules (chemokines and integrins) to facilitate their recruitment. Importantly each route orchestrated the acquisition of distinct phenotype and functional fate at the injured parenchyma of the differentiated monocyte-derived macrophages (M1/M2).

Conclusions: These results demonstrated an orchestrated homing of distinct monocyte-derived macrophage subsets (M1/M2) to the traumatized spinal cord parenchyma through different trafficking routes,

none of which is the assumed the leaky BBB. Such understanding has far reaching implications to both acute insults and other neurodegenerative conditions as it opens-up a potential novel reparative approach using selective intervention.

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Primary microglia lack strict regulation of inflammasome-mediated activation as compared to myeloid macrophages

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Objective: Myeloid cells can respond to intra- and extracellular danger/stress signals by inflammasome-mediated activation. This process consists of two steps: Toll-like receptor-induced production of pro-IL-1b followed by inflammasome-mediated cleavage and secretion of bioactive IL-1b. Inflammasome-mediated activation is strictly regulated by the induced expression of regulatory proteins and by inhibitory processes like autophagy. Recent studies describe that microglia are markedly different from other myeloid cells. They originate from a separate progenitor, are chronically exposed to the neural microenvironment and their activation may be regulated differently. The objective of this study was to determine the expression profile of inflammasome components in primary microglia and to compare inflammasome-mediated microglial activation and its regulation with other myeloid cells.
Methods: Primary microglia, bone marrow- and blood-derived macrophages (BMDMs) of adult rhesus macaques of the same donor were profiled for all NOD-like receptors (NLRs), inflammasome-associated adaptor proteins, caspases, and regulatory proteins by quantitative rt-PCR. Inflammasome function and kinetics were assessed by exposing LPS-primed microglia to ATP, Silica or MSU (danger-associated molecular patterns: DAMPs) followed by measuring the transcription of pro-IL-1b-encoding mRNA and the secretion of IL-1b.
Results: Primary microglia expressed NLRs (NALP1-3, NOD1/3/4, AIM2, IPAF, NAIP), adaptor proteins (ASC), caspases (1/3-5/7/8), and regulatory proteins (A20), and the expression profile closely resembled that of BMDMs. Priming of microglia with LPS induced high levels of pro-IL-1b-encoding mRNA, but IL-1b protein was only processed and secreted in response to subsequent stimulation with various DAMPs. Interestingly, in LPS-primed BMDMs the window for inflammasome-mediated activation was 4–6 hours. By contrast, LPS-primed microglia remained sensitive for inflammasome-mediated activation for at least 20 hours.
Conclusions: In conclusion, primary microglia express multiple inflammasome components closely resembling the expression profile of BMDMs and they can be induced to form functional inflammasomes. Importantly, primed microglia remain sensitive to inflammasome-mediated activation for much longer than other macrophages. We will present data on whether this reflects a difference in negative regulation of the inflammasome, or differences in autophagy or apoptosis sensitivity.

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LPS-induced microglial activation and neuroprotection against experimental brain injury is independent of hematogenous TLR4

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Intraperitoneal (i.p.) injection of the Gram-negative bacterial endotoxin lipopolysaccharide (LPS) elicits a rapid innate immune response. While this systemic inflammatory response can be destructive, tolerable low doses of LPS render the brain transiently resistant to subsequent injuries. However, the mechanism by which microglia respond to LPS stimulation and participate in subsequent neuroprotection has not been documented. In this study, we first established a novel LPS treatment paradigm where mice were i.p. injected with 1.0 mg/kg LPS for four consecutive days to globally activate CNS microglia. By using a reciprocal bone marrow transplantation procedure between wild-type and Toll-like receptor 4 (TLR4) mutant mice, we demonstrated that the presence of LPS receptor (TLR4) is not required on hematogenous immune cells but is required on cells that are not replaced by bone marrow transplantation, such as vascular endothelia, to transduce microglial activation and neuroprotection. Furthermore, by using a state-of-the-art 3-dimentional electron microscopy, we showed that activated microglia physically ensheath cortical projection neurons, which have reduced axosomatic inhibitory synapses from the neuronal perikarya. In line with previous reports that inhibitory synapse reduction protects neurons from degeneration and injury, we show here that neuronal cell death and lesion volumes are significantly reduced in LPS-treated animals following experimental brain injury. Taken together, our results suggest that activated microglia participate in neuroprotection and that this neuroprotection is likely achieved through reduction of inhibitory axosomatic synapses. The therapeutic significance of these findings rests not only in identifying neuroprotective functions of microglia, but also in establishing the CNS location of TLR4 activation.

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Ifit2 mediates protection from murine hepatitis virus induced encephalomyelitis through amplification of type I interferon expression

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Objective: Type I interferons (IFNα/β) provide a first line of defense against viral infections through the concerted action of hundreds of interferon stimulated genes (ISGs) and limit viral dissemination prior to the emergence of adaptive immune responses. Even though IFNα/β induction in the CNS is inherently low following infection with the dual hepatotropic and neutrotropic coronavirus, mouse hepatitis virus (MHV)-A59, it effectively limits viral spread and protects from mortality. However, the role of specific ISGs in mediating antiviral effects against CNS infections by MHV are largely unknown.
Methods: The present study determined the role of the ISG *Ifit2* in MHV-A59 induced encephalomyelitis using *Ifit2* −/− mice.
Results: Contrasting development of mild encephalitis in WT mice, nearly 60% of infected *Ifit2*−/− mice had more severe clinical disease and succumbed to infection between 6–8 days post infection (dpi).

Increased clinical symptoms in *Ifit2* −/− mice coincided with 2 log higher infectious virus and enhanced viral spread throughout the CNS parenchyma, including dissemination to the cerebellum and brainstem, areas not infected in WT mice. Surprisingly, expression of IFNα/β and consequently downstream ISGs such as *Ifit1, Ifit3*, *Pkr,* and *Isg15* were significantly reduced in the CNS of *ifit2* −/− mice harboring high loads of infectious virus. Resembling the *in vivo* phenotype, impaired IFNα/β induction was also observed in infected bone marrow derived macrophages derived from *Ifit2* −/− relative to WT mice.
Conclusions: These data suggest a novel role of *Ifit2* as a positive regulator in a feedback loop enhancing IFNα/β expression. We are further characterizing the role of *Ifit2* in modulating type I interferon responses in the CNS. This work was supported by NIH Grants NS064932 (CCB) and CA068782 (GCS).

B cells and antibodies

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Progressive encephalomyelitis with rigidity and myoclonus: a syndrome with diverse clinical features and antibody responses

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Objective: The aim is to better characterize progressive encephalomyelitis with rigidity and myoclonus (PERM) syndrome and identify novel PERM phenotypes.
Methods: Three cases were included to the study; 54-year old man presenting subacute onset of difficulty of walking and profuse sweating, 46-year old man with diplopia, dysphagia and gate ataxia and 67-year old man with subacute onset of amnesia. The clinical features and antibody status of PERM patients were investigated using immunoblots, cell based assays (CBA), radioactive immuno assays (RIA), protein macroarray and enzyme-linked immunosorbent assay (ELISA).
Results: Two patients with supratentorial involvement showed abnormal positron emission tomography (PET) or electroencephalography (EEG) findings. One patient was discovered to have renal cell carcinoma and protein macroarray revealed Ma3-antibodies. Another patient with voltage-gated potassium channel and glutamic acid decarboxylase-antibodies showed a good response to immunotherapy.
Conclusions: The heterogeneity of the immunological features suggests that PERM is caused by diverse pathogenic mechanisms. Seropositivity to well-characterized neuronal cell surface antigens might indicate a good treatment response.

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Effects of sex and age on antibodies to the acetylcholine receptor

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Objective: To determine the effect of sex and age distribution on **antibodies to the acetylcholine receptor** (AChR) seropositivity using data derived from patients with myasthenia gravis (MG) in an Argentinean cohort. The AChR autoantibody is a clinically validated serum biomarker to diagnose MG. Sex and age definitively influence MG onset, generally affecting women during the early life, although the prevalence of AChR positive patients in elderly age is being recently diagnosed.
Methods: Data of 119 individuals who tested positive for AChR antibodies were obtained from the Neuroimmunology Unit at Ramos Mejia Hospital, Buenos Aires, Argentina. A standard immunoprecipitation radioimmunoassay test was used. AChR antibody concentration above 0.5 nmol/L was regarded as positive.
Results: Out of 119 samples, 82 (68.9%) were female. The mean age of the patients was 37.4 years (range 9–81) and the mean AChR titer was 13.73 (DS 13.9). Most patients (94/119) were in the range of age between 13 and 60 years old, 54 of them (57.4%) were between 14 and 30 years. 3 (2.5%) were pediatric (≤12 years) and 21 (17.6 %) were elderly (>60 years). Pediatric patients were all female, with a mean AChR titer of 7.9 (DS 7.0). In the group of age between 13 and 60 years it was observed a high female prevalence (female to male ratio 3.6:1), and in patients between 14–30 years the female to male ratio was 3.15/1. The mean AChR titer in these patients was 14.09 (DS 14.5) and 14.34 (DS 15.3) respectively. In elderly patients a high male prevalence of positive AChR antibodies was found, male to female ratio 2.5:1. Mean AChR titer in this group was 12.55 (DS 12.16).
Conclusions: AChR-seropositivity occurs predominantly in women and particularly in individuals between 14 and 30 (45.3%). One in five seropositivity patients was in the extremes of age. In men, but not in women, AChR-IgG seropositivity increases exponentially beyond age 50. There was no difference between AChR titer relates to sex and age.

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Investigating novel autoantibodies in CNS demyelinating diseases

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Objective: The most common demyelinating diseases of the central nervous system (CNS) are autoimmune and include Multiple Sclerosis (MS), Neuromyelitis Optica (Devic's disease) and Acute Disseminated Encephalomyelitis (ADEM). Despite numerous studies trying to identify their primary autoantigens, only Neuromyelitis Optica is firmly associated with autoantibodies to aquaporin-4 (AQP4). Our aim was to study a cohort of patients with autoimmune demyelinating diseases and to identify possible new autoantigens
Methods: Sera (>500) from patients with suspected autoimmune demyelination were screened with immunofluorescence in mouse brain tissue in order to detect anti-neuronal or anti-glial staining. Primary astrocyte cultures were established and Western blot analysis was performed in order to further analyse positive staining.
Results: In 8 patients, (diagnosed with either MS/seronegative NMO/seropositive NMO), we observed an intense anti-astrocyte staining. We confirmed this observation by double immunofluorescence with anti-GFAP (Glial Fibrillary Acidic Protein antibody, a marker specific for CNS astrocytes) in both mouse brain tissue and cultured astrocytes. Western blot analysis in both brain and astrocyte extract

identified common reactivities in some patients. Immunoprecipitation experiments are under way in order to identify the antigen.

In 5 additional patients we observed intense staining in Purkinje cell bodies and dendrites. 4 of these patients were finally diagnosed as MS, while 1 was diagnosed as ADEM. Their common clinical finding was cerebellar ataxia. Three distinct staining patterns were identified. To determine the possible autoantigens in these patients we will perform double immunofluorescence experiments with candidate cerebellar antigens and we will also perform immunoprecipitation experiments.
Conclusions: Our preliminary findings indicate that novel autoantibodies are present in demyelinating CNS diseases and are directed either against glia (astrocytes) or neurons (cerebellum). The anti-astrocyte staining, observed in both MS and NMO patients, could uncover a common pathophysiological mechanism with astrocytes in the epicenter. The anti-cerebellar staining suggests that an uncommon but clinically important symptomatology in demyelinating disorders (cerebellar ataxia) may be further understood. In both cases, the respective autoantigens remain to be determined.

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Transciptome of B lymphocytes in multiple sclerosis

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Objective: Over the past two decades B cells have increasingly moved into the spotlight in multiple sclerosis (MS) research. So far it has not been performed a detailed study of the B cells transcriptome in MS. This may be a prerequisite for identifying the elements that B cells use to carry out their pathogenic role. The primary aim of this project was to analyze the whole B cell-transcriptome (coding and non-coding RNA) in MS context. We studied the differences between MS patients and controls in B cell transcriptome: 1) mRNA including alternative splicing; 2) human microRNAs (miR).
Methods: We studied peripheral CD19 + B cell subsets obtained from 10 MS patients, 10 healthy donors (HD), and 2 monozygotic twins discordant for MS. All affected individuals were treatment-naïve and all the investigations were performed at least three months after the last steroid therapy. Total RNA was extracted using miRNeasy Kit (Qiagen). Microarray data was performed using two Affymetrix's platforms: GeneChip-Human Exon and GeneChip-miRNA. The statistical analysis was performed by means of two different software packages, whose output results have been compared: Partek Genomic Suite (version 6.6) and our own analysis pipe-line based on EasyExon tools. In order to discover and understand the connections among the genes obtained from the analysis, and their position in other biological processes, a pathway analysis was performed using Ingenuity Pathway Analysis (IPA). The miR Analysis was performed by IPA microRNA Target Filter.
Results: Firstly, we found that EasyExon is much less permissive than Partek. So we focused on EasyExon results, in particular we selected 26 genes differentially expressed in MS compared HD ($p<0.03$ and $|\text{fold change}| \geq 1.5$). The list was then submitted to IPA and performed a pathway analysis that led us to choose seven, among the original 26 genes that were among those differentially expressed MS patients and HD. Furthermore, performing the miR analysis with IPA, it has emerged that 6 genes from our list are targeted by 9 miR differentially expressed in MS/HD groups.
Conclusions: Peripheral B cell compartment seems to be promising for the identification of mRNA and miR signature. We have identified an MS-related pattern, in terms of miR and/or genes.

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B-cell activation and treatment effects in multiple sclerosis

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Objective: It is assumed that disease activity in relapsing-remitting multiple sclerosis (RRMS) is mediated by systemically activated myelin reactive $CD4^+$T-cells. The beneficial effect of B-cell depletion in rituximab treated RRMS suggests that B-cells also have a role in the immunopathogensis of MS. Previous studies imply that dysregulated cytokine secretion by B-cells may promote the activation of T-cells. This study aimed to examine the relationship of myelin basic protein (MBP) induced B-cell and T-cell responses *ex vivo* and B-cell expression of cytokines involved in T-cell activation *in vivo* among untreated, interferon-beta (IFNb) treated and natalizumab treated MS patients and healthy volunteers (HV).
Methods: We obtained blood samples from HV ($n=13$) and from untreated ($n=13$), IFNb ($n=16$) and natalizumab treated ($n=14$) RRMS patients. By qPCR we measured mRNA expression levels of *IFNG*, *TNFA*, *IL1B*, *IL6*, *IL12A*, *IL12B*, *IL10*, *IL23*, *IL27*, *LTA*, *LTB* in B-cells. MBP specific B-cell and T-cell responses were analyzed in a CFSE proliferation assay.
Results: There was a strong association between $CD4^+$T-cell proliferation and B-cell proliferation induced by MBP among healthy individuals, which we did not find among MS patients. MBP induced proliferation of B-cells did not differ between the groups examined. Expression of *LTA* in B-cells was higher in IFNb treated than in untreated MS patients. B-cell expression of *TNF* and *LTA* expression was increased and *IL1B* and *TNF* expression decreased in natalizumab treated compared to untreated RRMS patients. B-cells from HV and from untreated MS patients did not show differences in mRNA expression levels.
Conclusion: Our findings suggest that MBP induced B-cell and T-cell responses *ex vivo* seem to be associated among healthy individuals but not among MS patients. This could indicate a dysregulated interaction of antigen-specifically activated B-cells and T-cells in MS. To what extent treatment induced changes in the gene-expression of B-cells contribute to the treatment efficacy needs to be examined in future studies.

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Maternal autoimmunity, brain-reactive antibodies and Autism Spectrum Disorder

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Objective: We propose that in some cases of Autism Spectrum Disorder (ASD), autoimmune susceptibility in the mother leads to the production of anti-brain antibodies that are transferred to the fetus during pregnancy and can alter fetal brain development, leading to ASD in the offspring. Epidemiologic studies have addressed an association between maternal autoimmunity and the development of ASD. Women with rheumatoid arthritis or celiac disease have an increased

risk for a child with ASD. Furthermore, antibodies specific for brain antigens have been found in the serum of some mothers of a child with ASD. When these antibodies were administered to pregnant mice or pregnant monkeys they caused behaviors in the offspring analogous to those seen in ASD.

Our aim is to predetermine whether a mother is at risk to have an ASD child due to her repertoire of autoantibodies and potentially to prevent those cases.

Methods: We screened 2789 sera of mothers of an ASD child and 693 sera of unselected women of child-bearing age for anti-brain antibodies using histology to mouse brain. Positive and negative sera from mothers with an ASD child were then analyzed for anti-nuclear antibodies and for possible association with known autoimmune susceptibility genes.

Results: Mothers of an ASD child were 5 times more likely to harbor anti-brain antibodies than unselected control women. Anti-nuclear antibodies, a measure of autoimmunity, were increased in serum of mothers of an ASD child harboring anti-brain antibodies compared to either mothers of an ASD child without anti-brain antibodies or unselected women of child bearing age. Risk alleles previously associated with rheumatoid arthritis or celiac diseases were present at higher frequency than expected in mothers with anti-brain antibodies and an ASD child.

Conclusions: This work could lead to an assessment of risk of having an ASD child through screening the serum of pregnant women for those with potentially pathogenic brain-reactive antibodies. This in turn offers the possibility of prevention of a proportion of ASD.

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A new sensitive ELISA for aquaporin-4 autoantibodies based on M23 isoform aquaporin-4

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Objective: Detection and measurement of serum aquaporin 4 autoantibodies (AQP4 Ab) are important in the diagnosis and management of neuromyelitis optica (NMO) and related disorders. AQP4 Abs are detectable by different methods including: indirect immunofluorescence (IIF) on tissue sections, AQP4 transfected cell-based assay (CBA), flow cytometric assay and bridge type ELISA. ELISA based on recombinant AQP4 M1 isoform (M1 ELISA) expressed in insect cells showed comparable sensitivity and specificity to commercially available CBAs. Also, CBAs based on AQP4 M23 isoform transfected cells (lacking first 22N-terminal amino acids) have greater sensitivity than those based on the M1 isoform. A new version AQP4 Ab ELISA based on M23 isoform AQP4 (M23 ELISA) was developed and evaluated.

Methods: Three assays, including M23 ELISA, M1 ELISA and a commercially available CBA were compared using 42 sera from patients with suspected NMO or NMO spectrum disorders.

Results:Of the 42 sera studied, 31 (74%) were positive for AQP4 Ab by M23 ELISA (values from 2.6 units/mL to 80 units/mL, mean 26 units/mL, median 6.8 units/mL). Of these 42 sera, 20 (48%, all positive by M23 ELISA) were positive in the M1 ELISA. 32 samples were also tested by CBA and 19 (59%) were positive compared to 21 (66%) positive in the M23 ELISA. There were 12 discrepant samples between M23 ELISA and CBA. 7 samples were positive in the M23 ELISA with AQP Ab values ranging from 2.6 to 46.7 units/mL but negative by CBA while 5 samples were positive by CBA but negative by ELISA. Furthermore, using a cut off for positive of 2.5 units/mL, 102/102 healthy blood donors and 62/62 patients with other autoimmune diseases were negative for AQP4 Ab by M23 ELISA.

Conclusions: The new version ELISA using M23 AQP4 provides a more sensitive assay for detecting AQP4 Ab and has good handling characteristics suitable for routine laboratory use worldwide.

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Antibodies against neurofascin correlate with disease severity in different models of experimental autoimmune encephalomyelitis

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Objective: The presence of oligoclonal bands in the cerebrospinal fluid and the efficacy of the treatment with B-cell depleting antibodies in some patients with multiple sclerosis (MS) provide evidence for the importance of antibodies in disease pathogenesis. Higher antibody levels against neurofascin, a protein localized at the nodes of Ranvier and oligodendrocytes, have been reported in one third of MS patients. Furthermore, antibody levels were more pronounced in secondary progressive MS than in relapsing-remitting MS. In addition, anti-neurofascin antibodies caused rapid worsening of experimental autoimmune encephalomyelitis (EAE), an animal model for MS, following systemic administration. In this study we aim to determine if anti-neurofascin antibodies correlate with EAE susceptibility and severity and if there is an epitope spreading to neurofascin during EAE development.

Methods: Susceptible DA rats were immunized with various myelin antigens such as myelin oligodendrocyte glucoprotein (MOG), myelin basic protein (MBP) and whole spinal cord homogenate (WSC). Rats were monitored for clinical signs of EAE up to 60 days post immunization and serum anti-neurofascin total IgG, IgG1 and IgG2b antibody levels were assessed at the onset and late phase of EAE by ELISA. Correlation of anti-neurofascin IgG levels with clinical EAE phenotypes was performed in 421 (DAxPVG)xDA rats immunized with MOG and monitored for 35 days.

Results: We detected anti-neurofascin antibodies only in the late stages of EAE induced with MOG suggesting that the epitope spreading occurs during disease course. Our preliminary analysis of the (DAxPVG)xDA population revealed higher anti-neurofascin IgG levels in the sick compared to the healthy rats at the late phase of the disease whereas anti-neurofascin antibody levels were almost non-detectable at the onset of MOG-EAE. Furthermore, rats with higher antibody levels against neurofascin displayed higher cumulative score, longer disease, greater weight loss and earlier onset of EAE compared to rats with lower antibody levels. Our preliminary analysis of animals immunized with WSC, also showed that anti-neurofascin antibodies are raised at a later time point in the disease.

Conclusions: There is a spreading of the immune response during MOG-EAE and WSC-EAE to neurofascin antigens secondary to the central nervous system damage. Anti-neurofascin antibodies correlate with susceptibility and severity of EAE and likely contribute to disease pathology.

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Active demyelination triggered by switching from azathioprine to fingolimod in a patient with multiple sclerosis characterized by recurrent optic neuritis: A case report

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Objective: We report a case of increased disease activity following change from azathioprine to fingolimod in a woman with multiple sclerosis characterized by recurrent optic neuritis.
Case Report: A 62 yo woman with multiple sclerosis first diagnosed in 1986 when she presented with an isolated pontine lesion was switched from azathioprine to fingolimod in November 2011 due to increased frequency of attacks of optic neuritis, the predominant manifestation of her disease for more than 20 years. She had tested negative for anti-NMO antibodies. Following a further attack of optic neuritis on fingolimod, widespread demyelination was observed on a subsequent MRI in April 2012. Previous MRIs had demonstrated lesions largely restricted to the pericollosal white matter and optic nerves, however, multiple new gadolinium-enhancing lesions were now seen within the right and left frontal lobes, as well as the right middle cerebellar peduncle. To date, no further clinical attacks have occurred following cessation of fingolimod and resumption of azathioprine.
Discussion: Therapies directed against B cells are gaining ground in the treatment of multiple sclerosis. Heterogeneity in the clinical phenotype and response to therapies in multiple sclerosis suggest it is possible that some forms of the disease are more responsive to treatment directed against antibodies. Although anti-NMO antibodies were not detected in our patient, the predominance of optic symptoms in her case might suggest the presence of an unidentified antibody at which treatment should be targeted. Azathioprine is known to be effective against some humoral-mediated CNS disorders, namely NMO. A recent study has demonstrated that fingolimod has little impact on B cells in the CSF or intrathecal IgG synthesis[1]. Thus, it is possible that active demyelination resulted from reduced suppression of B cell activity within the central nervous system when switching from azathioprine to fingolimod in our patient.
Conclusion: Caution should be exercised when changing patients from one immunotherapy to another, particularly with slightly atypical presentations of multiple sclerosis, necessitating close radiological and clinical observation.
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Cerebrospinal fluid levels of the "B-Cell Maturation Antigen" (BCMA, TNFSRF17) are increased in MS and correlate with B cells

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Objective: B cells play an essential role in the humoral immune response in neuroinflammation and serve as antigen presenting cells for T cells. The B-cell maturation antigen (BCMA, TNFRSF17) is a member of the superfamily of TNF receptors and preferentially expressed in B-cells. By interacting with the TNF family members APRIL (TNFSF13) and BAFF (TNFSF13B), BCMA plays an important role in B-cell homeostasis.

We are aiming to determine if multiple sclerosis (MS) disease modulates BCMA activity in the CNS.

Methods: We studied the expression of BCMA by cells derived from the cerebrospinal fluid (CSF) of untreated MS- and control patients by microarray hybridization. In addition, we quantified soluble BCMA and CXL13 protein as well as B-cell number in CSF samples by ELISA and flow cytometry, respectively.
Results: BCMA was >13 fold more strongly expressed by CSF cells of MS patients than by control patients' CSF cells (p-value: 6×10^{-5}). Active MS patients' CSF cells expressed BCMA approximately 4 times more strongly than those of inactive MS patients (p-value: 3.6×10^{-4}). Untreated MS patients had average BCMA CSF levels of 420 pg/mL and untreated control patients of 255 pg/mL.

Relative numbers of CD19 + B cells correlated well with BCMA CSF levels (Pearson correlation coefficient: 0.631, p-value: 4.9×10^{-3}) and with CXCL13 CSF levels (Pearson correlation coefficient: 0.765, p-value: 2.2×10^{-4}).
Conclusions: Our data suggest that BCMA may be an important factor for the accumulation of B cells in the CSF in the pathogenesis of multiple sclerosis. As BCMA CSF levels correlate strongly with B cell numbers in the CSF, BCMA may be a surrogate biomarker for CNS B-cell activity. CXCL13 is a chemokine discussed before to be important for B cell recruitment into the CNS. Hence, the strong correlation between CXCL13 and BCMA in CSF samples corroborates a probable link between BCMA CSF levels and the presence of B cells in CSF.

Soluble forms of other members of the TNF receptor superfamily were previously shown to have antagonistic rather than agonistic functions. So far, the role of soluble BCMA in contrast to membrane-bound BCMA was not addressed. We are currently trying to elucidate why MS patients have increased BCMA CSF levels.

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Diagnostic efficacy of the flow cytometric aquaporin-4 autoantibody assay: Comparison with conventional cell based assay

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Objective: The antibody to aquaporin4 (AQP4 antibody) is a disease-specific autoantibody to neuromyelitis optica (NMO). Though detection of AQP4 antibody can be sometimes essential in the diagnosis of patients with NMO, its sensitivity varies greatly according to the method of assay. We aimed to establish a highly sensitive and specific anti-AQP4 antibody in Korea using flow cytometry, to evaluate its diagnostic accuracy, and to compare it with that of the conventional cell-based assay (CBA).
Methods: Human embryonic kidney (HEK)-293 cell was transfected with human aquaporin-4 (M23) cDNA. Serum samples from patients with clinically definite NMO (n = 20), high risk for NMO group (n = 49), classic multiple sclerosis (n = 12), other idiopathic inflammatory demyelinating disease (other IIDDs) (n = 35), and negative controls (neurologic disease other than the IIDDs or healthy humans, n = 24) were tested both at the Seoul National University with flow cytometric anti-AQP4 antibody assay and at the Weatherall Institute of Molecular Medicine (John Radcliffe Hospital, Oxford, UK) with conventional CBA. Positive rates of flow cytometric anti-AQP4 antibody assay result in individual groups were assessed and compared with those of the conventional CBA. Receiver operating characteristics (ROC) analysis was adapted to optimize the cut off values of the flow cytometry, and Cohen's Kappa coefficient was measured for the agreement of these two assay methods.

Results: ROC curve for the mean fluorescence index (MFI) ratio of the flow cytometric assay and definite diagnostic criteria for NMO showed the areas under the curve (AUCs) of 0.953. The sensitivity of flow cytometry and conventional CBA were 96% and 91% respectively in definite NMO group, 21.7% and 25.0% in high risk for NMO group, 5.3% and 7.9% in other IIDDs group. Both assays showed no positive results in classic MS group and in negative controls. The kappa coefficient for these two tests was very high and was 0.761.
Conclusions: Both the flow cytometry and conventional CBA are highly specific and also sensitive in identifying patients with NMO, and these two assay methods showed a very good agreement. However, because a small number of patients (3.6%), showed disagreement of results, we suggest that complementary adaptation of those two highly sensitive antibody assays can produce the most accurate outcomes in identifying patients with anti-AQP4 antibody, especially those with low antibody titers.

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B cells influence T cell expansion during the initiation of inflammation in EAE

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Objective: A role for B cells in the CD4+ T cell response in multiple sclerosis (MS) and its animal model, experimental autoimmune encephalomyelitis (EAE), is supported by anti-CD20-mediated B cell depletion studies in both MS and EAE. However, the specific effects of B cells on T cell activation in the CNS prior to onset of disease are not understood. We used an adoptive transfer model to determine the effects of B cells during preclinical events in EAE.
Methods: MOG-specific Th1 and Th17 cells were adoptively transferred into wildtype (WT) or B cell deficient (μMT−/−) C3Heb/Fej mice, and CNS cells were isolated at multiple timepoints. The frequencies of different cell types were analyzed by FACS, and mRNA expression levels were determined by qRT-PCR. Numbers of MOG-specific IL-17- or IFN-γ-secreting T cells were quantified by ELISPOT.
Results: C3Heb/Fej μMT−/− mice have a significantly reduced incidence of EAE induced by adoptive transfer. MOG-specific T cells enter the CNS initially but do not expand in μMT−/− recipients compared to WT, although levels of proliferation are comparable in both recipients. Preclinical mRNA expression levels of many cytokines, chemokines, and adhesion molecules were upregulated to a significantly higher level in the WT CNS compared to μMT−/− recipient CNS, suggesting that the reactivation of and cytokine production by the initial wave of T cells may be defective in the absence of B cells, leading to reduced activation of the vascular endothelium and recruitment of additional T cells from the periphery. B cells were found to be the predominant MHC Class II+ cells in the non-inflamed CNS. B cells preferentially reactivated effector MOG-specific Th1 cells *in vitro* compared with Th17 cells, due to a lack of IL-1β production. Th1 cells induce classic EAE in WT recipients, but induce atypical EAE in μMT−/− recipients. The μMT−/− recipients have fewer IFN-γ producing T cells in the brain than WT recipients, resulting in higher Th17:Th1 ratios in the brain, correlating with the change in localization of inflammation to the brain.
Conclusions: These data support a role for B cells in the reactivation and expansion of T cells entering the CNS during the induction of EAE. B cells may be affecting T cell reactivation through direct antigen presentation or by secreting cytokines that promote T cell activity. Additionally, B cells may differentially influence the ratio of Th1 and Th17 cells that enter the CNS during the onset of EAE.

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A novel GM-CSF$^+$ TNF high IL-6high IL-10$^-$ human B cell subset is abnormally increased in patients with MS

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Introduction: B cell depletion therapy substantially decreases new inflammatory disease activity in patients with multiple sclerosis (MS). This treatment effect dose not apparently changes levels of auto-antibodies highlighting antibody independent mechanism(s) of B cell contribution to MS disease activity. Previous data from our lab showed that activated B cell soluble factors, presumably cytokines, can enhance pro-inflammatory T cell responses, and that these responses may be exaggerated in patients with MS. However, the phenotype, effector cytokine profiles, and frequencies of human pro-inflammatory B cells remain unknown. Here, we characterize a novel human pro-inflammatory cytokine producing B cell subset and implicate it in the patients with MS.
Methods: CD19+ B cells were purified from antecubital venous blood of healthy controls and matched (age, sex) untreated patients with relapsing remitting MS (RRMS). B cells were activated by CD40L-stimulation alone or in combination with B cell receptor (BCR) cross-linking, and/or TLRs ligands. Using multi-colour flow cytometry, B cell subsets were defined based on expression of single or combinations of cytokines (TNF , IL-6, IL-1β, IL-10 and GM-CSF) and compared between healthy controls and MS patients.
Results: Several distinct subsets of B cell were identified based on particular patterns of cytokine expression. Of particular interest is the description of a novel GM-CSF+ TNF high IL-6high IL-10- B cell subset (Beff). Previously described regulatory B cells (Breg) were found to be IL-10+ as expected and also TNF low IL-6low GMCSF−. Stimulation of B cells through CD40L together with BCR significantly induced Beff ($n=7$, $p=0.0196$) but decreased Bregs ($n=7$, $p=0.0068$). In contrast, presence of TLR9 signaling enhances Bregs ($n=7$, $p=0.0011$) while decreasing Beff ($n=7$, $p=0.0019$). Compare between healthy controls ($n=7$) and patients with MS ($n=11$) revealed that patients had significantly higher proportions of Beff ($p=0.0368$).
Conclusion: Our results define a novel pro-inflammatory human B cell subset which can be reciprocally regulated compared to regulatory B cells (Bregs), in an activation- and context-dependent manner. This pro-inflammatory B cell subset is over-represented in patients with MS and may represent a more selective therapeutic target that could obviate need for broad B cell depletion.

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Role of B cells in T cell-mediated Autoimmunity of the central nervous system

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Objective: The role of B cells in the pathogenesis of EAE and MS is incompletely understood. Recent studies with an anti-CD20 antibody therapy in MS patients have shown profound clinical efficacy without any significant effect on the total circulating antibody levels suggesting a role for B cells

beyond the production of autoantibodies. Other studies have proposed that antigen-specific B cells act as highly efficient APC, especially when whole protein is presented. Therefore, we studied the impact of antigen-specific B cells in the generation of pathogenic as well as regulatory T cells.
Methods:
Results: Very small amounts of recombinant MOG protein (rMOG) presented by MOG-specific B cells induced a strong proliferative response of antigen-specific T cells, which was accompanied by the production of high levels of IL-6, IL-10, and IL-17. In contrast, when MOG_{35-55} peptide was presented by B cells, responding T cells preferentially produced IFN-g. Furthermore, when B cells presented low concentrations of antigen, they promoted the conversion of antigen-specific $Foxp3^-$ CD4 T cells into $Foxp3^+$ regulatory T cells. On the other hand, presentation of higher concentrations of rMOG but not MOG_{35-55} completely abrogated Treg conversion. These observations were paralleled by substantially higher concentrations of IL-6 in the supernatant of rMOG stimulated cells. Interestingly, these effects were completely reversed when IL-6 deficient B cells were used.

These *in vitro* observations corresponded to our *in vivo* results using MOG-specific immunoglobulin knock-in mice (TH), in which 20–50% of the B cells express a MOG-specific B cell receptor (BCR). Immunization of TH mice with MOG_{35-55} or rMOG resulted in higher incidence and earlier onset of EAE compared to wild type mice as well as increased proliferation, IL-6, and IL-17 production by T cells in response to antigen. Interestingly, IL-23R-deficiency led to a complete abrogation of EAE in a spontaneous disease model in which MOG-specific T cell receptor transgenic mice (2D2) are crossed to TH mice, while deficiency of IL21R had no or little effect.
Conclusions: Myelin-specific B cells can initiate autoimmune responses by capturing even minuscule amounts of specific antigen via their antigen-specific BCR. As a consequence antigen-specific Th17 cells are generated, whereas the expansion of Tregs is inhibited most likely due to production of IL-6 by antigen-specific B cells.

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Moesin is a possible target molecule in cytomegalovirus-related Guillain-Barre syndrome

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Objective: The epitope in demyelinating Guillain–Barre syndrome (GBS), acute inflammatory demyelinating polyneuropathy (AIDP), remains unidentified. Outer surface of the Schwann cells was considered to be a target of an immune attack in AIDP. In this study, we used proteomic-based approach to reveal an immune target molecule in the pathogenesis of AIDP.
Methods: We searched the proteins recognized by AIDP patients' sera among the extracted proteins from Schwannoma cell lines by mass spectrometer after 2-dimensional electrophoresis and subsequent western blotting. Identified specific autoantibody was validated in the sera of GBS and normal controls.
Results: Autoantibody against moesin protein was identified only in the patients with AIDP after the cytomegalovirus (CMV) infection. We found 5 out of 6 CMV-related AIDP patients to have autoantibody against moesin, but none of the 15 AIDP patients without CMV infection and 2 of the 46 normal controls had it. Moesin protein was known to be present at distal tip of the Schwann cells and considered to play a crucial role in the myelinating process. Immunohistochemically detected moesin at distal tip of the Schwannoma cell lines was a target of the autoantibody in the patients with CMV-related AIDP.
Conclusions: Moesin is a possible immune target and play an important role in the pathogenesis of CMV-related AIDP.

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Rituximab treatment in a girl with voltage-gated potassium-channel associated FIRES (fever-induced refractory epileptic encephalopathy in school-aged children)

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Objective: Describe response to Rituximab treatment in a girl with Voltage-gated potassium-channel associated (VGKC) FIRES (fever-induced refractory epileptic encephalopathy in school-aged children)
Methods: This is a 6 year-old girl who presented with acute onset, status post febrile prodrome, of refractory multifocal status epilepticus requiring medically induced coma for approximately 1 month with high-dose barbiturates, Midazolam infusion, and inhaled Isofluorane. She had traveled to Vietnam 2 months prior. Her CSF profile was benign. Extensive infectious testing including full arboviral panel was negative. Her initial brain MRI had been normal. Repeat MRI one month into her illness showed bilateral hippocampal FLAIR hyperintensities extending into the thalami and posterior centrum semiovale.

She was found to have neuronal autoantibodies: VGKC 0.15 nmol/L (reference range ≤ 0.02). Thus, one month following presentation, she was treated with intravenous immunoglobulin (ivIG) 2 g/kg, Methylprednisolone 30 mg/kg/day iv for 5 days with a prolonged oral steroid taper. She then underwent plasmapheresis for 5 doses every other day. Her seizure control significantly improved, the timing of which seemed to correlate with her plasma exchange, though she had also required placement on the ketogenic diet along with a regimen of 5 antiepileptic medications. She had negative body tumor screen.

She remained encephalopathic, status post tracheostomy and G-tube placements. After a 4-month hospitalization, she was transferred to a rehabilitation facility. She continued to have a few brief seizures weekly. Five months after presentation she experienced recrudescence of seizures with some decrease in arousal. There was no intercurrent infection nor significant changes in her antiepilpetic drug troughs.VGKC antibodies which had decreased to zero post-pheresis were again detectable. Thus, she received a repeat Methylprednisolone pulse. She underwent repeat cycle of plasmapheresis.

Given this relapse, she was then treated with Rituximab 375 mg/m^2 iv weekly for four doses starting 7 months after her initial presentation, and she responded with complete B-cell depletion. She was weaned off her oral steroid taper shortly thereafter. She remains on monthly ivIG 1 g/kg, ketogenic diet 4.25:1 ratio, Lacosamide, Levetiracetam, Phenobarbital, and Phenytoin.
Results: One month later her VGKC was again detectable at 0.08 nmol/L despite complete B-lymphocyte depletion. Her epilepsy has stabilized and she has been able to wean some dosages of her medications. However her encephalopathy has seen only slow improvement: she still remains non-verbal with no clear communication; she can sit unsupported and hold herself standing with assistance.
Conclusions: We describe a pediatric case of relapsing VGKC-associated FIRES treated with Rituximab. There has been stabilization of her seizures and some modest improvements in her mental status. The VGKC antibody titers reappeared even while her B-cells were still depleted. These are likely still being produced by plasma cells, which

are unaffected by Rituximab. Whether earlier treatment or other immunosuppressive modalities might lead to better outcomes remains to be studied.

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Systemic autoimmunity in an Argentinean cohort of Neuromyeltis Optic patients and its clinical impact

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Objective: To study the autoantibody profile and associate autoimmunity and its impact on clinical features and disease severity of Neuromyelitis Optic (NMO) and its spectrum disorders (NMOSD).
Background: NMO has been associated with other clinical or serological markers of non-organ-specific autoimmunity. It is unknown if concomitant autoimmune disease or systemic autoantibody seropositivity confers a different prognosis in NMO/NMOSD patients.
Design/methods: We retrospectively reviewed patients with diagnosis of NMO/NMOSD at Neuroimmunology Unit, Ramos Mejia Hospital, Buenos Aires, Argentina, and the presence of autoimmune diseases and antibody positivity and their relationship with clinical characteristics and disease severity.
Results: Thirty-four (31 female, 3 male; 29 NMO, 5 NMOSD) were analyzed. Mean disease duration was 8 years (range: 3 – 17 years). Overall systemic autoantibody seropositivity was high: anti-nuclear antibodies 63% (17/27), anti-DNA 10 % (2/20) and anti-Ro and/or anti-La 37.7% (5/14). Autoimmune disease was present in 23.5 % (8/34). Of those who tested for NMO-IgG, 75 % (15/20) were positive; 53% (8/15) of these patients also had had presence of seropositivity of systemic autoimmunity and 20% (4/20) autoimmune disease. Mean annualized relapse rate and median Expanded Disability Status Scale (EDSS) score was not significantly different between systemic autoantibody-positive and autoantibody-negative patients, with or without concomitant autoimmune disease.
Conclusions: Our results suggest that concomitant systemic inflammatory diseases and systemic autoantibody positivity in NMO/NMOSD patients is a common feature. The presence of systemic autoantibody seropositivity or autoimmune disease does not translate to a worse clinical outcome in NMO/NMOSD patients.

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Identification of virus infection induced or modified cellular proteins as a target for humoral immune response in multiple sclerosis

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Objective: One of the hallmarks of multiple sclerosis (MS), is an increased intrathecal humoral immune response presenting as increased IgG index and oligoclonal bands (OCBs). Although in many of the infectious or autoimmune diseases of the central nervous system the bands have been shown to be specific for the cause, the specificity of the bands in MS, as well as the cause of MS have remained elusive. Virus infection can change the cell phenotype by altering the host cell gene expression or inducing post translational modifications of host cell proteins. Some of these modifications can lead to breakdown of immunogenic self tolerance and production of autoantibodies.
Methods: To test if MS cerebrospinal fluid (CSF) or serum contains antibodies to cellular proteins induced or modified by human herpesvirus 6 (HHV-6) infection, CSF and serum antibodies were used to immunoprecipitate cellular proteins in HHV-6, not mock, infected cell lysates. These proteins were separated in SDS-PAGE and bands were excised. Proteins were in-gel trypsinized and subjected to MALDI-TOF mass spectrometric analysis.
Results: We identified autoantibodies in MS CSF and serum to known autoantigens (e.g. histones) that were present only in HHV-6 infected cells.
Conclusions: This might suggest that virus infection can induce post translational modifications of histones or some other cellular proteins, which can lead to production of autoantibodies.

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Conditional MHCII expression reveals a selective role for B cell antigen presentation in autoimmune encephalomyelitis

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Objective: The activation and differentiation of effector CD4 T cells depends on interactions with major histocompatibility class II (MHCII)-expressing antigen presenting cells (APCs). While several APC populations coordinate CD4 T cell function, several lines of evidence indicate that antigen presentation by B cells is a critical element to the cellular immune response during immunity and autoimmunity. We have sought to determine the individual contribution of B cell antigen presentation to CD4 T cell priming and secondary responses in relation to neuro-inflammation.
Methods: To address the hypothesis that B cells are sufficient APCs for CD4 T cell responses, we have designed a new tool in which individual subsets of APCs are capable of conditionally expressing MHCII in vivo. We successfully targeted a stop sequence flanked by loxP sites to the MHCII β chain locus in mice in order to utilize the Cre/loxP system for conditional expression of MHCII, resulting in a mouse deficient in MHCII. Successful conditional manipulation was achieved using $CD19^{Cre}$ mice, restricting expression of MHCII to B cells.
Results: After intravenous or subcutaneous priming, sub-optimal proliferation and activation of CD4 T cells was observed in mice expressing MHCII only by B cells. Further, limiting MHCII expression to B cells constrained secondary CD4 T cell responses in vivo. We tested the sufficiency of B cell antigen presentation in directing autoreactivity in an animal model for multiple sclerosis, experimental autoimmune encephalomyelitis. B cell expression of MHCII alone was sufficient to support secondary autoreactive CD4 T cell responses targeting the central nervous system, but only when B cells were highly efficient at recognizing target antigen.
Conclusions: These results demonstrate a novel capacity to conditionally express MHCII in vivo. Further, they highlight the selective capacity for B cell antigen presentation to initiate and propagate CD4 T cell autoreactivity targeting the central nervous system.

Biomarkers

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Myelin basic protein and neurofilament as biomarkers of treatment response in a Phase II add-on trial of rituximab for relapsing multiple sclerosis

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Objective: Myelin basic protein (MBP) and neurofilament (NF) levels are markers of tissue destruction to myelin sheaths and axons, respectively. We evaluated these biomarkers in predicting outcomes in multiple sclerosis (MS) subjects treated with rituximab, a B cell depleting monoclonal antibody, in combination with platform agents.
Methods: Clinical and imaging data were evaluated from a phase II trial of rituximab as an add-on agent. Patients were categorized into ideal responders (n = 10), intermediate responders (n = 11), and non-responders (n = 9) based on clinical measures and contrast enhancing lesions (CEL) after treatment. Ideal responders had no CEL and stable or improved clinical exams after treatment. Levels of MBP and phosphorylated heavy chain NF were determined at weeks 0 and 24 using ELISA (Beckman Coulter and BioVendor, respectively). Statistical analyses were conducted using nonparametric Kruskal-Wallis rank tests and Spearman correlation coefficient (r_s) by rank.
Results: CSF MBP levels correlated strongly with the number of CEL [Spearman r (r_s) = 0.609, p = 0.002] and volume of CEL (r_s = 0.520, p = 0.009), but did not correlate with clinical markers (p = 0.784 with EDSS). CSF NF correlated with EDSS (r_s = 0.479, p = 0.018), but not with MRI (p = 0.187 with number of CEL; p = 0.436 for volume of CEL). CSF levels of MBP trended towards correlating with NF (r_s = 0.405, p = 0.050). Serum levels of MBP and NF were mostly below detection limits. At week 24 after treatment, subjects had lower levels of CSF MBP (p = 0.046) and NF (p = 0.067). These differences were driven by subjects who had been pre-identified to be ideal responders to treatment with rituximab; changes were not significant in the intermediate responders or non-responders. Baseline CSF levels of MBP (p = 0.191) and NF (p = 0.629) at week 0 did not predict treatment response to rituximab. Subjects that responded best to treatment had more CEL (p = 0.037) and lower EDSS scores (p = 0.036).
Conclusions: A significant drop at 24 weeks in the CSF levels of MBP and NF was associated with good response to treatment with rituximab and may warrant future study. CSF MBP correlated with inflammatory CEL, while NF correlated with clinical measures, as has been reported previously. In this add-on study, treatment with rituximab was most beneficial in patients who were less disabled and with greater numbers of inflammatory lesions.

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Novel diffusional kurtosis imaging metrics of demyelination and neurodegeneration

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Objective: Diffusion weighted MRI (DWI) is sensitive to microscopic injury in normal appearing white matter (NAWM) in MS patients and in experimental models of demyelination but it is not specific to demyelination and axonal degeneration. We propose to use Diffusional kurtosis imaging (DKI) a novel clinically feasible DWI method to probe non-Gaussian diffusion tissue properties. Using DKI we will quantify intra- and extra-axonal diffusivities, the axonal water fraction (AWF) and the tortuosity of the extra-axonal space in relapsing-remitting MS (RR-MS) patients and will investigate the feasibility of this novel method in murine experimental models.
Methods: Thirty two patients with RR-MS and 19 matched controls underwent MRI at 3 T using a TRSE sequence for DKI: 30 diffusion directions, b = 0, 1000, 2000 s/mm^2, in-plane resolution 2.7 × 2.7 mm^2. DKI parametric maps were calculated, and then used to derive WM parametric maps of the intra-axonal diffusivity *D*axon, extra-axonal axial and radial diffusivity, *De*,|| and *De*,$^{\perp}$, tortuosity α, and AWF. Using tract-based spatial statistics (TBSS), skeletonized voxel-wise analysis was performed of the DKI and WM metrics using FSL. In addition, regions of interest (ROI) were drawn of the corpus callosum genu and splenium to determine between group differences for each parameter. Three12 week-old male C57BL/6 mice underwent MRI at 7.4 T using a 2D DWI gradient and spin echo sequence with 30 diffusion directions and b = 0, 1300 and 2100 s^2/mm, in-plane resolution of 60 × 60 μm. DKI parameters were measured in the corpus callusum.
Results: TBSS showed a widespread pattern of significant changes in all DKI parameters of RR-MS patients compared to NC. Of the WM parameters, significant changes were observed in De, , the tortuosity α, and the AWF, whereas Daxon and De,|| did not significantly differ between groups. The ROI-based analysis showed similar results in both the genu and splenium of the corpus callosum. The study in the wild C57BL/6 mice showed that the method is feasible in small animals with an acquisition time of 60 minutes.
Conclusions: Changes in the WM-parameters (as obtained from DKI) may provide more insight in the specific underlying disease processes occurring in the NAWM of RR-MS: The decrease in the AWF, tortuosity and the increase in *De*, can be explained by demyelination, while the lack of change in Daxon suggests an absence of intra-axonal injury in the NAWM. The method is feasible in mice and the new DKI-metrics will be validated in experimental models of lysolecithin-induced demyelination.

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Neuron specific enolase (NSE) as a valid marker for neuronal damage in murine models of ischemic stroke and EAE

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Objective: Immunity and inflammation are not only key players in the pathophysiology of experimental allergic encephalomyelitis (EAE) but also in ischemic stroke. In the murine middle cerebral artery occlusion (MCAO) stroke model common methods for analyzing brain tissue damage are vital staining with TTC (2,3,5-triphenyltetrazolium chloride), immunohistochemical methods and magnetic resonance imaging (MRI). While TTC and immunohistochemical approaches are fast and reliable methods the further exploration of the analyzed tissue is restricted. In contrary MRI monitoring allows a noninvasive analysis but

is rather expensive and time consuming. Standard methods for detection of the neuronal damage in EAE are histological examination and the neurological score.

In this study we aimed to validate whether plasma levels of neuron specific enolase (NSE), a protein excusively espressed by neuronal cells, correlates with the degree of cerebral damage in stroke in EAE.

Methods: Mice underwent temporary occlusion of the middle cerebral artery for one hour followed by reperfusion (MCAO) or induction of EAE with MOG35-55 peptide. In stroke experiments we correlated infarct size measured by MRI and TTC with the plasma NSE levels at d1, d3 or d7 of reperfusion. In EAE experiments we correlated plasma NSE levels with the neurological score at d15, d21 or d30. Plasma NSE levels were detected by ELISA (IBL, Hamburg, Germany).

Results: In the stroke model there was a good linear correlation between NSE plasma levels and infarct size measured by TTC staining and MRI ($r > 0.5$) for d1 and d7 and a high correlation ($r > 0.7$) for d3.

In the EAE model plasma NSE levels showed a high correlation ($r > 0.7$) at d15 and even a good correlation ($r > 0.5$) at d21 and d30.

Conclusions: Taken together we provide evidence that plasma NSE levels correlate significant with the degree of neuronal damage in animal models of chronic and acute injury of the central nervous system. NSE is therefore a valid and simple experimental tool allowing to assess the degree of neuronal injury in a non invasive approach offering the opportunity to carry out further biochemical or morphological experiments with the CNS tissue samples.

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Myeloid microvesicles as a prognostic biomarker in CIS patients converting to MS: A 2 year-prospective study

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Objective: The aim of the study is to explore the role of Myeloid Microvesicles (MMVs) – emerging new biomarkers of tissue damage and disease activity – as a possible prognostic biomarker for early MS conversion in CIS patients and to understand their potential biological role studying their correlation to other clinical and neuroradiological markers.

Methods: This is a prospective study on 54 ClS patients (60%female, median age 30 years, median EDSS at onset 1.5, median disease duration 7 months) recruited at San Raffaele Hospital in 2 years. Patients underwent lumbar puncture for diagnostic reasons and 500 μL of CSF were analyzed by flow cytometry (stained for AnnexinV, IB4) in order to detect MMVs. 30 age matched healthy-controls were also evaluated for MMVs presence.

Results: First, we confirmed that CIS patients have higher MMVs than healthy controls (ROC curves of CIS vs HC: sensibility of 85% and specificity of 100% with cut-off value 1.6 MV/μL)Patients earlier-converters (converting to Clinically Defined MS – according to 2010 McDonald criteria – in 1 to 3 months) have higher MMVs (median 34.6 MV/μL) in comparison to one-year converters or two-year converters or non-converters($p < 0.001$). Interestingly, 2 patients who underwent a progressive course of the disease have definitely higher MMVs.

MMVs rate directly correlate with EDSS both at onset and at follow-up (Spearman $r = 0.68$, $p < 0.001$). An association with axonal damage has to be demonstrated.

No correlation with oligoclonal bands, disease duration or type of clinical presentation, was evident. Correlation with neuroradiological data (DTI, fMRI) on a subgroup of CIS patients is ongoing.

Conclusions: MMVs are able to identify earlier converters to MS and may represent a prognostic biomarker.

Patients presenting with higher MMVs more frequently developed an established disability in comparison to those with lower MMVs. We may suppose that MMVs may reflect disease activity rate and axonal damage. Their role in neurodegeneration has to be determined.

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Myeloid Microvesicles as potential biomarker in probable Alzheimer disease and other dementia patients

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Objective: To assess the amount of microglial microvesicles in the CSF of patients affected by Alzheimer Disease (AD) and Mild Cognitive Impairment (MCI), in comparison to age-matched healthy controls and other dementia and prospectically correlate it with clinical, laboratory and neuroradiological data.

Methods: 200 patients (80 AD, 50 MCI, 20 Frontotemporal Dementia, 30 other dementia; 20 healthy controls) were recruited at San Raffaele Hospital in 18 months. Patients underwent CSF, MRI and neuropsychological assessment analysis. 200 μL of CSF were analyzed by flow cytometry to detect Microglial MVs

Results: An increased number of Microglial MVs is found in the CSF of AD patients, in comparison to MCI and healthy control patients. FTD patients had an even higher MMVs level. Among FTD patients those with higher MMVs were patients affected by Semantic Dementia, while BvFTD have lower MMVs. Microglial MVs in AD correlated with disease duration and CSF B-Amiloid/Tau while no differences were found for age, sex, MMSE, symptoms at onset, educational level. Homocystein blood levels significantly correlated with MMVs. For MCI no correlations with any markers was evident. Interestingly, the subgroup of MCI-converters to AD within one year ($n = 28$) showed higher MMVs. A correlation with hippocampal atrophy and MMVs is evident in AD. Correlations for other, more sensitive multiple neuroradiological parameters is ongoing, in order to find early, better markers of disease progression.

Conclusions: We proposed Microglial Microvesicles as an additional novel biomarker – to be combined with other early markers – to characterize the course of AD and the probability of MCI to convert to AD. The role of MMVs in differentiating FTD subtypes has to be confirmed.

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Analysis of Peripheral blood mononuclear cell phenotypes of patients with relapsing-remitting multiple sclerosis under fingolimod treatment (0.5 mg, oral dose): A prospective stu

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Objective: The inhibition of lymphocyte egress from secondary lymphoid tissues by Fingolimod (inhibitor of sphingosine-1-phosphate-receptors) leads to a pronounced lymphopenia. So far, specific immunological changes in the peripheral venous blood, sera or PBMCs respectively, have only been analyzed in small patient numbers. The influence of fingolimod on peripheral blood mononuclear cell (PBMC) subpopulations in patients with RRMS over a 6-month treatment period was analyzed and potential surrogate markers that may be associated with a clinical response to treatment were explored.
Methods: 450 RRMS patients receiving 0.5 mg fingolimod on a daily oral dose were included in this prospective, multi-centre, single-arm, open-label study. Peripheral venous blood samples were obtained at several timepoints (baseline, 1, 4 and 6 months respectively). Leucocyte surface marker expression was analysed in a flow cytometric analysis (CD3, CD4, CD8, CD16, CD19, CD34, CD45RA, CD56, CCR7 surface markers were determined). Additionally patient sera samples were screened for their immunoglobulin levels (IgG, IgM, IgA, IgE) and the presence of various cytokines by ELISA assays.
Results: The analysis of the first 250 patients revealed a decrease in the relative number of CD45+, CD4+ and CD19+ positive cells, which maintained at low levels for the follow-up. The decrease in the CD4+/CD8+ ratio is due to the reduction of CD4+ T cells. CD4+ cells however, were never found to be depleted. Also cell numbers of naïve T cells (CCR7+/CD45RA+) were decreased to a sixth of the level before treatment and stays at low level for the whole 6-month period. In contrast effector memory T cells (CCR7-/CD45RA-) were increased. Also IgG levels decrease in the 6-month treatment by approx. 11%. IgM and IgA are just slightly decreased, while IgE shows no variation in the 6-month treatment. For the majority of the cytokines tested so far, no significant alterations were found. First results on the viral load during treatment show no alteration. The study is still ongoing and data for the majority of the patients will be available.
Conclusions: Fingolimod induces an intended strong and reversible effect predominantly on CD4+ for the time of the treatment. Naïve T cells are reduced, whereas effector memory T cells are increased. Biomarkers predicting clinical efficacy and/or safety signals have not been identified yet and require further prospective studies.

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Blood adipokines and their association to disability status and progression index in multiple sclerosis: A three-year follow up study

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Objective: Multiple sclerosis (MS) is a chronic inflammatory disease of central nervous system that affects young adults and leads disability. Blood biomarkers which indicate a specific biological/pathological process are essential to confirm or reject a diagnosis, to anticipate the outcome and to check the efficiency of therapy in a particular patient. In the present study, we determined the 3-year follow-up levels of adipokines including leptin, adiponectin, adipsin and resistin in the sera of patients with MS and analysed their association to various clinical parameters including disease activity and progression.
Methods: The study was a 3-year prospective follow-up study that included 60 MS patients, 20 subjects with clinically isolated syndrome (CIS) and 30 healthy controls who underwent neurological examination for 3 years. Plasma adipokine levels were measured using enzyme immunoassay.
Results: At the baseline and 1-year follow-up, CIS patients showed higher levels of resistin than RRMS group ($p<0.05$). Comparison between the CIS and CIS converted group revealed that the baseline levels of adiponectin increased in those subjects who converted to CDMS ($p<0.01$). The highest levels of adipsin were found in progressive subtypes, while in RRMS adipsin levels remained low during the whole 3-year period ($p<0.05$). In RRMS group, the levels of adipsin seemed to correlate positively with disability status and progression index (PI) ($p<0.05$). In PPMS group at the 2 yr follow-up, the levels of leptin seemed to correlate with the PI ($p<0.05$).
Conclusions: Our data suggest that leptin, adipsin and resistin might contribute to the development of disability and progression of the disease, while adiponectin seems to be a candidate predicting conversion of CIS to MS. These adipokines are important in MS pathogenesis and might be potential biomarkers.

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Switch-associated protein 70 (SWAP 70) antibody levels are associated with clinical relapse in multiple sclerosis

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Objective: To identify an antibody biomarker for multiple sclerosis (MS) that can be used as a predictor of MS relapses.
Methods: MS patients' sera were screened by a protein macroarray derived from human fetal brain cDNA library (hEX1). Sera of 90 consecutive relapsing remitting MS (RRMS) patients and age-matched 145 Behçet's disease (BD) patients, 40 infectious meningoencephalitis patients and 70 healthy controls were screened by ELISA for serum antibodies against the selected clone.
Results: Sequencing of the clone with the highest signal intensity revealed switch-associated protein 70 (SWAP70) as a potential target autoantigen in RRMS. ELISA studies showed high-titer SWAP70-antibodies in 21 (23.3%) RRMS and 7 (4.8%) BD patients. SWAP70-antibodies were more likely to be found positive in sera obtained during or shortly after a relapse.
Conclusions: RRMS and BD might share common pathogenic mechanisms associated with SWAP70 functions. Detection of SWAP70-antibodies during the attack period might suggest that these antibodies are involved in MS relapse pathogenesis. Serum SWAP70-antibody detection may be utilized as an easy-to-use MS relapse predictor.

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Genetic biomarkers for preferable individualized selection of interferon-beta or glatiramer acetate for disease-modifying treatment in multiple sclerosis patients

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Objective: Several immunomodulatory strategies are being actively implemented in therapeutic intervention in multiple sclerosis (MS). Long-term medication with specific first-line disease modifying treatments (DMTs) interferon-beta (IFNb) and glatiramer acetate (GA) has been shown to improve MS course. In search of biomarkers for selection of preferable individual DMT we performed the comparative pharmacogenomic analysis of immune response genetic variants and their combinations in MS patients treated with IFNb or GA. Unified set of genes, encoding proteins which are involved in the mechanisms of action of IFNb and/or GA and identical clinical criteria of treatment response in Russian MS patients were used.
Methods: 253 unrelated MS patients resident of the Moscow region treated with IFN-beta and 285 MS patients treated with GA were studied. The patients who were event-free during no less than 2 years on DMT were considered as optimal responders (Rs), other patients were considered as non-optimal responders (NRs). We compared the distribution of alleles/genotypes/allelic combinations of investigated genes among IFNb Rs versus GA Rs and among IFNb NRs versus GA NRs. The allelic combinations, of which carriage was associated with discriminative DMT response, were identified using APSampler algorithm with subsequent validation by means of the exact Fisher's (p_f) and permutation (p_{perm}) tests. Functional polymorphisms in the following candidate genes were studied: *DRB1* HLA class II, *IFNB1* (rs1051922), *IFNAR1* (rs1012335), *IFNG* (rs2430561), *TNF* (rs1800629), *TGFB1* (rs1800469), *IL7RA* (rs6897932), *CCR5* (rs333) and *CTLA4* (rs231775).
Results: Carriage of allelic combinations composing of *CCR5*, *DRB1*, *TGFB1* and *IFNAR1* genes was different between IFNb Rs and GA Rs ($p_f = 0.00054–0.03$; $p_{perm} = 0.004–0.024$), whereas carriage of allelic combinations composing of *CCR5*, *CTLA4* and *IFNAR1* genes was different between IFNb NRs and GA NRs ($p_f = 0.00078–0.02$, $p_{perm} = 0.017–0.058$). Some discriminative composite markers, of which carriage in Russians is beneficial for MS individuals on IFNb treatment whereas detrimental for those on GA treatment were identified.
Conclusions: Our study provides an option for identification of promising prognostic composite genetic markers for IFNb or GA treatment selection for individual MS patients.

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MicroRNA profiling in human EDTA plasma identifies a unique profile in Multiple Sclerosis patients compared to healthy controls

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Objective: Multiple sclerosis (MS) is an immune cell mediated autoimmune disease. There is little information available regarding disease stage specific biomarkers and predictors of response to any MS therapy. Identification of biomarker that could identify the stage and type of MS and that could predict a response to therapy, is invaluable, and would be the first step towards individualized medicine. Altered expression of cellular microRNA (miRNAs) has been reported previously in MS compared to controls. We investigated differential expression of miRNAs in EDTA plasma samples from MS patients compared to controls.
Methods: Expression of 368 miRNAs was tested in EDTA plasma obtained from 19 MS patients (10 relapsing remitting MS (RRMS), and 9 secondary progressive MS (SPMS)) and healthy control (HC, 9 subjects). This screening step was used to identify miRNAs that were differentially expressed in SPMS to RRMS and among various group comparisons. We selected 23 miRNAs from initial screening experiment that were further tested using quantitative real time PCR on a separate set of 100 MS patients (50 RRMS and 50 SPMS) and 32 HCs to validate results obtained from screening results.
Results: We found differential expression of miRNAs in MS patients compared to HCs plasma samples. We identified miRNA variables in plasma that could differentiate SPMS or RRMS from HCs. Most importantly, we identified miRNAs in plasma that could differentiate a progressive patient from a relapsing remitting patient.
Conclusions: In summary, our results establish a blood-based test to measure miRNAs in plasma that could be used to study disease or therapy related biomarkers in MS patients.

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Single cell pStat1 activation, a promising biomarker to evaluate neutralizing antibody effects in interferon-beta treated multiple sclerosis patients

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Objective: IFNb is a major first line therapy in MS, but available preparations are immunogenic and can induce the production of neutralizing antibodies (NAbs) that reduce treatment efficacy. Routine testing for NAbs with *in vitro* assays is recommended. However, reported NAb titers in sera of patients are not necessarily transferable to clinical outcomes. Therefore, a personalized *in vivo* assay is needed to evaluate the importance of NAbs in individual patients. The hypothesis was to test if phospho-specific flow cytometry (phosphoflow) can measure the activation of the IFNb/Stats signaling pathway in immune cell subtypes after administration of IFNb *in vivo* and to test if an inappropriate response to IFNb due to NAbs can be detected and quantified *in vivo*.

The suitability of the assay was tested against IFNb inducible gene expression changes, including Mx1.
Methods: An 8 parameter phosphoflow single cell assay was developed to identify immune cell subtypes in blood and quantify intracellular phosphorylated (activated) Stats transcription factors before IFNb injection and at several time points thereafter and to assess NAb effects *in vivo* (t = 15 min–24 h; n = 10). In addition, IFNb specific gene expression changes (whole blood mRNA) and IFNb concentrations (sera) were measured. Data was subjected to PCA, PLSR, and Ffmanova.
Results: IFNb preparations were significantly different in their Stats signaling signature in immune cells *in vivo* and NAbs significantly affected this pattern in cell subtypes. Phosphorylation of Stat1 in T cells and monocytes was predictive of NAb class *in vivo*. Gene expression patterns were significantly affected by NAbs *in vivo*, but had lower predictive values than Stats. IFNb concentrations in sera were variable but increased after INFb injection in most patients.
Conclusions: Phosphoflow can quantify the activation of the INFb/Stats pathway in immune cell subtypes and detect NAb effects *in vivo*. This single cell based assay may be more accurate than gene expression changes in whole blood to predict NAbs effect *in vivo*. The *in vivo* results support our previously published *ex vivo* phosphoflow data that identified Stat1 as a promising biomarker for NAbs effects in individual patients.

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The role of Fetuin-A in neuroinflammatory responses in multiple sclerosis

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Objective: We recently identified Fetuin-A as a cerebrospinal fluid (CSF) biomarker of multiple sclerosis (MS). Elevated CSF Fetuin-A was highly correlated to active disease in MS patients. In addition, therapeutic response to natalizumab correlated with a significant reduction in CSF Fetuin-A. Fetuin-A, a cystatin-like serum protein, was also found to be aberrantly expressed in neurons in and around demyelinated lesions in MS and in EAE. Because mice lacking Fetuin-A showed reduced severity of EAE, we hypothesize that Fetuin-A may play a direct role in MS pathogenesis. The aim of this study was to examine the role of Fetuin-A in mediating neuroinflammatory responses in MS.
Methods: As an *in vitro* model of neuroinflammation, SH-SY5Y neuroblastoma cells were co-cultured with LPS-stimulated macrophages in the absence or presence of Fetuin-A. Growth and viability were measured by Alamar Blue assay. As an *in vitro* model of T cell polarization, naïve CD4 + T cells were isolated from PBMCs and polarized into Th1, Th2, Th17, and Treg cell populations in the absence or presence of Fetuin-A. Gene expression changes were measured by quantitative real time PCR. Protein expression and secretion was measured by flow cytometry, immunocytochemistry, and ELISA.
Results: We found that Fetuin-A potentiates the neurotoxic response of neuroblastoma cells to inflammation mediated by activated macrophages, correlating with uptake of Fetuin-A. In T cell polarization experiments, Fetuin-A promoted Th1 polarization from naïve T cells, which correlated with an increase in Th1-specific gene expression and cytokine secretion. In contrast, Fetuin-A had no effect on Th17 or Treg polarization.
Conclusions: These results indicate that Fetuin-A promotes pro-inflammatory responses in *in vitro* models of neuroinflammation. These findings are consistent with the role of Fetuin-A as a CNS biomarker of active MS and suggest potential mechanisms by which Fetuin-A may play a role in MS pathogenesis.

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MS disease related variables and its impact on gray matter volume and cortical thickness

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Nordic multiple sclerosis (MS) patients are in 95% of cases positive for oligoclonal IgG bands (OCB) in the cerebrospinal fluid (CSF). OCB-positive and OCB-negative patients are similar with regard to clinical characteristics—and indistinguishable with regard to fulfillment of the Barkhof criteria on brain magnetic-resonance-imaging (MRI). Intriguingly, OCB-negative MS is not associated with the best known MS risk factor—the HLA class II allele *DRB1*15*—but rather with the *HLA-DRB1*04* allele. Intrathecal IgG synthesis has been reported to be associated with cortical lesions in clinically isolated syndromes and early relapsing-remitting MS. *HLA-DRB1*1501* was shown to be associated with lower grey matter fraction and lower brain parenckymal fraction.
Objective: To investigate which of the variables, *HLA-DRB1*15 and HLA-DRB1*04* genotypes, OCB, gender, disability, patients age, and treatment length and type, have the biggest impact on cortical thickness and gray matter volume in MS patients.
Methods: For this retrospective–explorative study we chose 25 MS patients attending Stockholm MS Center and selected according to the following criteria: (1) they had been examined with brain MRI according to a MS specific protocol (2) their OCB status was known; and (3) they had been genotyped for *HLA-DRB1*. Genotyping was performed by sequence-specific PCR using a commercial kit. Gray matter volume and cortical thickness were analyzed using FreeSurfer software on MP-RAGE T1 weighted sequences. Principal component analysis (PCA) and regression analysis was conducted to find out which of the covariates has strongest impact for regional and global atrophy development.
Results: Statistics is currently conducted to address the objectives.
Conclusions: This study gives a better understanding the impact of HLA and OCB status on grey matter volume and cortical thickness

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CSF biomarker development for oxidative stress in patients with multiple sclerosis

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Background: Multiple sclerosis (MS) is an immune-mediated disease of the central nervous system (CNS). While inflammation predominates in relapsing-remitting MS (RRMS) patients, it becomes less important in progressive stages of MS, where current immunomodulatory therapies provide no clinical benefit. One of the mechanisms that may contribute to neurodegeneration in subjects with long-lasting CNS demyelination is intrathecal mitochondrial dysfunction, associated with decreased production of ATP and enhanced mitochondrial generation of reactive oxygen species (ROS). Resulting oxidative stress may drive further

mitochondrial dysfunction, leading to progressive neurodegeneration. This pathogenic mechanism could be hypothetically inhibited by therapies that have a potential to improve mitochondrial function, such as idebenone. However, another source of ROS in the CNS of MS patients is oxidative burst of the myeloid cells. Therefore, the purpose of this study was to compare CSF biomarkers of oxidative stress and the biomarkers of activation of myeloid lineage in a large cohort of MS patients and other inflammatory (OIND) and non-inflammatory (NIND) neurological diseases controls in order to assess mitochondrial versus myeloid source of ROS in different patient subgroups.
Methods: ELISA assays were optimized to quantify, in a blinded fashion, the concentrations of 4-hydroxynonenal (4-HNE) histidine adducts as biomarker of oxidative stress and CXCL13 and IL-12p40 as biomarkers of activated myeloid lineage in a large cohort ($N = 192$) of untreated subjects who presented for the diagnostic work-up of possible neuroimmunological disorder.
Results: 4-HNE concentrations were significantly elevated in the CSF of MS patients compared with NIND controls ($p < 0.05$). Among MS subtypes, RRMS and particularly PPMS patients showed significantly higher CSF levels of 4-HNE compared with NIND controls. In contrast CXCL13 and IL-12p40 levels were selectively elevated in RRMS and OIND subjects, but not in PPMS cohort.
Conclusions: Our result suggests that while oxidative burst of myeloid cells contributes significantly to intrathecal oxidative stress in RRMS patients, mitochondrial dysfunction is more likely source of ROS in the PPMS subjects. This validates PPMS as ideal cohort for testing of therapeutic efficacy of idebenone. Phase II clinical trial of idebenone in PPMS is currently in progress.

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RNA sequencing analysis of intrathecal exosomes isolated from multiple sclerosis patients

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Objective: Multiple sclerosis (MS) is one of the most common causes of neurological disabilities in young adults. Multiple physiological and pathological processes in central nervous system (CNS) were observed in MS autopsy tissue. Unfortunately, there are no reliable tools to quantify these different intrathecal processes in living subjects. Cerebrospinal fluid (CSF) represents an invaluable diagnostic window to the CNS, thanks to its function in removal of brain metabolites by draining interstitial fluid. While CSF cells and soluble factors represent a traditional target of investigation, CSF contains also a specific type of microvesicles – exosomes – released by different cell types and loaded with a protein and RNA cargo, suggesting their role as mediators of intercellular communication. The objective of this study is to investigate the nature of the exosomal RNA load using an unbiased approach.
Methods: Using RNA sequencing we investigated the RNA content of CSF exosomes in a total of 5 groups of patients, pooling 10 patient samples per group. Three groups represented primary progressive MS, relapsing–remitting MS, and secondary progressive MS. Two control groups of non-MS patients represented non-inflammatory neurological diseases and other inflammatory neurological diseases. The resulting sequence data were aligned to human exome, as well as to the databases of human herpes viruses and human endogenous retroviruses (HERV).
Results: RNA sequencing analysis confirmed the presence of sequences originated from cellular mRNA in CSF exosomes; this analysis is currently ongoing. No RNA sequence derived from human herpes viruses family was found in exosomal RNA in either of 5 groups of patients. In contrast, we identified a large number of sequences mapping to HERV family. Over 18,000 sequence reads aligned to 120 HERVs, represented by 3883 different loci. However, comparison of the expression levels for each HERV among 5 analyzed groups did not reveal any significant differences.
Conclusions: Exosomes isolated from CSF contain RNA that originates from both, protein coding and non-coding regions of human genome, including a large portion of RNA sequences derived from HERVs. Several HERV transcripts have been detected in the CNS, frequently in the context of neuroinflammation. Although our study identified a presence of many HERV transcripts in exosomes derived from CSF, we didn't find any correlation between HERV expression and MS disease status.

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Single nucleotide polymorphisms in relapsing-remitting multiple sclerosis in Serbian patients: Disease susceptibility and interferon beta treatment response biomarkers

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Objective: Recombinant interferon (IFN)-β therapy is most widely used to reduce disease activity in patients with relapsing-remitting multiple sclerosis (RRMS). However, up to 50% of patients continue to have relapses and worsening disability despite therapy. Since reliable biomarkers with predictive value for natural history of disease and clinical response to therapy are still lacking, our aim was to analyze the association of selected single nucleotide polymorphisms (SNPs) in genes coding for cytokines or their receptors with presumed pathogenic role in MS with disease susceptibility and response to IFN-β therapy in RRMS patients in Serbia.
Methods: We assessed SNPs in IFN-γ (rs2430561), tumor necrosis factor (TNF)-α (rs1800629), interleukin (IL)-17F (rs11465553), p40 subunit of IL-12/23 (rs3212227) and IL-23 receptor gene (IL-23R, rs2201841) in 121 consecutive RRMS patients starting IFN-β therapy and 166 healthy controls. Response to IFN-β was defined after two-year follow-up: patients without relapses and no progression in the Expanded Disability Status Scale (EDSS) score were considered responders (R, $n = 69$), while those with relapses and progression in disability were considered nonresponders (NR, $n = 52$). SNPs were determined by qPCR using custom-made oligonucleotides (IFN-γ) or TaqMan® SNP genotyping assays (other SNPs).
Results: Distribution of IL-23R CC and CT genotypes was different in RRMS patients compared to healthy controls (5.8% vs. 15.7% and 47.9% vs. 38%, respectively; $p = 0.022$). In contrast, the frequency of C and T alleles of IL-23R SNP did not differ between the groups. Genotype distribution and allele frequencies of other 4 SNPs were also similar in RRMS patients and healthy subjects.

None of the analyzed SNPs correlated with the response status of the RRMS patients. However, when R were compared with poor

responders (defined as 2 or more relapses and a 6-month sustained increase in the EDSS score of at least one point), we found the difference in the frequencies of G and A alleles of −308 TNF-α SNP (89.7 and 10.3% vs. 79 and 21%, respectively; $p = 0.042$). Disparity was also observed in TNF-α genotype distribution, although it did not reach statistical significance ($p = 0.057$).
Conclusions: Our results suggest that IL23R and TNF-α SNP genotyping might help in diagnosis and prediction of clinical response to IFN-β therapy in RRMS patients, but the studies with larger cohorts of patients are needed to evaluate their potential use as biomarkers.

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Pharmacogenomics biomarkers in multiple sclerosis: Transcriptional profiling of the B cell response to interferon-beta

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Objective: Interferon beta (IFN-B) is a widely used therapy in multiple sclerosis (MS), however, up to 50% of the patients are poor responders (PR) to the drug. Currently there are no predictive biomarkers for IFN-B response in clinical use. Limited data is available on the B cell response to IFN-B. Our aim was to characterize the gene expression response to IFN-B in B cells, using lymphoblastoid cell lines as model for B cells, and to identify gene expression patterns associated with the mode of clinical response to IFN-B in multiple sclerosis (MS) patients.
Methods: Lymphoblastoid cell lines (LCLs) from MS patients classified as either excellent responders (ER, $n = 9$) or PR ($n = 8$) were used as an experimental model for B cells. LCLs are known to retain many B cell properties, as well as inter-donor variability in gene expression. LCLs were cultured for 4 hrs with or without IFN-B and genome wide expression analysis was performed in Illumina BeadChip (HumanHT-12 v4). Differentially expressed genes (DEGs) and significantly enriched functional pathways between the experimental groups were identified using two-way analysis of variance and Ingenuity Pathways Analysis.
Results: We identified > 100 DEGs following IFN-B exposure (False Discovery Rate (FDR) ≤ 0.05). Among the list we found DEGs that had not been previously associated with IFN-B response in other cell types and validated 5 of them by Real-Time RTPCR on additional LCLs. Network analysis revealed that most of the IFN-β response DEGs map to known canonical IFN pathways. Interestingly, some of the genes belong to functional pathways known to be involved in MS pathology and therapeutics, such as the sphingosine-1-phosphate signaling pathway. Comparison of the gene expression patterns between untreated LCLs from ER and PR (FDR ≤ 0.05) yielded 11 DEGs, most of which were mapped to several IFN-related canonical pathways.
Conclusions: The novel IFN-B response genes identified in LCLs from MS patients suggest the existence of B cell-specific IFN-B response pathways. Further analyses in primary B cells of these genes and of DEGs identified as associated with the MS patients' clinical response to IFN-B would contribute to validation of their importance for tailored MS therapeutics.

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Extracellular Actin Scavenger System as a diagnostic/prognosis tool in multiple sclerosis

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Objective: Since multiple sclerosis (MS) is an heterogeneous and multifactor disease, the study of inter-individual differences in cerebrospinal fluid (CSF) proteome may contribute to the discovery of novel markers useful for diagnosis, disease subtype categorization and progression monitoring, as well as for the development of new therapeutic agents. Indeed, personalized investigations may highlight new biomarkers of MS subtypes linked to potentially different pathological mechanisms. To this aim, we focused on the study of individual CSF samples collected from a "homogenous" population of female relapsing-remitting multiple sclerosis subjects.
Methods: A blind proteomic study was performed analysing by bi-dimensional electrophoresis (2DE) the CSF from 24 treatment-naïve MS patients declared as relapsing-remitting RRMS at the moment of the lumbar puncture. Clinical data of each patient were accurately recorded and patients follow up was monitored for about 2 years after the lumbar puncture.
Results: On the basis of the percentage volume of spots, a hierarchical clustering analysis was performed and four spots enabling to separate patients in subsets strictly correlated to the aggressiveness of the disease were highlighted. Surprisingly, by mass spectrometry analysis we found that these spots contain gelsolin (GS) and vitamin D binding protein (DBP), both belonging to the extracellular actin scavenger system. Further analysis highlighted an opposite trend of two spots corresponding to two different DBP isoforms, suggesting the involvement of a post translational modification (PTM). Preliminary experiments combined to 2DE position of the spots indicate the two isoforms correspond to a different glycosylation status of the DBP. Moreover, DBP isoforms appear to be able to discriminate the only subject whose RRMS diagnosis was not confirmed.
Conclusions: These findings seems very promising because they can have a diagnostic/prognostic value and help the clinicians to early establish the rate of disease progression. Moreover, they may represent novel therapeutic targets and be useful to elucidate new pathological mechanisms. Further analysis on purified DBP will be crucial to characterize the structure of DBP isoforms and to study the enzymes involved in the post-translational modification process. These data are protected by an Italian patent filing procedure (No. MI2012A000865; holders: FISM and Bertolotto A).

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Hu antibodies are present in smokers with chronic obstructive pulmonary disease without cancer or neurological disease

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Objective: Chronic obstructive pulmonary disease (COPD) and smoking are risk factors for lung cancer. It was studied if the paraneoplastic

antibodies, anti-Hu and anti-CRMP5, were associated with cancer in smokers with or without COPD.
Methods: Hu and CRMP5 antibodies were measured in sera from 552 smokers (≥10 pack-yrs); 379 of these had COPD and 300 blood donors served as controls. The paraneoplastic antibodies were measured by a radioimmunoprecipitation assay (RIPA). The positive sera were also tested by indirect immunohistochemistry and line blot with recombinant proteins. The 552 smokers were matched with data from the Norwegian Cancer Registry, and the hospital medical records from the patients with paraneoplastic antibodies were reviewed. The index serum samples were collected in 2006, and the mean follow-up time of the smokers with paraneoplastic antibodies was 4.4 years (range 2.5–5.7 years).
Results: The IP assay showed that 5/379 (1.3%) COPD patients had Hu antibodies and 1/379 (0.3%) COPD patients had CRMP5 antibodies. Only the COPD patient with the highest IP assay index had detectable Hu antibodies also by immunohistochemistry and line blot. None of the smokers without COPD had such antibodies. None of the controls had Hu antibodies, but 2/300 (0.7 %) had CRMP5 antibodies. The Hu and CRMP5 antibodies remained positive during an observation time of more than 5 years. No cancer and no relevant neurological disease have been recorded in the Cancer Registry or medical records up till date. The total cancer frequency in smokers with COPD was 57/379 (15 %).
Conclusions: Hu antibodies were not significantly more frequent in smokers with COPD than in blood donors ($p = 0.07$, Fisher's exact test). Hu antibodies were not associated with cancer or neurological disease in COPD and are therefore not always paraneoplastic. It cannot exclude that indolent cancer has been present and then been discarded by the immune system in these patients.

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Novel cerebrospinal fluid and serum autoantibody targets for Clinically Isolated Syndrome

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Objective: Multiple Sclerosis (MS) is a chronic inflammatory, autoimmune disease of the central nervous system, characterized by demyelination of neurons. Clinically Isolated Syndrome (CIS) is a possible first manifestation of MS and a subgroup of these CIS patients will develop MS. Cerebrospinal fluid (CSF) of both CIS and MS patients is characterized by the presence of oligoclonal band antibodies. However, little is known about the antigens to which these antibodies are directed. The aim of this study was to identify novel antigens for CIS and explore their prognostic potential to predict conversion to MS.
Methods: To identify novel antigenic targets for CIS, a phage display technique, called serological antigen selection (SAS), was applied on CSF from 4 CIS patients, who developed MS. Two cDNA phage display libraries suitable for SAS have been constructed, which were derived from normal and MS brain, respectively. Antibody reactivity towards candidate CIS antigens was tested in CSF and serum from patients with CIS ($n = 123/108$), MS ($n = 65/n = 44$) and other (inflammatory) neurological diseases ($n = 75/n = 38$) as well as in serum from healthy controls ($n = 44$).
Results: Using SAS, a panel of 6 novel CIS candidate antigens was identified, all derived from selections with the normal brain cDNA library. Apart from antibody reactivity in CSF from CIS patients used for the SAS technology, antibody reactivity was also detected in CSF from additional CIS patients and relapsing–remitting (RR) MS patients. Serum antibody reactivity was significantly increased in CIS and RR-MS as compared to healthy and neurological controls ($p = 0.03$). For 2 antigens prognostic potential could be demonstrated, because the frequency of both CSF and serum antibody positive patients was higher in CIS patients who converted to MS as compared to CIS patients without conversion.
Conclusions: A panel of 6 novel CIS antigens was identified to which antibody reactivity was primarily detected in CIS and RR-MS as compared to controls. Prognostic potential to predict conversion to MS could be demonstrated for 2 antigens. Future research is needed to study the role of these antigens in CIS and MS pathogenesis.

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Response gene to complement-32 as a biomarker of relapses in multiple sclerosis

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Objective: Response gene to complement (RGC)-32 is a novel molecule that plays an important role in cell proliferation but its role in multiple sclerosis (MS) has not been investigated. Our objective was to investigate the expression of RGC-32 in MS brain and in the peripheral blood mononuclear cells (PBMCs) and correlate this expression with clinical activity in patients with relapsing remitting MS.
Methods: We first examined the expression of RGC-32 in MS brains by immunohistochemistry and correlate its expression with that of inflammatory cells ($CD3^+$, $CD4^+$,$CD68^+$ cells), glial cells (ionized calcium biding adapter molecule 1 positive cells) and astrocytes (glial fibrillary acidic protein positive cells). To gain more insight into the role played by RGC-32 in MS, we then examined the expression of RGC-32 mRNA in relation to disease activity in PBMCs using real time PCR and correlated its expression with that of FasL mRNA. To silence RGC-32 expression, PBMCs were transduced with RGC-32 shRNA lentiviral particles (Santa Cruz Biotech).
Results: We found that RGC-32 was expressed by $CD3^+$, $CD68^+$, and glial fibrillary acidic protein positive cells in MS plaques. Expression of RGC-32 was not confined only to the MS plaques but was also present in normal adjacent white and gray matter areas, indicating a widespread distribution of cells expressing RGC-32. Our data also indicated that RGC-32 mRNA expression is up-regulated in PBMCs of stable patients with RR MS when compared with healthy controls ($p < 0.0001$). In contrast, during periods of clinical exacerbation, RGC-32 mRNA expression was significantly decreased in these patients when compared to that in stable patients ($p < 0.0001$). RGC-32 expression was also found to be correlated with that of FasL mRNA during relapses ($r = 0.89141$, $p = 0.0002$). In addition FasL expression by CD4+ cells was significantly reduced after RGC-32 silencing, indicating a role for RGC-32 in the regulation of FasL expression.
Conclusions: This decrease in RGC-32 expression might be useful in predicting disease activity in patients with relapsing-remitting MS.

Our data suggest that RGC-32 plays a dual role in MS, both as a biomarker of disease activity and as a regulator of FasL expression in CD4 T-cells.

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The frequency of inflammatory activity (intrathecal oligoclonal bands and Gd-enhancement in MRI) in German and Turkish MS cases

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Background and objectives: Multiple sclerosis (MS) is a demyelinating neurodegenerative disease of the central nervous system (CNS). As the most consistent immunological finding, detection of oligoclonal band (OCB) in the cerebrospinal fluid (CSF) is an important tool for the diagnosis of MS and clinically isolated syndrome (CIS). The frequency of OCB was found to be different in various countries suggesting a possible influence of environment and/or of genetic background. In addition, B cell activity markers including CSF-OCB are found to be higher during periods of disease activity as detected by MRI. In our study, we

a) compared the frequency of intrathecal OCB in patients with CIS and MS from Germany and Turkey, and
b) evaluated the correlation between intrathecal OCB and the presence of Gd-enhancing lesions in MRI.

Patients and materials; This retrospective collaborative study was performed in Ulm University and Cerrahpasa Medical School, Neurology Departments. Total number of patients included is 277 (164 German, 113 Turkish patients). Subgroups of patients are CIS, relapsing remitting MS (RRMS), secondary progressive MS (SPMS) and primary progressive MS (PPMS). Mean age at onset was 34 years in Germans and 30 years in Turks. Isoelectric focusing and immunoblot method was used to detect CSF OCBs. MRI scan (1.5 T) and CSF collection were performed prior treatment with steroids.
Results: The frequency of OCB was found to be similar in both groups (78% in Germans, 76% in Turks), and the occurrence of simultaneous Gd- enhancing lesions was found to be different as follows: 65.6% in Germans and 46.4% in Turks.
Conclusion: The frequency of CSF OCB occurs similar in two populations with different genetic backgrounds. The inflammatory activity as detected by MRI appears causally unrelated to inflammation as reflected by CSF OCB.

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IRF5 and IRF8 genotypes, relapses and progression in multiple sclerosis patients treated with interferon-beta

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Objective: A proportion of patients with relapsing–remitting multiple sclerosis (RRMS) treated with interferon-beta respond well to therapy, whereas others continue to have relapses and are developing irreversible neurological deficits over time. Interferon response factor (IRF)5 and IRF8 mediate the induction of endogenous type I interferons in response to viral infections, and previous studies have demonstrated that polymorphisms in the *IRF5* and *IRF8* genes are associated with the response to treatment with interferon-beta in RRMS. We studied whether three single nucleotide polymorphisms (SNPs) in the *IRF5* gene and two SNPs in the *IRF8* gene are associated with relapse risk and risk of disease progression in Danish patients treated with interferon-beta.
Methods: We studied 497 Danish patients (159 men, 337 women), who were initiated on their first treatment with interferon-beta from December 1995 to March 2008. Median time of follow-up was 54 months. Clinical data were obtained from the Danish MS Treatment Registry. The annualized relapse rate (ARR), the time to first relapse (censored after two years) and the time to a confirmed 1 point increase in the EDSS score (censored after five years) were recorded for all patients.

DNA was obtained from venous blood samples and the SNPs rs2004640, rs3807306 and rs3807306 in the *IRF5* gene and rs13333054 and rs17445836 in the *IRF8* gene were analysed using TaqMan allelic discrimination. Primers and probes were obtained from Applied Biosystems (Foster City, CA, USA).
Results: The median number of relapses in the two years before start of therapy was 2 (range 0–8). During two years of follow-up 298 patients (60%) suffered a relapse. Patients with more than two relapses in the two years prior to treatment had a higher relapse risk on treatment (log-rank test: $p<0.001$; hazard ratio 1.512, 95% confidence interval 1.198-1.909). There was no relationship between the genotype of the *IRF5* and *IRF8* SNPs and the time to first relapse or the ARR on treatment with interferon-beta. During follow-up for up to five years, 155 patients (31%) had a sustained 1 point increase in the EDSS score. Patients with more than two relapses in the two years prior to treatment had a higher risk of sustained EDSS progression (log-rank test: $p=0.045$; hazard ratio 1.39, 95% confidence interval 1.004-1.924). There was no relationship between the genotype of the *IRF5* and *IRF8* SNPs and risk of sustained EDSS progression.
Conclusions: Patients with high disease activity prior to treatment with interferon-beta have an increased risk of breakthrough disease activity and sustained EDSS progression on treatment. The studied SNPs in the *IRF5* and *IRF8* genes are not associated with relapse risk or disease progression in Danish patients treated with interferon-beta.

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Identification of a novel autoantigen and a diagnostic marker of autoimmune hypophysitis

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Objective: Autoimmune hypophysitis (AH) is a neuroendocrine disorder with various degrees of pituitary dysfunction. Histology shows chronic inflammation with infiltration of lymphocytes and plasma cells. Lymphocytic infundibuloneurohypophysitis (LINH) is one type of AH, in which lymphocyte infiltrated in the neurohypophysis accompanied with central diabetes insipidus due to the insufficient arginine vasopressin (AVP) secretion. Pathogenesis of LINH as well as the autoantigen is unknown. The differential diagnosis between LINH and other pituitary diseases that cause DI such as pituitary tumor is sometimes difficult, resulting in the misdiagnosis. Thus, the aim of the present study is to identify pathogenic pituitary autoantigen of LINH and develop the non-invasive diagnostic marker biomarker of LINH.

Methods: We performed an immunoprecipitation-shotgun liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis for screening the pathogenic pituitary autoantigens of LINH. The purified IgG from sera and the extract of the pituitary posterior glands were mixed, immunoprecipitated, and the antigens were screened with shotgun proteomics methods with LC-MS/MS.
Results: We identified several autoantigen candidates and performed the antigen-based immunoblotting. Anti-76 kD protein antibodies were detected in serum samples from 6 of the 7 patients with LINH (86%). The specificity of 76 kD-autoantibody immunoblotting was confirmed by absorption studies, in which preabsorption of the serum samples with recombinant 76kD completely blocked the band migrating at 76 kDa. The anti-76 kD protein antibodies were detected in 1 of the 10 patients with DI due to other causes such as pituitary tumor (10%) and were detected in 1 of the 9 healthy controls (11%). In addition, we found that a 76 kD protein that is known to be involved in vesicle transport was colocalized with AVP neurons in hypothalamus and posterior pituitary where the sites of the disease are. We subjected rat pituitaries to immunostaining with serum samples. Serum samples from patients with LINH identified posterior pituitary, whereas there was no staining of the posterior pituitary by serum samples from controls. Moreover, double immunostaining analysis revealed that serum samples from patients recognized AVP neuron as well as 76 kD proteins. These findings suggest that the 76 kD protein is involved in the pathogenesis of LINH.
Conclusions: In conclusion, 76 kDa protein appears to be an autoantigen involved in LINH. Autoantibodies against 76 kD protein appear to be diagnostic for LINH.

Blood brain barrier

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Laquinimod Enhances Central Nervous System Barrier Functions

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Objective: The blood–brain barrier (BBB) is a complex structure of tightly joined capillary endothelial cells (ECs) restricting passage of cells and molecules into the CNS. Disruption of the BBB and trafficking of auto-reactive T cells from the systemic compartment into the CNS are considered important and early events in the development of multiple sclerosis (MS) lesions. Leukocyte transmigration across the BBB requires the sequential activation and interaction of numerous molecular effectors expressed by BBB-ECs including selectins, chemokines, cytokines and cell adhesion molecules. Evidence of the anti-inflammatory role of the immunomodulator laquinimod for the treatment of MS arose mainly from animal studies, where it was shown to inhibit the development of experimental autoimmune encephalomyelitis. However, the effects of laquinimod on the BBB remain to be established. In this study, we sought to evaluate whether laquinimod provides clinical benefit by decreasing BBB activation and leakage, as well as immune cell migration to the CNS.
Methods: To model changes in the characteristics of the BBB, we employed an *in vitro* model of the human BBB. We determined the effect of laquinimod on the permeability of human BBB-ECs grown in primary culture and measured by ELISA the array of chemokines and cytokines released by BBB-ECs in response to laquinimod treatment.
Results: The transendothelial electrical resistance of confluent monolayers of human BBB-ECs significantly increased when exposed to laquinimod, possibly through an upregulation in the expression of tight junction molecules. Laquinimod also induced tightness of other CNS barriers, namely meningeal and choroid plexus ECs. Furthermore, laquinimod decreased BBB-EC activation by reducing their secretion of proinflammatory mediators, including monocyte chemoattractant protein, interferon-gamma-inducible protein 10 and IL-6, and by downregulating the expression of activated leukocyte cell adhesion molecule (ALCAM) on the surface of the brain endothelium. Finally, laquinimod treatment significantly restricted the trafficking of T_H1 and T_H17 lymphocytes across BBB-ECs, two crucial effectors of disease in MS.
Conclusions: These data suggest that laquinimod may exert its therapeutic activity in part by promoting tightness of brain EC monolayers and by restricting the migration of T cells across the BBB, through attenuation of the proinflammatory cytokine/chemokine cascade induced by the brain endothelium in MS.

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Proteinkinase C beta regulates Blood Brain Barrier Integrity and T-Cell Proliferation in Experimental Autoimmune Encephalomyelitis

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Objective: Disruption of the blood–brain barrier (BBB) is a hallmark of acute inflammatory lesions in multiple sclerosis (MS) and its animal model experimental autoimmune encephalomyelitis (EAE). This disruption may precede and facilitate the infiltration of encephalitogenic T cells. The signaling events that lead to this BBB disruption are poorly understood but appear to involve dysregulation of tight junction proteins such as claudins. Pharmacological interventions aiming at stabilizing the BBB in MS might have therapeutic potential.
Methods: Efficacy of LY-317615 in the EAE model was studied in two actively immunized mouse models as well as in adoptively transferred EAE. Tight junction proteins were detected by immunohistochemistry and tight junction morphology was investigated by electron microscopy. BBB integrity was functionally tested in vitro by T-cell migration assays through a brain endothelial cell layer and in vivo by evans blue injection as well as 2-photon microscopy of dextran leakage. T cell proliferation was measured by 3H-thymidine incorporation, T cell cytokine secretion was measured by ELISA and intracellular flow cytometry.
Results: The orally available small molecule LY-317615, a synthetic bisindolylmaleimide and selective inhibitor of protein kinase C beta (PKC-beta), which is clinically used for the treatment of cancer, suppresses the transmigration of activated T cells through an inflamed endothelial cell barrier, where it leads to an induction of the tight junction molecules zona occludens (ZO)-1, claudin-3 and claudin-5. Treatment of mice with established EAE with LY-317615 ameliorates inflammation, demyelination, axonal damage and clinical symptoms. While LY-317615 dose-dependently suppresses T cell proliferation and cytokine production independent of antigen specificity, its therapeutic effect is abrogated in a mouse model requiring pertussis toxin, indicating that the anti-inflammatory and clinical efficacy is mediated by stabilization of the BBB. Histological and functional analyses have shown that this is a

direct effect of LY-317615 on endothelial cells while T cells treated with LY-317615 do not show altered migration behavior.
Conclusions: Our data demonstrates the involvement of endothelial PKC-beta in stabilizing the BBB in autoimmune neuroinflammation and suggests a therapeutic potential of BBB stabilizing PKC-beta as a novel therapeutic approach for MS.

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Blood brain barrier alterations and neuro-inflammation after single or repetitive exposures to primary blast using a high-intensity focused ultrasound model of mild traumatic brain injury (mTBI)

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Objective: It is unclear if exposure to a mild blast results in neuropathology or long term neurocognitive deficiencies. High-intensity focused ultrasound (HIFU) exposure was utilized as a model to exposure the rodent brain to a non-impact pressure alteration and subsequent assessment was employed to evaluate neuropathology or neurocognitive performance.
Methods: In the HIFU model, a transducer-generated wave travels through water and a conduction medium is used to ensure efficient coupling of the ultrasonic energy through the skull. All animals were anesthetized with 5% Isoflurane for 2–3 min and the scalp region was cleared of fur using Nair. Animals assigned to the HIFU-exposure condition were placed on a platform above the HIFU transducer and exposed to a one-millisecond pulse. Animals were exposed to either a single exposure or to 3 exposures that were spaced 24 h apart.
Results: Using male C57/Bl6 mice, we have demonstrated an immediate neuro-inflammatory response with activation of the endothelium within the blood brain barrier. An increased permeability of the barrier to low molecular weight molecules below 40 kD was seen early but was resolved by 24 h after exposure. Immunohistochemical analysis of brain sections indicated that the expression of the neuroinflammatory markers, C3 and IBA-1, were up-regulated within 2 h of exposure, while GFAP wasup-regulated at 24 h post exposure. However, other acute signs of injury were not evident, such as neuronal death, hemorrhage, or defects in motor function. Long term effects were assessed by open field analysis, Zero Maze, Novel Object Recognition and Novel Location tests in animals after three exposures to HIFU at 24 h intervals. Open field and Zero Maze results at 24 h and 7 days post exposure indicated an increase in the time spent in the open space and decreased anxiety.
Conclusions: These results indicate repetitive exposure to HIFU produced changes in long term cognitive functioning and patterns of activity. The data is suggestive of a link between the immediate neuroinflammatory response and long term neurocognitive function due to primary blast-like pressure exposures.

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CXCL1 can be regulated by interleukin-6 and promotes granulocyte adhesion to brain capillaries during bacterial toxin exposure and encephalomyelitis

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Objective: Granulocytes generally exert protective roles in the central nervous system (CNS), but recent studies suggest that they can be detrimental in experimental autoimmune encephalomyelitis (EAE), the most common model of multiple sclerosis. While the cytokines and adhesion molecules involved in granulocyte adhesion to the brain vasculature have started to be elucidated, the required chemokines remain undetermined.
Methods: CXCR2 ligand expression was examined in the CNS of mice suffering from EAE or exposed to bacterial toxins by quantitative RT-PCR and in situ hybridization. CXCL1 expression was analyzed in IL-6-treated endothelial cell cultures by quantitative RT-PCR and ELISA. Granulocytes were counted in the brain vasculature after treatment with a neutralizing anti-CXCL1 antibody using stereological techniques.
Results: CXCL1 was the most highly expressed ligand of the granulocyte receptor CXCR2 in the CNS of mice subjected to EAE or infused with lipopolysaccharide (LPS) or pertussis toxin (PTX), the latter being commonly used to induce EAE. IL-6 upregulated CXCL1 expression in brain endothelial cells by acting transcriptionally and mediated the stimulatory effect of PTX on CXCL1 expression. The anti-CXCL1 antibody reduced granulocyte adhesion to brain capillaries in the three conditions under study. Importantly, it attenuated EAE severity when given daily for a week during the effector phase of the disease.
Conclusions: This study identifies CXCL1 not only as a key regulator of granulocyte recruitment into the CNS, but also as a new potential target for the treatment of neuroinflammatory diseases such as multiple sclerosis.

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Human brain microvascular endothelial cells present viral peptides to HLA-class I-restricted CD8 T cells

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Objective: CD8 T cells specifically recognize pathogen-derived peptides presented by MHC class I molecules. As key components of the blood–brain-barrier, brain microvascular endothelial cells (BMVECs) constitutively express MHC class I; however, their role in class I-restricted antigen presentation and consequent antiviral immunity in the CNS is poorly understood. Previous *in vivo* studies demonstrated that entry of murine antiviral CD8 T cells into the CNS was associated with viral uptake and class I-restricted peptide presentation by BMVECs. These findings have yet to be confirmed in a human *in vitro* model. The aim of this study was to investigate whether human BMVECs can present viral peptides to CD8 T cell clones *in vitro*, facilitating antiviral immunity in the CNS.
Methods: Human primary and transformed BMVECs were chosen to express the HLA A*02 and/or A*03-MHC class I alleles. IFNγ pre-stimulated BMVECs were loaded with synthetic peptides representing A*02 and A*03-restricted viral epitopes and exposed to antigen-specific human CD8 T cell clones. Antigen-specific activation was measured in static tissue culture by intracellular cytokine

staining and IFNγ-ELISA. To evaluate activation and adhesion under shear force, confocal video-microscopy in conjunction with a Fluxion Bioflux optical microfluidic tissue culture system was used.
Results: T cell clones produced IFNγ in response to cognate peptide presentation by primary and transformed BMVECs. In preliminary experiments, cognate peptide loading of IFNγ-pre-stimulated BMVECs enhanced both adhesion and activation under conditions of shear flow. Furthermore, significantly shorter crawling distances were observed in specific T cell clones that recognized BMVECs loaded with their specific peptides versus control peptides.
Conclusions: The ability of human BMVECs to present viral peptides to CD8 T cells highlights their potentially beneficial role in host immune defense against viral infection in the CNS. Our findings also suggest a possible therapeutic target for blocking the MHC-dependent activation of viral antigen-specific CD8 T cells.

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PSGL-1 and E/P-selectin mediated rolling of encephalitogenic T cells is not required for T cell invasion into the CNS during EAE

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Objective: In multiple sclerosis and in its animal model experimental autoimmune encephalomyelitis (EAE), immune cells migrate across the endothelial blood–brain barrier (BBB) and gain access to the CNS. The involvement of P-selectin glycoprotein ligand 1 (PSGL-1) and of its endothelial ligands E- and P-selectin in this process has been controversial. Although intravital fluorescence videomicroscopy (IVM) studies have provided evidence for P-selectin glycoprotein ligand (PSGL-1) and its endothelial ligands E- and P-selectin in mediating the rolling of encephalitogenic T cells in inflamed leptomeningeal brain vessels during EAE, functional absence of PSGL-1 or E/P-selectin failed to influence immunopathogenesis of EAE. In the present study we demonstrate by means of IVM that PSGL-1 and E/P-selectin are essential for mediating rolling but not initial capture of encephalitogenic T cells in inflamed spinal cord microvessels during EAE. Lack of T cell rolling resulted in significantly reduced numbers of T cells firmly adhering in the inflamed spinal cord microvessels. Surprisingly, additional blocking of α4-integrins on PSGL-1-deficient encephalitogenic T cells did not abrogate T cell capture. Our data demonstrate that T cell rolling is not required for T cell migration across the BBB during EAE. Thus our observations suggest an important role of not yet defined adhesion molecules in mediating T cell capture to spinal cord microvessels initiating PSGL-1 and E/P-selectin independent T cell extravasation across the BBB during EAE.

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Challenging the dogma of neutrophil invasion after ischemic stroke in mouse and men

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Neutrophil extravasation into brain parenchyma and release of their abundant proteases is commonly held to be a cause of neuronal death and reperfusion injury following an ischemic insult. Yet stroke therapies targeting neutrophils are largely ineffective. To address this discrepancy we studied the temporo-spatial localization of neutrophils after transient ischemia in murine middle cerebral artery occlusion (tMCAO) and human stroke.

Using specific markers that distinguish neutrophils (Ly6G) from monocytes/macrophages (Ly6C) and that define the cellular and basement membrane boundaries of the neurovascular unit (NVU), confocal microscopy revealed that neutrophils rarely enter the CNS parenchyma.

Regardless of tMCAO duration, neutrophils were restricted to the meninges and the luminal surfaces or perivascular spaces of cerebral vessels. Neutrophil localization did not correlate with altered vessel permeability or expression of endothelial cell adhesion molecules known to be involved in neutrophil arrest and diapedesis (P-selectin, ICAM-1, VCAM-1, JAM-C). *In vitro* studies confirmed that hypoxia fails to induce neutrophil migration across the blood–brain barrier under re-established flow conditions. Results were corroborated in 25 rare human stroke specimens collected at defined time points after infarction. Our observations identify the NVU as the potential site of neutrophil action after CNS ischemia and suggest reappraisal of targets for therapies to reduce reperfusion injury after stroke.

Clinical research

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Myasthenia gravis. A retrospective argentine hospital-based study

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Objective: Myasthenia gravis (MG) is an autoimmune disorder of neuromuscular transmission. It is a relative uncommon disease, with prevalence estimated approaching 20 per 100000 worldwide. Data from Argentina are not available.
Methods: Records of patients with MG, follow up at Neuroinmunology Unit in Ramos Mejia Hospital, from June 2009 to May 2012 were review. Demographic, clinical and immunological features were evaluated.
Results: Out of 86 MG patients, 56 (65.1%) were female. Except ten patients (6 from Paraguay, 2 from Peru, 1 from Bolivia and 1 from Italy), the others cases were native argentine patients. The mean age of onset was 38.2 years (range 3 to 81 years) and the mean disease duration was 9.1 years (range 0–57). 80.2% (65/81) were AChr Ab-positive. In patients without AChr antibodies, 1/4 specimens tested was anti-MuSK positive. 5 patients (5.8%) were diagnosed younger than 12 years old, all females. Twenty six cases (30.2%) had late-onset (LO) MG (onset age > 50 years). This group was characterized by male predominance (male to female ratio 1.6/1), which was accentuated in the subgroup whit onset age older than sixty (male to female ratio 4.3/1). Repetitive nerve stimulation was done in 63 patients, 40 of them (63.4%) showed an abnormal decrement. The first main symptom was ptosis and diplopia (57%) but only 9 patients (10.5%) persisted as ocular MG. 45.3% (39 patients) MG patients had moderate disease (MGFA III) and 8.1% (7 patients) had myasthenic crisis. In the LO MG subgroup, 50% (13/26) presented moderate or severe disease (MFGA III–V) at worst status, compared to 71.6% (43/60) in early onset (EO) MG (onset age < 50). Also, relative to EO MG patients, LO MG patients required more frequently pyridostigmine as the only treatment along the disease, 38.4% (10/26 patients) vs. 23.3% (14/60 patients). Among 22 patients who underwent thymectomy, 7 patients had thymic tumors (9%), 1 of them presented thymolipoma. 35.2 % (25/71) presented associated autoimmune diseases, hypothyroidism was the most frequent. MG familial was diagnosed in 2 patients (2.3%).
Conclusions: MG patients of our population had clinical characteristics quite similar than previously reported in studies performed in other countries. However, as particular features, late onset MG seems had

less severe disease, with less frequency of myasthenic crisis and immunosuppression requirements. The male predominance, already reported, was greater than expected in the LO group.

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NMO-spectrum disorders in Turkish population: Clinical, demographic characteristics and the presence of NMO-Ig G

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Objective: Neuromyelitis optica (NMO) is a rare inflammatory, demyelinating disorder of central nervous system (CNS), that predominantly affects the optic nerves and the spinal cord. Serum antibodies to aquaporin-4 are involved in pathogenesis of NMO-spectrum disorders. Testing for NMO IgG is recommended for the patients with classic NMO, transverse myelitis (TM) with longitudinally extensive spinal lesion, severe or relapsing optic neuritis (ON) and opticospinal MS (OSMS). The aim of our multicenter study was to describe the demographic and clinical characteristics of NMO-spectrum disorders in Turkish population.
Methods: Data was collected from five different medical centers, 91 patients were included. Number of patients' in subgroups are as follows: 29 classical NMO, 38 ON with no MS typical brain lesions, 21 TM, 3 optico-spinal MS (OSMS). Indirect immunofluorescence method using commercial kits was used for detection of NMO-Ig G.
Results: Female predominance was revealed in whole group (n: 67 females, 24 males, 2.8:1). The mean age at disease onset was 30 (min 11, max 62). The mean follow up was 6 years for both NMO and ON, 5 years for TM and 3 years for OSMS patients. In classical NMO group, either ON or TM was detected as an initial symptom in 12 cases (41%) for each. Both ON and LETM simultaneously occurred in 4 patients (14%). The mean duration between the first and second attacks was 1.29 years. Fourteen of classical NMO patients had normal cranial MRI, while only one patient showed typical MS lesions. No oligoclonal band (OCB) was detected in classical NMO patients. The presence of NMO-IgG was investigated in 23 classical NMO patients and 10 of them were positive (43.5%). Only one of 15 patients with TM was positive for NMO IgG, while 14 patients with ON tested for NMO-Ig G were all negative. The more frequent onset symptom in seropositive NMO patients was TM (60%), while ON as an initial symptom was more common in the seronegative group (64%). The correlation between clinical course and NMO-Ig G seropositivity was also evaluated.
Conclusions: Because the incidence of NMO-spectrum disorders are low, data regarding different aspects of the disease is lacking. We collected data to describe the characteristics of Turkish patients diagnosed with NMO-spectrum disorders. Our findings revealed lower NMO-Ig G positivity rate and absence of OCB in Turkish NMO cases. Correlation between the status of NMO-Ig G, OCB and clinical features has also been evaluated.

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Anti-NMDA-receptor encephalopathy: Clinical presentation in Thai patients

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Objective: To describe clinical manifestations in patients with the diagnosis of NMDA-receptor encephalopathy in Thailand.
Methods: Patients whose sera or CSF were positive for NMDA receptor-IgG were identified retrospectively from the Neuroimmunology Laboratory data base of the Prasat Neurological Institute (PNI). Sera and CSF were tested from June 2010 to April 2012 by indirect immunofluorescence on substrates of mouse cerebral cortex/hippocampus and cerebellum and HEK293 cells transfected with the NMDA receptor NR1 subunit (cell-based assay; Euroimmun). All PNI patients' medical records were available for review, but only general demographic data and tumor association were available for non-PNI patients.
Results: 45 seropositive patients were identified amongst 2044 tested; 37 were female (82%) and 8 were male (18%). Median onset age was 15 years (range 5–53 years). Ovarian teratoma was identified in only one patient (age 25 years). No tumor was found in a male patient. Among 14 PNI patients, the most common initial clinical manifestations were cognitive deficit, aggressive behavior and hallucinations (79%). Generalized seizure occurred in 78% of patients at the disease onset. EEG revealed epileptic discharge in 14% of patients with decreased level of consciousness (mostly generalized slow wave). Abnormal movements developed in 71%: 80% had orofacial dyskinesia and 60% had limb dyskinesia. Autonomic disturbances were documented in 93%: instability of heart rate or low grade fever. No patient had hypoventilation or died. MRI brain abnormalities were found in 64% (temporal and or frontal and insular lobe). NMDA receptor antibody was detected in all CSFs and in 75% of sera. The early indicators for disease improvement were heart rate stabilization and reduced abnormal movements. Mean time to clinical recovery after immunosuppressive treatment was 22 days. Median time of cognitive recovery to baseline was 6 months. Three of 7 patients followed more than 3 months had recurrent attacks.
Conclusions: NMDA-receptor encephalopathy in Thailand predominantly affects young patients (6–15 years old) and male patients are affected more commonly than reported in previous studies. Ovarian teratoma was detected infrequently.

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Neutralizing antibodies against Interferon-beta in neuromyelitis optica patients

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Objective: Neuromyelitis optica (NMO) is believed to be an autoimmune disease mediated by antibodies specific for aquaporin-4 (AQP4). Interferon-beta (IFNß) is a first-line therapy for relapsing-remitting multiple sclerosis. It has been clinically reported that IFNß treatment worsens the clinical status of NMO patients. IFNß therapy may facilitate the production of various autoantibodies and of IFNß-neutralizing antibodies (NAbs). Therefore, we investigated if administration of IFNß was accompanied by development of IFN-neutralizing antibodies and of other autoantibodies in NMO patients.

Methods: A retrospective case series with clinical and radiological follow-up of 35 NMO patients from a population-based cohort were studied. Within the cohort 6 patients originated from a group with a diagnosis of multiple sclerosis, who received IFNß therapy before the diagnosis of NMO. An immunofluorescence assay was used to determine AQP-4 antibodies. Nabs were determined by a commercially available virus neutralisation assay.
Results: All 6 NMO-patients were AQP 4-antibody positive and all patients after IFNß treatment developed NAbs independent of age, sex, disease duration. Four patients had serological or clinical signs of other autoimmunity. The patients all had magnetic resonance imaging (MRI) activity in CNS, high relapse rate, and progression in Expanded Disability Status Scale (EDSS) score. Further studies of this cohort are in progress.
Conclusions: All NMO patients treated with IFNß were NAbs positive suggesting that NMO patients are particularly prone to develop IFNß-neutralizing antibodies Nabs after IFNß therapy. B cells are recognized as a major player in NMO pathogenesis and it may be speculated that IFNß increases B cell activity and antibody production and aggravates NMO disease activity.

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Investigation on epitope regions for anti-synapsin Ia antibody in patients with PPMS

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Background and objective: We had found a novel IgG anti-neural antibody, anti-synapsin Ia antibody, in a patient, who was clinically diagnosed as primary progressive multiple sclerosis (PPMS). In Western blot using rat brain membrane fraction as antigen, sera from other three patients clinically diagnosed as PPMS also showed positive bands with the same mobility as one detected in the first patient. To elucidate the clinical significance of the antibody and establish an efficient assay system, we confirm the antibody specificity using recombination proteins and investigated on the epitope regions.
Methods and results: We cloned a rat *synapsin Ia* cDNA with the same coding sequence as one registered in the GeneBank (accession number NM_019133) from a rat cDNA library by combination of PCR and synthetic DNA. Synapsin Ia was not well expressed as GST fusion protein in *E. coli*, and the mobility of the product band on SDS-PAGE suggested that full length of the protein was not expressed. Reaction with the patients' sera was not clearly detected. We divided synapsin Ia into three parts; N-terminal one-third, middle one-third, and C-terminal one-third, and expressed them as GST fusion proteins. N-terminal and middle parts were well expressed and the product appeared to be complete judging from the molecular size on SDS-PAGE. However, C-terminal one, which mainly consists of proline-rich region of the protein, was not well expressed and incomplete, even using *E. coli* strain engineered to rescue codon bias. In Western blot, serum from one patient reacted with the middle part, and another patient's serum reacted with incomplete products of the C-terminal part. Sera from the other two patients did not reacted with any products of the three parts.
Conclusions: We confirmed serum IgG antibody against synapsin Ia in two of four patients with PPMS. One patient's IgG reacted with epitope in the middle parts of the protein, and another reacted with epitope in the C-terminal one-third. Though no reaction was detected in the other two patients, the negative study might be due to the incomplete expression of the C-terminal region so far. Further approaches to this part are necessary.

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Complement-activating capacity of IgG antiganglioside antibodies in Guillain–Barré and Fisher syndromes

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Objective: In addition to pathological studies using human nerve specimen, several ex vivo and in vivo studies have indicated that antiganglioside antibody-induced complement activation in nerve membranes is a key process causing nerve damage in Guillain–Barré syndrome (GBS) and Fisher syndrome (FS). Clinical outcome in GBS and FS are likely to be based upon the extent of nerve damage, which may be influenced by the complement-activating capacity of antiganglioside antibodies. The purpose of this study was to investigate complement activation by antiganglioside IgG antibodies in GBS and FS, and to examine its association with IgG subclass and clinical features.
Methods: IgG antiganglioside antibodies in sera from 35 GBS and 19 FS patients were used as primary antibodies and healthy sera samples were used as the complement source. Formation of C5b-9 complex (a terminal complex in the complement activation pathway) was determined by rabbit polyclonal antibodies to C5b-9 complex with ELISA. Based on the IgG subclasses of antibodies to single gangliosides or ganglioside complexes determined by ELISA, the subjects were divided into IgG1-dominant (GBS, $n=17$; FS, $n=7$) and IgG3-dominant (GBS, $n=18$; FS, $n=12$) groups.
Results: In GBS, antiganglioside antibody activity positively correlated with anti-C5b-9 activity ($p=0.028$). Antibodies to GM1 or GalNAc-GD1a were more frequent in IgG1-dominant GBS. In IgG1-dominant GBS, anti-C5b-9 activity was associated with peak disability graded on the Hughes scale ($p=0.015$). The pure motor variant of GBS was significantly more frequent in IgG1-dominant (71%) than in IgG3-dominant (33%). In FS, there were no significant correlations among anti-C5b-9 activity, antiganglioside antibody activity, and peak disability.
Conclusions: In IgG1-dominant GBS, antiganglioside antibody activity was directly associated with complement-activating capacity and may be correlated with the extent of complement-mediated nerve damage, which influences the level of disability.

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An investigation into Adaptive Autoimmunity in Severe Traumatic Brain Injury

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Objective: The term “beneficial autoimmunity” in traumatic brain injury has been described in recent years, in particular in reference to rodent work. Moalem et al. (1999) described a beneficial effect of Myelin Basic Protein (MBP) specific T cells on injured retinal ganglion cells in rats. Hauben et al. (2000) described improved recovery when these cells were transferred into rats with spinal cord contusion injuries. The neuroprotective effect of passively transferred anti-MBP T cells was decreased after blockade of neurotrophin receptor activity

(Moalem et al. 2000), suggesting the beneficial effect might be mediated via these neurotrophins.

This project is applying similar experiments to humans. Cox et al. (2006) studied CD4+ T cell proliferation to MBP in severe traumatic brain injury and suggested a more favourable outcome in those who showed proliferation. This project considered proliferation of T-cells to several central nervous antigens. Then to determine benefit, the production of neurotrophins, in particular brain-derived neurotrophic factor (BDNF), from these T-cell cultures was analysed. This data is correlated with clinical parameters.

Methods: 50 ml blood was taken from healthy volunteers and from patients 72 h, 10 days or more than 6 months after severe traumatic brain injury. Proliferation of T-cells was investigated with carboxyfluorescein succinimidyl ester (CFSE) using flow cytometry after 5 days in culture: with MBP, neuron specific enolase or S100 beta. These were compared to Concanavalin A and unstimulated PBMC cultures. Supernatants from these cultures were analysed with ELISA to determine levels of BDNF.

Results: Patients continue to be recruited and the results analysed, but early results suggest no difference in T-cell proliferation between the healthy volunteers and patients. However, BDNF secretion by PBMC is increased in patients, particularly at an early timepoint after injury. Secretion was greatest to myelin basic protein.

Conclusions: Although there does not appear to be a difference in T cell proliferation between the groups, there does appear to be an increase in BDNF secretion to CNS antigens by the patients. This may suggest that cells specific to CNS antigens have a role in repair following severe traumatic brain injury in humans and hence this "autoimmunisation" is conferring benefit.

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Clinical features of CIDP with antibodies to LM1 and LM1 containing ganglioside complexes

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Objective: LM1 is the predominant ganglioside in human peripheral nerve myelin. We recently reported the presence of antibodies to LM1 and LM1-containing ganglioside complexes in a part of the patients with CIDP. Those antibodies may be associated with the pathogenesis of CIDP. We here investigated clinical features of the patients with the antibodies.

Methods: Serum IgG antibodies to LM1, a mixture of GM1 and LM1 (GM1/LM1), and that of GD1b and LM1 (GD1b/LM1) were examined in 75 consecutive patients with CIDP using ELISA as described previously. Clinical features of the CIDP patients with the above antibodies in the present series and those in our previous report were investigated and compared with those of the antibody-negative patients.

Results: Of the 75 patients with CIDP, eight patients had at least one of the IgG antibodies to LM1, GM1/LM1 complex or GD1b/LM1 complex. Among the eight patients, two had antibodies to LM1, three had anti-GM1/LM1 complex antibodies, one had anti-GD1b/LM1 complex antibodies and the remaining two had antibodies to both GM1/LM1 and GD1b/LM1 complexes. Eighteen patients with the LM1-associated antibodies (eight in the present series and 10 in our previous report) were more elderly than the 67 antibody-negative patients ($p<0.01$). Cranial nerve deficits were observed in none of the antibody-positive patients but in 25% of the antibody-negative patients ($p<0.05$). Ataxia was observed significantly more often (67%) in the antibody-positive patients than in the antibody-negative patients (33%) ($p<0.05$).

Conclusions: In humans, LM1 is contained more in the dorsal root than in the cranial nerves. No cranial nerve deficits and frequent ataxia in the CIDP patients with antibodies to LM1 and LM1-cotaining complexes may be associated with the distribution of LM1 antigen. The mechanism of the production of the LM1-associated antibodies may be age-dependent. Those antibodies to LM1 and LM1-containing complexes are possible markers for a subclass of CIDP.

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Characteristic Feature on MRI Spine differentiating Neuromyelitis Optica from Multiple Sclerosis

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Background: The advent of NMO antibody has permitted a much clearer separation between Neuromyelitis Optica (NMO) and Multiple Sclerosis and has permitted clarification of the differences in MRI imaging between NMO and Multiple Sclerosis. For example, we now know that the presence of cerebral Demyelinating lesions does not rule out NMO.

Methods: We did a retrospective, rater blinded review of 29 cases of NMO and 30 cases of MS using the criteria of the combination of a long (more than 3 vertebral level), continuous and a central cord location as criteria for NMO and more peripheral and patchy appearance for MS.

Results: Using these criteria, the raters were able to distinguish the two conditions with a good degree of confidence, particularly when the imaging was done at the time of an acute cord attack.

The sensitivity for diagnosis of NMO for the two readers was 86.2% with a specificity of 93.3% for reader A and 96.4% with a specificity of 78.6% for reader B with a kappa of 0.72.

Conclusion: There are significantly different lesion parameters that allow the distinction on spinal cord imaging between MS and NMO with a moderately high degree of confidence. We can now fairly establish a distinguishing diagnostic feature between Multiple Sclerosis and Neuromyelitis Optica based on the location of the lesion as evident on the MR Imaging of the spine.

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ImmVar Project: Genetic architecture of leukocyte gene expression in healthy humans

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Background/objective: The Immune Variation (ImmVar) project aims to establish a reference atlas with which to explore the genetic architecture of variation in immune gene expression. Here, we present an overview of the ImmVar project's *ex vivo* data.
Methods: We have sampled peripheral blood from 670 fasting subjects of the Brigham & Women's Hospital PhenoGenetic Project, a living tissue bank of healthy subjects (see poster by Von Korff et al.). In one aspect of the ImmVar project, we have purified naïve CD4+ T cells and CD14+CD16− monocytes by flow cytometry to generate a transcriptome-wide profile of each cell type using the Affymetrix HuGene ST1.0 array.
Results: Fasting blood samples were collected from 164 African-American, 155 East Asian, and 377 Caucasian subjects in a systematic, rigorous manner between 07:30 AM and 08:30 AM, over the course of 18 months. These subjects have been genotyped genome-wide using the Illumina Infinium HumanOmniExpressExome BeadChips which includes rare variants from 12,000 exomes as well as common coding variants from the whole genome. Here, to illustrate the utility of this new resource, we report the validation of correlations between SNPs associated with multiple sclerosis (MS) susceptibility and the expression of nearby genes in an interim analysis of the data: in 4/15 loci, we confirm, using CD4+ T cell and CD14+ monocyte data from healthy subjects, the same relation between an MS risk allele and gene expression that is observed in data collected from peripheral blood mononuclear cells of MS subjects.
Conclusion: This interim analysis of just 10% of the sampled subjects already illustrates the utility of the ImmVar project's resource: it provides a powerful atlas within which to explore the relation of genetic variation to variation in immune gene expression. Once completed, these and other aspects of ImmVar project will serve as a robust foundation for *in silico* analyses exploring the functional consequences of disease-associated variants.

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Identification of active zone protein ERC1 as an additional antigen in Lambert–Eaton myasthenic syndrome — A case report

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Objective: To describe a patient with Lambert-Eaton myasthenic syndrome (LEMS) and antibodies against ERC1.
Methods: To identify new LEMS associated small cell lung cancer (SCLC) markers, we used LEMS serum and a SCLC cell line to immunoprecipitate antigens. One tumor-negative LEMS patient showed strong immunoreactivity against a 120 kDa protein. Mass-spectrometry identified this protein as ERC1. A recombinant ELISA assay and a cellular assay expressing GFP-tagged full length ERC1 were used to confirm the presence of auto-antibodies against ERC1 in this patient, other LEMS patients and controls.
Results: We describe a non-tumor patient with LEMS, positive for antibodies against VGCC who also has serum antibodies against ERC1. Testing of 58 LEMS patients including 9 VGCC-antibody negative LEMS patients, 48 myasthenia gravis patients, 84 control patients with other diseases and 12 healthy controls revealed no other positive results.
Conclusions: ERC1 is a new, but rare, antigen in LEMS. No SCLC could be demonstrated in this non-smoking patient. Thus, it is unlikely that ERC1 auto-antibodies are caused by an anti-tumor immune response. This new auto-antibody against a presynaptic intracellular protein is possibly the result of structural damage to presynaptic axons in LEMS patients, and an example of "epitope spreading" secondary to the immune response against VGCC's.

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Repetitive nerve stimulation in myasthenic syndromes: Diagnostic yield and relation to disease severity

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Objective: Repetitive nerve stimulation (RNS) is an important part of the diagnostic workup of patients suspected of neuromuscular junction disorders. Comparison of different forms of myasthenia and relation to disease severity could further define the role of this diagnostic test and increase our understanding of the pathophysiological mechanism.
Methods: We retrospectively studied all patients who underwent repetitive nerve stimulation from 1999 to 2009 in our center. RNS was performed at 1, 3 and 5 Hz in abductor digiti minimi (ADM), orbicularis oculi and nasalis muscles. Sensitivity and specificity were determined for all myasthenic syndromes. We also studied the relation between Myasthenia Gravis Foundation Association (MGFA) disease severity scores and outcome parameters, including compound muscle action potential (CMAP) amplitude and decrement.
Results: A total of 284 patients were studied, including 144 patients with myasthenia gravis, 53 patients with Lambert-Eaton myasthenic syndrome (LEMS) and nonmyasthenic controls. Sensitivity was 68.3 %, 66.7 %, 30.3% and 100% in anti-AChR, anti-MuSK and seronegative myasthenia gravis and LEMS, respectively. Specificity of RNS to differ patients from patients with other neuromuscular diseases and controls was 98.6%. The initial CMAP amplitude of the ADM muscle correlates negatively with increasing disease severity for both myasthenia gravis and LEMS, with a slope of −0.75 and −1.14 respectively in transversal and −0.34 and −0.64 in longitudinal analysis.
Conclusions: The diagnostic yield of RNS is generally good in clinically affected patients with myasthenia gravis and excellent in LEMS. The initial CMAP amplitude relates to disease severity, which has important implications for our understanding of the pathophysiological mechanism. We conclude that in patients with severe myasthenic disease, part of the neuromuscular junctions are no longer only "fatiguable" after repetitive stimulation, but already blocked at the first moment of testing.

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Increased Hassall's corpuscles in myasthenia gravis Patients carrying thymic hyperplasia

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Objective: The thymus is implicated as a site that triggers autoimmunity in myasthenia gravis (MG). The medullary region in the thymus plays a key role to prevent autoimmunity and to establish self-tolerance in T cells. The thymic medulla contains Hassall's corpuscles (HCs), an epithelial structure without established functions. We examined the morphology and number of HCs in the thymus of MG patients.
Methods: Multicolor immunohistofluorescence analysis of involucrin, CD4, and CD8 was performed in the thymic sections obtained from MG (+) (n = 25) and MG (−) patients (n = 23). Corticomedullary architecture of the thymus could be visualized by the predominant localization of CD4CD8 double-positive thymocytes in the cortex and CD4CD8 single positive thymocytes in the medulla. The number and size of involucrin (+) HCs per medulla (μm^2) were measured with ImageJ software. The area of HCs was calculated by tracing the periphery of involucrin (+) HCs.
Results: Epithelial cells of HCs were identified to be involucrin (+). The number of involucrin (+) cells per unit medullary area (μm^2) was significantly larger in MG patients who exhibited the thymic hyperplasia than MG patients who did not carry the thymic hyperplasia. Involucrin (+) cells in MG patients with the thymic hyperplasia tended to be large in shape and distribute around germinal centers.
Discussion: Involucrin (+) HCs are thought to represent terminally differentiated stage of medullary thymic epithelial cells (mTECs). We speculate that altered differentiation of mTECs may be associated with the thymic hyperplasia and the onset of MG.
Conclusion: The density and size of Involucrin (+) HCs are increased in MG patients carrying thymic hyperplasia.

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In vivo achievable concentrations of idebenone are unlikely to inhibit oxidative burst of immune cells

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Objective: Markers of oxidative stress have been observed in the pathology studies of brain tissue from the multiple sclerosis (MS) patients. There are two potential sources of reactive oxygen species (ROS) in the intrathecal compartment of MS subjects: 1. Oxidative burst of the immune cells and 2. Failing mitochondria. Because most of the mitochondrial defects observed in MS tissue point towards functional deficiency of electron transport chain (ETC.) complex I, we are currently investigating whether therapy with idebenone may improve generation of ATP by mitochondria in demyelinated tissue by bypassing dysfunctional complex I, as it does in Leber's hereditary optic neuropathy. However, idebenone is also considered an anti-oxidant. Therefore, we wanted to determine if in vivo achievable concentrations of idebenone can effectively inhibit oxidative burst of the immune cells.
Methods: Fresh whole blood samples from healthy donors, untreated MS patients and idebenone treated PP-MS patients participating in IPPOMS clinical trial were subjected to osmotic lysis of red blood cells. The remaining white blood cells were pre-treated with 20nM (N = 17) or 120nM (N = 20) of idebenone and 10 μM diphenylene iodonium (DPI; N = 37), a NADPH oxidase inhibitor, which served as our positive control. The oxidative burst of granulocytes, monocytes and lymphocytes was determined by longitudinal measurements (every 5 min for 30 min) of fluorescence intensity of immune cells after addition of Fc-oxyburst reagent, which increases green fluorescence upon oxidation by H_2O_2 generated by oxidative burst.
Results: Neither 20 nM, nor 120 nM concentration of idebenone had significant inhibitory effect on oxidative burst. These concentrations correspond to peak CSF or peak plasma concentrations of free idebenone achievable with 2250 mg/day dosing. In contrast, DPI significantly inhibited oxidative burst of all three cellular subtypes.
Conclusions: It is unlikely that in vivo achievable concentrations of idebenone can significantly inhibit oxidative burst of immune cells. Therefore, if idebenone therapy results in the inhibition of intrathecal oxidative stress, such effect has to be attributed to the inhibition of ROS formation by failing mitochondria.

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First interim results on treatment satisfaction and pharmaco-economic data comparing Fingolimod and first-line therapies in multiple sclerosis patients in Germany (PANGAEA and PEARL)

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Objective: Fingolimod is licensed in the European Union as escalation treatment for patients with relapsing–remitting multiple sclerosis (RRMS). It is orally available and its efficacy has been demonstrated in a large clinical trial program, but data from everyday settings are not yet available.
Methods: To investigate the safety and efficacy of fingolimod in daily practice, a large national 5-year non-interventional study (NIS) is being conducted (PANGAEA) including subset pharmaco-economic (PE) data over 2 years. A further NIS (PEARL) collects analogous data in a first-line setting, i.e. Interferon-beta (IFN-beta) and Glatirameracetate (GA) treatment, allowing comparison of treatment satisfaction and health resource utilization between fingolimod and first-line therapy. 3-month interim-data will be presented.
Results: Already 1574 patients in the PEARL study and 168 patients of the PE-subset of the PANGAEA study have completed month 3. The populations are comparable regarding gender and age with a slight difference in disease duration (8.8 vs 7.2 years, fingolimod vs first-line). As expected fingolimod patients exhibit a higher baseline disease activity compared to IFN-beta and GA (relapse rate: 1.2 ± 1.18 vs. 0.5 ± 0.87; EDSS: 3.0 ± 1.70 vs. 2.3 ± 1.52; mean ± SD). 98.8 % of the fingolimod patients but only 66.1 % of the patients with first-line therapy found their medication easy to take (easy: 5.4 vs 26.6 %; very easy 6.6 vs 20.0 %; extremely easy 86.7 vs 19.5 %, fingolimod vs first-line). For 97.0 % of the fingolimod patients and only 69.5 % of the first-line patients, it is convenient to take their medication as instructed (convenient: 5.4 vs 26.9 %; very convenient 7.8 vs 21.9 %; extremely convenient 83.7 vs20.6 %, fingolimod vs first-line). Twice as many patients receiving fingolimod are overall extremely satisfied with their medication (42.2 vs 21.4 %).

The median number of days absent from work was three times lower in patients receiving fingolimod (5 ± 6.45 vs 14 ± 26.54; days ± SD).
Conclusions: These first results suggest that patients are more satisfied with fingolimod, which is taken orally once daily, compared to injectable first-line IFN-beta and GA. This might translate to better compliance and effectiveness, which still has to be shown. Apart from that, the lower count of days absent from work under fingolimod hold a

chance of reducing health costs caused by MS-related sick-leave. More data will be needed to prove these assumptions.

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Integrating genetic and environmental risk scores into an algorithm to predict multiple sclerosis susceptibility

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Objective: The goal of the Genes and Environment in Multiple Sclerosis (GEMS) study is to test the efficacy of a predictive algorithm that incorporates genetic susceptibility and environmental exposure data into a single estimate of risk of developing MS. If validated, this algorithm will enable the development of prospective cohorts to study the transition from health to MS in high-risk individuals such as first-degree relatives who have a 20–50 fold increase in lifetime risk.
Methods: The GEMS study will recruit 5,000 first-degree relatives between 18 and 50 years of age. All participants will donate saliva for DNA extraction and complete a questionnaire of medical and exposure history. Using this information, each participant will be assigned a genetic and environmental risk score (GERS). The GERS incorporates non-genetic risk factors such as gender, history of smoking and infectious mononucleosis, and at least 54 genetic variants. Subjects with high and low GERS will undergo blood biomarker profiling and brain magnetic resonance imaging (MRI) scans.
Results: Subject recruitment has been the most successful by means of internet-based strategies such as Facebook and the National Multiple Sclerosis Society website. As of May 2012, the GEMS study has enrolled 1108 first-degree relatives of MS patients, including 224 males and 854 females. Approximately 770 saliva samples have been genotyped. Subjects with the 100 highest and 100 lowest GERS will be tested further (blood biomarkers and brain MRI) to identify signs of early disease progression.
Conclusions: In MS, the first neurological symptom occurs well after the onset of the disease process. Early initiation of disease treatment therapies and lifestyle changes can delay disease progression. Thus, there is a pressing need to develop a clinically deployable algorithm that incorporates genetic susceptibility, environmental exposure, and other data to predict an individual's risk of MS in high-risk persons such as family members of MS patients.

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Aquaporin-4 antibodies in Korean patients with idiopathic inflammatory demyelinating diseases of the central nervous system

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Objective: To evaluate the diagnostic utility of aquaporin-4 antibodies (AQP4-Ab) for idiopathic inflammatory demyelinating diseases (IIDDs) of the central nervous system in Korea.
Methods: In total, 153 consecutive patients with IIDDs from three major hospitals in Korea were included. All were tested for AQP4-Ab using a cell-based assay at the John Radcliffe Hospital, Oxford, UK. Patients were evaluated for neuromyelitis optica (NMO), other NMO spectrum disorder (OTHER NMOSD), and multiple sclerosis (MS) according to the 2010 International panel criteria.
Results: Fourteen patients fulfilled the criteria for NMO, 64 were identified as OTHER NMOSD, 25 fulfilled the criteria for MS and 50 had either CIS or topographically restricted IIDD. Patients with NMO required a mean follow-up duration of 37.3 month before experiencing both optic neuritis and myelitis. AQP4-Ab test positivity in patients with OTHER NMOSD was only 21.9%. Subgroup analysis revealed that the test positivity was relatively high in myelitis with typical brain lesions of NMO group (75%) and recurrent longitudinally extensive myelitis (LETM) group (46.7%), but low in monophasic LETM group (5.7%), and recurret or bilateral simultaneous optic neuritis group (20%). About 40% of patients with limited manifestations of NMO would have fulfilled the 2010 International panel criteria for MS, without the antibody test results. After exclusion of NMO and OTHER NMOSD, Korean patients with MS were AQP4-Ab-negative and had similar characteristics to those of Western patients. Some patients were AQP4-Ab-positive (4% of patients who had features of neither NMO, OTHER NMOSD, nor MS), but were not identified using the current criteria.
Conclusions: The AQP4-Ab assay can be crucial in the differential diagnosis of IIDDs in Koreans, because it facilitated the early diagnosis of most NMOs, showed low positivity in Korean patients with OTHER NMOSD, could prevent limited NMO patients from being misdiagnosed with MS. In addition, because some IIDD patients who did not have features of NMO or OTHER NMOSD can show the positive test results, clinical suspicion for the atypical manifestation of NMO or aquaporinopathy is needed.

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Factors associated with the AQP4-Ab positivity in patients with longitudinally extensive transverse myelitis (LETM): Possible role of asymptomatic VEP abnormality

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Objective: To evaluate the factors associated with the aquaporin-4 antibodies (AQP4-Ab) positivities in patients having isolated longitudinally extensive transverse myelitis (iLETM) without optic neuritis in Asian population.
Methods: Fifty patients with iLETM, who did not meet the criteria for definite NMO and did not have symptoms of optic neuritis, were included from three major hospitals in Korea. All were tested for AQP4-Ab using a cell-based assay at the John Radcliffe Hospital, Oxford, UK.

Factors as gender, age, Kurtzke extended disability status scale (EDSS) at nadir, EDSS$\geq$6 after intravenous methylprednisolone (IVMP) treatment for acute relapse, visual evoked potential (VEP) abnormality without symptoms of optic neuritis, radiologic characteristics, and other laboratory characteristics were assessed.

Results: Of 50 patients with isolated LETM, 9 (18%) showed positive AQP4-Ab test results. Asymptomatic VEP abnormality, poor response to acute IVMP treatment, severe disability at relapse, as well as female gender and relapsing disease course were significantly associated with AQP4-Ab positive test results in patients with LETM. However, age of onset, presence of asymptomatic brain lesion, maximal length of spinal cord involvements, and cerebrospinal pleocytosis were not associated with the AQP4-Ab test results.
Conclusions: A considerable number of patients with AQP4-Ab-positive LETM who did not have recognizable optic symptoms might have sub-clinical optic nerve involvement , which could facilitate the early diagnosis of definite NMO. The presence of AQP4-Abs in LETM patients may assist prediction of long-term prognosis, disability at acute attack, and the need for a second-line acute-phase treatment, such as plasmapheresis due to the insufficient treatment response to steroids. In addition, LETM patients with these characteristics should be strongly encouraged to undergo AQP4-Ab testing to facilitate precise diagnosis and appropriate treatment.

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Thymoma following thymectomy of hyperplastic thymus in young onset MG patients

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Objective and methods: It is extremely rare for young onset myasthenia gravis (MG) patients to have thymoma following removal of hyperplastic thymus. We present two AChR-MG patients who developed thymoma years after thymectomy, and highlight the clinical manifestations that heralded the thymoma.
Results: Case 1: a 20 year-old female with generalized MG received extended transternal thymectomy (thymic hyperplasia). After 14 years of stable, mild MG on chronic steroid therapy, she got worse (respiratory infections needing ventilation and plasma exchange). She also developed alopecia totalis, oral candidiasis and recurrent genital Herpes simplex infections. Thymoma was removed, but her MG and associated manifestations did not improve, and patient died six years later with a severe pneumonia and respiratory failure. Retrospectively, she was found to have high levels of anti-IL22, IL-17A and IL-17F antibodies. Case 2: a 27 year-old male with generalized MG was treated with immunosuppression and extended transternal thymectomy (thymic hyperplastic). MG was initially difficult to treat, needing plasma exchanges, but improved slightly with cyclosporine. Seven years after the MG onset, he presented with spread skin lesions (paraneoplastic exfoliative erythoderma) and worsened myasthenic symptoms with generalized severe bulbar and respiratory involvement. Imaging and histology revealed thymic carcinoma with pleural dissemination. He had severe immunodeficiency (CD4 + = 99) and severe nosocomial infections (*P. aeruginosa*, CMV and HBV hepatitis). Thoracic radiotherapy 30 Gy was followed by chemotherapy, but patient died of massive pulmonary thromboembolism.
Conclusions: These two cases illustrate not only clinical expression of thymic neoplasms, but signs of the underlying complex immunodeficiency associated to the production of auto-antibodies to interleukins.

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Dysregulation of adrenergic/dopaminergic pathways in circulating lymphocytes of CIS patients

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Objective: The occurrence of dysregulated dopaminergic receptor (DR)- and adrenoceptor (AR)-operated pathways in circulating lymphocytes has been extensively documented in patients with multiple sclerosis (MS). Treatment with interferon-β seems to restore lymphocyte DR and AR responsiveness, suggesting their involvement in the therapeutic response to immunomodulating treatments. It is however uncertain whether such dysregulations develop as a consequence of MS or precede its clinical manifestations. We therefore decided to investigate DR/AR-operated pathways in circulating lymphocytes from subjects with clinically isolated syndrome (CIS) and to assess the relationship of possible dysregulations with subsequent progression to MS.
Methods: We planned to enroll into a multicentric longitudinal study 50 ambulatory patients with CIS. Patients are studied at baseline and after 6 or 12 months. A sample of venous blood is obtained and circulating lymphocytes are isolated. Expression of mRNA for β_2-AR, DRD2 and D5, as well as for tyrosine hydroxylase (TH) is evaluated in total lymphocytes and in purified CD4+CD25− T effector cells (Teff) and in CD4+CD25+ T regulatory cells (Treg). The production of dopamine, noradrenaline and adrenaline by cultured lymphocytes is assessed by means of HPLC-ED. A group of 15 age- and sex-matched healthy subjects (HS) is enrolled as control.
Results: So far, we enrolled 18 CIS patients and 14 HS. Lymphocytes from CIS patients express higher levels of TH mRNA both at rest and after stimulation with PHA 10 μg/mL ($P<0.01$ in both cases). Stimulus-induced increase of β_2-AR mRNA in cells from CIS patients is significantly less than in cells from HS ($P<0.01$). In CIS patients, Teff have higher TH mRNA levels and Treg have higher DRD5 mRNA levels in comparison to cells from HS ($P<0,01$ in both cases). Assay of lymphocyte catecholamines is ongoing.
Conclusions: Preliminary data suggest that in CIS patients circulating lymphocytes have dysregulated DR/AR-operated pathways. Adrenergic dysregulation in lymphocytes and Teff might represent a compensatory mechanism, while dopaminergic dysregulation in Treg – also in view of the inhibitory role of DR in these cells – might underlie an early impairment of Treg function. Enrollment and follow up of all scheduled subjects will allow to establish the relevance of these findings as well as their eventual relationship with the clinical progression of MS.

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Analysis and comparison of cellular intrathecal and peripheral immune responses in patients with multiple sclerosis

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Objective: Multiple sclerosis (MS) is an immune-mediated disorder of the central nervous system (CNS). Neither the antigenic target of MS, nor the cell population that mediates CNS tissue destruction has been defined. Therefore, the objective of this study was the determination of antigen (Ag)-specificity and phenotype of $CD4^+$ and $CD8^+$ T cells in the cerebrospinal fluid (CSF) of patients with MS in comparison to subjects with other neurological diseases.
Methods: Intrathecal immune reactivity is investigated by an Ag-recognition assay based on co-cultures of dendritic cells, pre-loaded with complex Ag and autologous CSF and peripheral T cells. Ag-specificity and phenotype of T cells are determined by a combination of a 7-day primary proliferation assay with an intracellular cytokine staining confirmation assay after overnight re-stimulation with identical Ag. Ten different Ag are being tested, but the current report focuses on reactivity to human herpes viruses Epstein-Barr Virus (EBV), Human Herpes Virus-6 (HHV-6) and Cytomegalovirus (CMV).
Results: So far, 15 patients were analyzed for T cell reactivity to CMV, 25 for HHV-6 and 27 patients for EBV reactivity. In the blood 53% of the patients responded to CMV, 36% to HHV-6 and 19% to EBV lysates. 33% of patients had intrathecal reactivity to CMV, 64% to HHV-6 and 81% to EBV. The mean stimulation index (SI, calculated with TNF-α/IFN-γ^+ $CD4^+$ T cells) of intrathecal EBV reactivity was significantly higher (SI = 101) than in periphery (SI = 17). Similar results were obtained for HHV-6 (SI of 14 in CSF vs.4 in periphery) and CMV (SI of 565 in CSF vs.166 in periphery) albeit without reaching statistical significance.
Conclusions: Intrathecal T cell reactivity to human herpes viruses is common and robust. While high intrathecal reactivity to CMV and HHV-6 can be explained by their neurotrophic character, these findings are unexpected for EBV, which is known to be latent in B cells, but not necessarily in the CNS. We continue performing these assays in a blinded fashion and plan to unblind the diagnostic category once we analyze at least 100 subjects. Then, we will be able to answer the question whether T cell responses to different human herpes viruses differ between MS subjects and controls.

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Uveitis in patients with multiple sclerosis

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Background and objective: Multiple sclerosis (MS) is an inflammatory demyelinating disorder of the central nervous system (CNS). Uveitis may be seen in about 5–10% of the patients with MS. Neuro-Behcet's disease is frequently encountered in our region and cause uveitis and MS-like clinical findings in most patients. Therefore, in isolated uveitis patients, it is crucial to determine the clinical and laboratory findings indicating high risk for MS development. In this study we evaluated the cases with MS and uveitis in terms of clinical course, radiological and laboratory findings.
Patients and methods: There were 6300 patients seen between 1993 and 2011 in Multiple Sclerosis and Demyelinating Disorders Unit in Istanbul Medical Faculty, Department of Neurology. Their files were retrospectively screened; a total of 41 cases (35 women, 6 men) had had uveitis. In Group I, there were 21 cases with MS and uveitis; in Group II, there were 20 cases who had uveitis and MS-like radiological and laboratory features, without clinical evidence of neurological involvement.
Results: In Group I, uveitis was the initial symptom before the neurological findings in the majority of the cases. Mean interval between the onset of uveitis and neurological symptoms was 6.7 years (1–17). All patients had relapsing remitting MS, and the mean EDSS was 2.2 (0.0-4.0). Moreover in Group I, the mean progression index was 0.32. Twelve Group I patients were under interferon-beta treatment. In Group II, all patients had intermediate uveitis and were sent for neurological consultation in search of an underlying demyelinating disease. All had periventricular T2 hyperintense lesions on cranial MRI, and oligoclonal bands in the CSF. In both groups uveitis had a good prognosis.
Conclusions: MS can be associated with intermediate uveitis, most commonly uveitis being the initial symptom of MS. This is easily distinguishable from anterior uveitis observed in Behcet's disease patients. Clinical course and EDSS scores of the patients with MS seem to be relatively mild. However, this needs to be verified in larger prospective series.

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Clinical features, treatment, and outcome of 568 patients with anti-NMDA receptor encephalitis

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Objective: To report the clinical features and suggest treatment guidelines for anti-NMDA receptor encephalitis.
Methods: Cohort study, analysis of demographics, symptom onset, treatment, and long-term follow-up. Demographic and clinical features were compared using Chi^2 tests; risk factors and treatment-determining outcome were analyzed with Cox regression.

Results: 81% were female. Median age was 21 years (range 1–85; 37%<18, 5%≥45 years). 39% had a tumor (96% teratomas). 54% of females≥12 years had ovarian teratoma(s) versus 6%<12 years; 6% of males (all adults) had a tumor. In patients<12 years old the most frequent initial symptoms were seizures, abnormal behavior, and movement disorders (36%, 33%, and 14%), while in adults were abnormal behavior and memory problems (65% and 14%). Within the first month, 88% of the patients had ≥4 of the following characteristic symptoms (psychiatric features, memory, speech disorder, seizures, dyskinesias, decreased level of consciousness, autonomic instability, or hypoventilation), while only 1% had a monosymptomatic illness. Movement disorders are more frequent in children (88% vs 72%, $p<0.0001$), while central hypoventilation and memory deficits are more common in adults (79% vs 69%, $p=0.001$ and 40% vs 21%, $p<0.0001$). Atypical symptoms like cerebellar ataxia or hemiparesis occur predominantly in young children.

Immunotherapy (93%) and tumor removal (when appropriate) resulted in full recovery or substantial improvement in 79% of the patients at 24 months follow-up; 11% died. Early treatment led to better outcome, while the need of ICU admission associated with a worse outcome ($p<0.0005$ for both). Among patients treated with immunotherapy, 53% responded to 1st line treatment (steroids, IVIg and/or plasma exchange), 98% of them with good outcome. If 1st line treatment failed (47%), 2nd line immunotherapy (rituximab or cyclophosphamide) significantly improved outcome compared with repeating 1st line drugs or no further treatment (76% vs 55%, $p=0.01$). Relapses occurred in 13%, more frequently in patients without teratoma. Most relapses were milder than the original episode.
Conclusions: Anti-NMDA-receptor encephalitis is a severe but treatable disorder of predominantly young individuals. Prompt treatment improves outcome. If initial immunotherapy fails, second-line treatment is usually effective. 79% of the patients have good outcome although the process of recovery can take more than 24 months.

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Clinical course and immunomodulating therapy in anti-TPI antibody-positive acute cerebellar ataxia

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Back ground and objective: Immunomodulating therapies are applied to post-infectious acute cerebellar ataxia (ACA), but the choice of therapeutic methods was still empirical. We reported that serum IgM antibody against triosephosphate isomerase (TPI) was found in approximately 35% of patients with ACA, and that this antibody was related with preceding Epstein-Barr virus (EBV) infection. We studied clinical course and response to immunomodulating therapy in anti-TPI antibody positive ACA.
Methods: The chronological change of clinical manifestations and the antibody titers, and the effect of immunomodulating therapies were investigated in six anti-TPI antibody-positive patients with ACA.
Results: Three patients have been treated with some immunomodulating therapy. Corticosteroid therapies were first administered in all these patients, but there was no obvious improvement. In two of these patients, intravenous immunoglobulin therapy (IVIg) was done following the steroid pulse therapy, and significant improvement was observed after starting IVIg in the both patients. The other three patients did not receive immunomodulating therapy, and the neurological symptoms improved gradually in the natural course. Chronological changes of the anti-TPI antibody titers were examined in two patients. In one patient, who received IVIg following the steroid pulse therapy and was discharged on the 32nd disease day with almost-full recovery, the antibody titer peaked on the 3rd disease day, decreased rapidly along with the therapies, and became negative in serum obtained about two month after the onset. In the other one, who was left to natural course without immunomodulating therapies, the antibody titers peaked on acute phase, decreasing gradually, but weak reaction still remain on the 225th disease day. He was discharged on the 60th disease day with remaining trunk ataxia, and needed about seven months of recuperation.
Conclusions: ACA is generally recognized to be a self-limiting disease with a good prognosis in the natural course, but symptom also could remain for more than half a year in some patients. There is no established immunological marker to choose the therapy to alleviate nadir and to shorten disease duration. Although our observation could not tell which could be truly effective, conbination of steroid-pulse and IVIg or sole IVIg, it suggests that serum anti-TPI antibody might be a immunological marker of responder for such therapies including IVIg.

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The PhenoGenetic Project: A living biobank with which to advance investigations of the genetic and environmental architecture of immune variation

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Objective: The PhenoGenetic Project is an ongoing study aimed at examining a large collection of healthy individuals in order to understand how genetic variation, as well as environmental factors such as intestinal microbes, alters the function of the immune system.
Methods: All participants complete a health history questionnaire and provide a blood sample on enrollment. This initial sample is used to isolate DNA, serum, plasma and peripheral blood mononuclear cells that are kept in a frozen archive. Thus, our study has developed a large biobank that serves as a resource for a variety of immunogenetic projects. Research groups are able to draw from the 1744 enrolled donors to obtain fresh blood, urine and stool samples from subjects of interest based on phenotypic and genotypic characteristics.
Results: Over 30 different research groups have utilized the PhenoGenetic biobank. Blood samples from this tissue bank have contributed to projects that illustrate different paradigms in which the resource can be used, including (1) screening the phenotypic and functional profile of NK cells in over 100 randomly selected subjects to identify the functional consequences of multiple sclerosis (MS) susceptibility loci such as MAPK1 (see poster by Kaliszewska et al.), (2) phenotyping a subset of subjects homozygous for a specific MS susceptibility allele such as those found in the CD6 and TNFRSF1A loci, (3) supporting the large-scale RNA profiling of over 600 healthy subjects to establish an atlas relating genetic and RNA variation in the immune system (ImmVar project, see poster by Lee et al.), (4) using stool samples provided by the PhenoGenetic Project healthy subjects as control samples to discover elements of the gut flora that may be enriched patients with Relapsing-Remitting MS and Secondary Progressive MS.
Conclusions: By enrolling a large population of healthy individuals our biobank is able to facilitate the genetic and environmental dissection of immunologic traits.

Innate immune system

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Secondary progressive multiple sclerosis is associated with an altered monocyte phenotype

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Objective: Secondary progressive multiple sclerosis (SPMS) is characterized by accumulating disability, nerve damage and signs of innate immune activation. Recently we showed that expression of VEGF-A in cerebrospinal fluid (CSF) cells was reduced in MS patients compared to controls, irrespective of disease course (Iacobaeus et al., Plos One 2011). However, in peripheral blood mononuclear cells (PBMC), VEGF-A was reduced only in SPMS, while relapsing-remitting MS (RRMS) were similar to controls. Expression of VEGF-A was expressed mainly by monocytes in the PBMC pool. The objective of this study was to further explore the finding of reduced VEGF-A in PBMC and to further characterize the monocyte profile of SPMS patients.
Methods: Expression of different monocyte markers was determined in PBMC obtained from SPMS and RRMS patients (n = 168) and controls with other non-inflammatory diseases (n = 116) using real time PCR. Flow cytometry was used to determine the proportion of different monocyte subsets in whole blood from MS patients and healthy controls (HC) (HC n = 5; RRMS n = 4; SPMS n = 8). Genome-wide mRNA expression and DNA methylation profiling, using Affymetrix Gene 1.1 ST Array (HC n = 16; RRMS n = 15; SPMS n = 17) Illumina Methylation450 BeadChip (HC n = 13; RRMS n = 10; SPMS 13) respectively, was performed on $CD14^+$ magnetic bead sorted cells from matching samples.
Results: SPMS patients displayed lower expression of the monocyte markers CD14, CD11b and CD163 in PBMC compared to all other groups. In CSF cells, all transcripts were downregulated in MS, irrespective of disease course, compared to controls. Flow cytometry revealed a trend towards a lower number of monocytes in SPMS, with a relatively higher proportion of inflammatory monocytes in whole blood. Microarray expression showed lower expression of VEGF-A and several other transcripts in CD14+ cells from SPMS, compared to RRMS. Interestingly, preliminary methylation data indicate changes in DNA methylation between HC and MS, of which some are sustained or increased in SPMS.
Conclusions: SPMS patients display an altered peripheral blood monocyte phenotype, which include reduced expression of VEGF-A. These findings support the notion of innate immune changes in SPMS and should be further evaluated for therapeutic and/or biomarker purposes.

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Immunoregulatory role of Natural killer cells in Multiple Sclerosis

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Objective: Multiple sclerosis (MS) is an autoimmune central nervous system (CNS) disease characterized by inflammation, demyelination, axonal/neuronal loss and gliosis. Although the etiology of the disease is not defined clearly, there are findings emphasizing the prominent role of the immune system. Natural killer (NK) cells are innate lymphocytes with cytotoxic property that also play an immunoregulatory role in the pathogenesis of autoimmune diseases. Several studies showed that NK cells works at the border of innate and adaptive immunity, to prevent the induction, propagation and activation of autoimmune T cells. Because of the discrepancy regarding the results of studies on NK cells, the actual role of them in MS has not revealed yet. We aimed to study the cell frequency and effector functions of NK cells in patients subgroups diagnosed as a clinically isolated syndrome(CIS), relapsing–remitting MS(RRMS) (naive and treated with disease-modifying drugs: DMDs) and secondary-progressive MS (SPMS) with no treatment.
Methods: Primarily, surface expression of CD3, CD4, CD8, CD19, CD16 and CD56, and proportions of NK cell subsets were detected in peripheral blood samples of patients and healthy subjects. Peripheral blood mononuclear cells were stimulated with or without hrIL-2, hrIL-12 or hrIL-4. After 24 h cell culture, IFN-γ, IL-10 and IL-22 contents of $CD56^{bright}CD16^-$ and $CD56^{dim}CD16^+$ NK cell subsets were measured by flow cytometry. NK cell cytotoxic activity was measured using erythromyeloblastoid leukemia cell line K562, and the frequencies of NK cell subsets were detected after 72 h of culture. The results were compared with age and gender-matched healthy subjects.
Results: In RR-MS patients receiving DMDs, SP-MS and CIS increased IFN-γ levels were detected in $CD3^-CD16^+CD56^{dim}$ NK cells, while IFN-γ levels were normal in the treatment naïve relapsing remitting patients. In comparison with control group, higher CD16+CD56 dim, IL-10 and IL-22 levels in $CD56^{dim}CD16^+$ and $CD56^{bright}CD16^-$ NK cell were detected in patients.
Conclusions: There was no difference between the levels of IL-10 and IL-22 secretion by NK cells in patients' groups, however IFN-γ secretion was found higher except treatment naïve RRMS patients. Although our preliminary results suggest that NK cells have a suppressive function on the pathogenesis of MS by releasing cytokines, further studies including more patients are needed to verify.

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Modulation of microglial activation via adenosine A3 receptors

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Objective: Toll-like receptor (TLR)-induced activation of microglia is tightly regulated by different mechanisms that are constitutively present or acquired during inflammatory conditions. Extracellular adenosine can modulate TLR-induced activation through adenosine receptor (ADORA)-mediated signaling. Four ADORA subtypes have been described that can either increase ($A_{2A}R$ and $A_{2B}R$) or decrease (A_1R and A_3R) intracellular cAMP levels. The expression pattern of the subtypes (the ADORA-code) thus orchestrates the cellular response to adenosine. This study was aimed to elucidate the effects of ADORA-mediated signaling on TLR-mediated activation of microglia.
Methods: Primary microglia from adult rhesus macaques were used for gene expression profiling and in functional assays.
Results: Activation of microglia dramatically altered the ADORA-code, sensitizing microglia to $A_{2A}R$-mediated inhibition of TLR-induced cytokine responses. We revealed that, in resting microglia in particular, $A_{2A}R$-mediated inhibitory signaling is effectively counteracted by

A_3R-mediated signaling. In addition, our most recent data demonstrates that A_3R-mediated signaling, evoked by endogenously produced extracellular adenosine in an autocrine or paracrine way, is essential to trigger transcription of TLR-induced IL-12, IL-23 and MCP-1 mRNA.
Conclusions: We have identified A_3R as an attractive therapeutic target to modulate innate immune responses. Inhibiting A_3R-mediated signaling will affect both resting as well as activated microglia by different mechanisms of action. These data and the roles of ADORAs in both the initiation and resolution of neuroinflammation will be discussed.

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Phosphatidylserine alters the functional properties of myelin-phagocytosing macrophages by activating PPARs

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Objective: Foamy macrophages, containing degenerated myelin, are abundantly found in active multiple sclerosis (MS) lesions. Recent studies have described an altered phenotype of macrophages after myelin phagocytosis. However, mechanisms by which myelin affects the phenotype of macrophages and how this phenotype influences lesion progression remain unclear.

Myelin damage and oligodendrocyte apoptosis leads to exposure of phosphatidylserine (PS) on the outer leaflet of the plasma membrane. As apoptotic cell clearance by macrophages via PS induces an immunosuppressive phenotype in macrophages, we investigated the impact of PS on the functional properties of myelin-phagocytosing macrophages.
Results: In this study we show that PS modulates the phenotype of macrophages treated with myelin through the activation of peroxisome proliferator-activated receptors (PPARs). Furthermore, uptake of PS by macrophages, after intravenously injected PS-containing liposomes (PSLs), suppressed the production of inflammatory mediators and ameliorated experimental allergic encephalomyelitis (EAE). The protective effect of PSLs on EAE was paralleled with reduced demyelination and CNS infiltration of immune cells and a decreased splenic cognate antigen specific proliferation. Interestingly, we found that the expression of PPAR response genes was upregulated in active human MS lesions.
Conclusions: Collectively our data show that myelin modulates the phenotype of macrophages via a PS-induced PPAR activation, which may subsequently affect lesion progression in demyelinating diseases such as MS.

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Regulation of increased pro-inflammatory cytokine mRNAs in short-term slices of cerebral cortex by distinct anti-inflammatory compounds

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Objective: Suppressing the synthesis of inflammatory mediators in the central nervous system (CNS) pharmacologically is an attractive therapeutic approach for many neurological illnesses such as traumatic injury, stroke, infection, and neurodegenerative or autoimmune diseases. Yet, the uniqueness and complexity of the CNS make discovering effective drugs very difficult, and results from dissociated cell systems often don't predict in vivo efficacy. As a means to bridge the gap between dissociated microglia cultures and whole animal models of neurological disease, our research laboratory has assessed the utility of short-term slices of CNS tissue for screening anti-inflammatory molecules.
Methods: Slices of cerebral cortex from adult female mice were explanted in culture medium for up to five hours. Up-regulation of mRNAs for many inflammatory mediators was demonstrated, while the cellular microenvironment is largely maintained. Distinct anti-inflammatory compounds were either added directly to the explant medium or injected systemically in mice prior to resection of cortical tissue. Abundance of IL-1beta and TNF-alpha mRNAs in slices were measured and normalized to beta-actin mRNA levels. Anti-inflammatory activity for each compound in slices was compared to effects observed in microglia and/or macrophage cultures.
Results: Glucocorticoid receptor agonists inhibited the increased cytokine mRNA in slices of cerebral cortex whether added in explant medium or injected into mice previously. Minocycline, thalidomide, propentofylline, and clenbuterol were not inhibitory in cortical slices. Paradoxically, cytokine mRNAs were enhanced by some anti-inflammatory compounds.
Conclusions: Our results demonstrate that the (1) increased expression of cytokine mRNAs in short-term CNS slices can be regulated pharmacologically and (2) some anti-inflammatory molecules have conflicting effects in microglia cultures and slices. Further evaluation with a small library of anti-inflammatory molecules is warranted to confirm the utility of short-term explants of CNS tissue as a drug discovery research tool for neurological diseases.

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Circulating dendritic cells of multiple sclerosis patients are dysregulated and their frequency is correlated with MS-associated genetic risk factors

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Objective: Dendritic cells (DC) are widely known as professional antigen-presenting cells and provide an important link to the adaptive immune system where they regulate the balance between immunity and tolerance. Alternations in the DC compartment can ultimately lead to the induction or perpetuation of autoimmune diseases such as multiple sclerosis (MS). This study aims to identify alterations in DC phenotype and functionality in MS. Moreover, the contribution of genetic risk factors to DC alterations was determined.
Methods: An *ex vivo* analysis of myeloid (mDC) and plasmacytoid DC (pDC) was carried out on peripheral blood of MS patients ($n = 104$) and age- and gender-matched healthy controls (HC, $n = 112$). Frequencies and expression of costimulatory (CD80 and CD86) and migratory molecules (CD62L, CCR5 and CCR7) were investigated. Interleukin (IL)-12p70 and interferon (IFN)-α secretion was measured following Toll-like receptor (TLR) challenge. Study subjects were genotyped for HLA-DRB1*1501 and IL-7R α.
Results: A significant decrease of circulating pDC was found in peripheral blood of patients with chronic progressive MS (CPMS) compared

to relapsing-remitting (RR) MS and HC. No differences in blood frequencies of mDC were found between different study groups. Both mDC and pDC of MS patients show shifts in the expression of CD86, CCR5 and CCR7 indicating that activation and migratory patterns of DC change during MS. Moreover, RRMS patients showed a reduced upregulation of CD86 on pDC and enhanced IL-12 production by mDC after TLR ligation, indicative of altered DC responsiveness. Treatment of MS is associated with a decrease of CD62L-positive mDC and pDC. HLA-DRB1*1501 carriers have reduced frequencies of circulating mDC as compared to non-HLA-DRB1*1501 carriers. Moreover, patients not carrying the protective IL-7Rα haplotype 2 have lower frequencies of pDC in the peripheral blood, indicating that genetic risk factors may impact the DC compartment of MS patients.
Conclusions: Our data indicate that circulating DC subsets undergo changes in phenotype and functionality during MS disease. This study further provides evidence that MS-associated genetic risk factors such as HLA-DRB1*1501 and absence of IL-7Rα haplotype 2 have an impact on the DC compartment and thereby may contribute to the induction and/or maintenance of autoimmune responses.

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Role of the innate antiviral response of trigeminal ganglia neurons in control of herpes simplex virus replication

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Objective: Herpes Simplex virus type 1 (HSV-1) is a persistent pathogen that cycles between an acute/lytic infection and a latent state within sensory neurons of the trigeminal ganglia (TG). The control of acute HSV-1 replication, and the maintenance of latency by TG neurons is crucial for prevention of disease pathology. The factors needed for this control remain largely unknown. Mouse and human studies show that the global innate anti-viral response is critical for controlling HSV-1 replication and disease. Little is known, however, about the importance of these antiviral pathways in the control of HSV-1 replication in neurons. We therefore hypothesize that innate immunity of TG neurons is a critical factor in the control of acute HSV-1 replication and in the establishment and maintenance of latency.
Methods: We used an in vitro model of HSV-1 infection of cultured TG neurons derived from adult mice. Acute HSV-1 replication was measured over time in neurons isolated from WT, IFNαβγR −/− (interferon receptor), STAT1 −/−, STING −/− or TLR3 −/− mice. Previous studies have shown TG neurons to be largely non-permissive to productive HSV-1 infection. We therefore sought to measure the permissivity of TG neurons by quantifying cells that were both infected and expressing late proteins (permissive infection) compared with infected cells with no evidence of late gene expression (non-permissive).
Results: Growth of HSV-1 was not altered in neuronal cultures deficient in IFNαβγR, STAT1, STING or TLR3 demonstrating that that these molecules are dispensable for control of acute replication in vitro. Viral late gene expression appeared comparable in all WT and KO infected cells, suggesting that neurons lacking these innate immune components were equivalent to WT in terms of permissivity to productive viral infection.
Conclusions: Contrary to our hypothesis, components of the innate antiviral response may be dispensable for efficient control of HSV-1 acute replication in TG neurons, suggesting other mechanisms for control. This also implies a large role for antiviral signaling in non-neuronal cells, as the drastic phenotype seen in mice lacking antiviral signaling cannot be explained by lack of control at the neuronal level. Ongoing studies are investigating the role of antiviral signaling in TG neurons and the maintenance of HSV-1 latency using an in vitro model.

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Neutrophil depletion affects Dark Agouti experimental autoimmune encephalomyelitis

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Objective: Neutrophils have been shown to own several immunoregulatory properties. Nevertheless few attention has been given to their contribution to both Multiple Sclerosis and Experimental Autoimmune Encephalomyelitis (EAE) etiopathogenesis and progression.

We aim to investigate the potential role of neutrophils during EAE sensitization and chronic phase in Dark Agouti (DA) EAE.
Methods: Chronic EAE was induced in DA rats by intrafootpad injection of syngenic spinal cord homogenate in incomplete Freund's adjuvant. Neutrophils were depleted by means of intraperitoneal injection of anti-rat neutrophil serum (aNEU) at different disease time course, i.e. either during the sensitization and the chronic phase of the disease. The animals were weighted weekly and aNEU effect was first evaluated on clinical score while deeper analysis were performed on blood formula, spleen morphology and spinal cord cytokine, chemokine and myelin basic protein expression.
Results: aNEU had no effect on clinical score when neutrophil depletion was performed during the chronic phase, i.e. from 14 to 35 days post EAE induction (dpi). When aNEU was administered during the EAE sensitization phase up to 8dpi, no difference in mean clinical score could be revealed between EAE and aNEU-treated EAE rats up to the disease peak, i.e. 14 dpi. However a significant improvement in clinical condition could be reported soon after the disease peak so that a chronic course was transformed into an acute/monophasic one. aNEU treatment induced a faster body weight gain while it did not affect blood formula changes related to EAE course. Moreover while aNEU treatment did not counteract EAE effects on both spleen microscopic morphological changes and Treg (FoxP3) content, it mainly affected the expression of both pro- and anti-inflammatory cytokines in the three main spinal cord traits (cervical, thoracic, lumbar) while relative low effect was observed on both MCP-1 and myelin basic protein expression.
Conclusions: Neutrophil depletion transformed a chronic EAE model into an acute/monophasic one and resulted in cytokine expression changes in spinal cord mainly referred to the cervical trait.
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KIR2DL2 expression alters natural killer cells response to herpesvirus infection in multiple sclerosis patients

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Objective: Multiple sclerosis (MS) is an autoimmune chronic inflammatory disease of the Central Nervous System characterized by demyelination and axonal damage, initiated and mediated by autoreactive T cells. Perturbations in immune-modulatory networks that include Th2 cells, regulatory T cells and Natural Killer (NK) cells are thought to be in part responsible for the relapsing–remitting progression of the disease. However, the mechanisms at the basis of the development and the progression of MS disease remain uncertain. Epidemiological studies indicate that exposure to an infectious agent, in combination with genetic predisposition, could be implicated in MS pathogenesis. It is proposed that the development and the perpetuation of MS autoimmunity could involve the presence of a latent infection in which the microorganism persists in a subclinical form that can periodically reactivate. This condition is typical for herpesviruses that have been evidenced with an increased frequency in MS patients.

We evaluated the role of NK cells towards herpesvirus infection in MS patients. In particular we were interested in possible NK cell functional differences defined by KIR–HLA genotype repertoire.
Methods: Peripheral blood mononuclear cells from MS patients and controls were treated with CpG sequences and infected in vitro with Herpes simplex virus 1 (HSV1). Samples were analysed for viral yield, TLR9 pathways by RT PCR Array System, cytokine secretion by Searchlight Chemiluminescent cytokine array, NK cell activation by CD107a expression and cytotoxicity assay and killer immunoglobulin-like receptors (KIR) expression by flow cytometry.
Results: CpG treatment promoted an unexpected sensitivity to herpesvirus infection in a subset of MS patients. TLR9 pathways did not show defects while NK cells presented decreased degranulation and cytotoxicity and up-regulated the inhibitory KIR2DL2 receptor. The presence of the KIR2DL2 ligand, HLA-C1, is fundamental to reduce the NK cell activation in the presence of a viral infection.
Conclusions: These results are the first direct proof of the implication of KIR2DL2 receptor in the control of NK cell activation towards herpesvirus infection in MS patients, supporting the role of viral infection in creating an environment more susceptible to the maintenance of an autoimmune disease in the presence of a specific genetic background.

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A genetic and functional characterization of tumor necrosis factor regulation in rat antigen presenting cells

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Objective: Tumor necrosis factor (TNF) is a pleiotropic cytokine affecting the activity of numerous cells through a wide variety of pathways controlling among others inflammation, apoptosis and cell activation. A tight regulation of TNF is essential for inducing proper host inflammatory responses while restricting self-aggression and promoting tissue repair. We have previously shown that TNF production is genetically regulated by a locus on rat chromosome 4, *Eae26* (Gillett et al., J Immunol, 2010). Polymorphisms in *Eae26* regulate TNF production in macrophages and susceptibility to various inflammatory diseases in rats including experimental autoimmune encephalomyelitis (EAE), a model for multiple sclerosis (MS). We now sought to characterize mechanisms underlying this genetic regulation.
Methods: To characterize the effect of *Eae26* we are comparing susceptible DA rats with the DA.PVG-*Eae26* congenic strain that contains resistant *Eae26* alleles on the DA background. We have characterized the phenotype of different antigen presenting cells (APCs). Macrophages and dendritic cells were differentiated from bone marrow cells with MCSF, GMCSF, GMCSF/IL-4 or rhFLlt3l. We have also studied intraperitoneal (i.p.) macrophages, blood monocytes and microglial cells from adult central nervous system (CNS). The impact of *Eae26* on activated APCs was measured with ELISA (TNF), griess reaction (NO), flow cytometry and ability to activate co-cultured T cells. Intra-regional recombinants, in combination with sequence variations and gene expression, have been utilized to identify the gene(s) responsible for *Eae26* effect.
Results: Our results indicate that besides known TNF regulation, *Eae26* also regulates NO production and expression of CD86 in APCs. This regulation was specific to stimulated bone marrow derived dendritic cells (GMCSF/IL-4), i.p. macrophages and blood monocytes. In contrast, we observed no effect of *Eae26* on MCSF derived macrophages, splenic DCs and CNS microglial cells. MCSF and GMCSF/IL4 bone marrow derived APCs from DA.PVG-*Eae26* induced lower T cells proliferation than the DA APCs. This was not the case for rhFLT3l derived APCs. Furthermore this difference was abrogated when we used peptide as antigen instead of the whole protein.
Conclusions: These results suggest that *Eae26* regulates the phenotype of infiltrating macrophages and monocytes, which in turn modulates EAE susceptibility.

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Investigation of brain endothelial cell lines as models of the blood–brain barrier

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Objective: In this study we aim to characterize two brain endothelial cell lines, GPNT and bEND5, with regards to innate immune function in order for them to be used for in vitro studies of the blood–brain barrier during activation of innate responses
Methods: Intracellular signalling molecules were quantified by Western blot and cytokine release by ELISA, respectively.
Results: In the rat GPNT cell line a rapid increase (5–15 min) in MAPK p38 phosphorylation was detected. In these cells the release of the chemotactic cytokine CINC-1 was increased 5-fold by 1 ng/ml of LPS compared to untreated control. In the bEND5 cells neither p38 phosphorylation or JNK phosphorylation was observed. However, KC release was comparable to CINC-1 release. None of the tested histones (H1, II AS or VIII-S) induced KC release
Conclusions: These results warrant further investigation of these cell lines for their suitability for in vitro studies of the role of TLR4 signalling in neutrophil transmigration.

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Enteric neurons express alpha-synuclein mRNA and functional Toll-like receptors

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Objective: Several psychiatric and neurological disorders such as Parkinson disease are associated with intestinal problems. Toll-like receptors (TLRs) on mucosal immune cells and epithelium play an important role in the pathophysiology of intestinal inflammatory responses. There are limited reports about the expression of TLRs on enteric neurons. We hypothesize that TLRs on enteric neurons play an important role during intestinal inflammation associated with Parkinson's disease. Therefore, we have studied the expression and function of TLRs in relation to alpha-synuclein on the IM-FEN cell line of neurons of the enteric nervous system.
Methods: The IM-FEN enteric neuronal cell line was used to study the expression and function of TLRs. Immunohistochemistry was used to determine expression of the neuronal proteins Protein Gene Product 9.5 (PGP9.5), peripherin, class III β-tubulin and Hu-antigen D (HuD), hallmarks of a neuronal phenotype. TLR mRNA expression and alpha-synuclein (AS) mRNA expression was determined by qPCR. Functionality was determined by exposure of the cells to KCl and lipopolysaccharide (LPS), a TLR4 ligand. After stimulation, TLR and AS mRNA expression was determined by qPCR.
Results: In cultured enteric neuronal cells, bearing PGP9.5, peripherin, class III β-tubulin and HuD, mRNA expression was found for TLR-2, -3, -4, -5 and -7. Exposure of the enteric neuronal cells to KCl for 24 h increased TLR7 and AS mRNA expression. In addition, exposure of the enteric neuronal cells to the TLR4 ligand LPS resulted in decreased AS mRNA expression.
Conclusions: The IM-FEN enteric neuronal cell line expressed TLRs -2, -3, -4, -5, -7 and AS mRNA, and TLR4 protein. TLR4 is functional; 24 h exposure of the enteric neuronal cells to LPS resulted in a decreased expression of AS mRNA. Hyperpolarization of enteric neuronal cells through KCl exposure resulted in increased TLR7 and AS mRNA production, possibly indicating upregulation of TLR7 and AS protein expression.

IM-FEN cells are therefore a good model to study the role of TLRs on enteric neurons and the role of enteric neurons in psychiatric and neurological disorders.

This study is part of the Utrecht University Focus en Massa program

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TLR induced type I interferon signaling in regulation of glial and inflammatory response in CNS

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Objective: Our goal is to understand the glial signaling that controls inflammatory responses in the central nervous system (CNS). This glial response can play both a detrimental and beneficial role. Toll like receptor (TLR) signaling is implicated in responses to pathogene or endogenous signals. TLR signaling mediate immune response by inducing cytokines, including type I interferons (IFN), known to exert anti-inflammatory effects. IFN-beta, a member of the type I IFN family, is currently used as a therapeutic for multiple sclerosis. Type I IFNs signal through a common receptor, IFNAR. The aim of the present study was 1) to investigate the *in vivo* response of microglia and astrocytes to CNS administration of TLR ligand/agonist, and 2) to examine whether this response involves type I IFN signaling.
Methods: Mice were administered TLR ligand/agonist by injection to the cisterna magna. We analyzed leukocyte entry to the CNS at 6, 18 and 72 h post injection by flow cytometry, and their localization by immunostaining. Astrocytes and microglia were sorted by FACS and gene expression was measured using quantitative real time-PCR.
Results: Injection of ligand/agonist for TLR2, 3 and 4 resulted in infiltration of CD45+ leukocytes after 18 h, determined to be the optimal time. Immunostaining showed parenchymal localization of infiltrating cells in cerebellum. FACS sorted astrocytes expressed equivalent levels of TLR3 mRNA to microglia but lower level of TLR2 or 4. Microglia and astrocytes were induced by TLR3 and 4 signaling to express interferon regulatory factor 7, which regulates the induction of type I IFN. Injection of ligand/agonist for TLR2, 3 and 4 led to increased level of glial CXCL10 gene expression. Together these results suggest the involvement of type I IFN signaling. However, unlike CXCL10 gene expression, that was dependent on IFNAR signaling, TLR-induced leukocyte infiltration was not affected in IFNAR deficient mice.
Conclusions: These studies point to a role for TLR signaling in the innate glial response that regulates CNS inflammation.

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Impaired suppressor function of CD56bright NK cells in early multiple sclerosis

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Objective: Multiple sclerosis (MS) pathogenesis might be linked to impaired function of regulatory subsets of immune cells. In vitro, activation of NK cells with IL-27 or IL-2 or treatment with the anti-CD25 monoclonal antibody can induce suppressor function of $CD56^{bright}$ NK cells, with mechanisms, which are not fully explained but might involve lytic enzymes such as perforin and granzyme K, while the $CD56^{dim}$ NK cell subpopulation is believed to maintain prominent cytotoxic feature. Aim of the present study was to assess the suppressor function of $CD56^{bright}$ and $CD56^{dim}$ NK cells isolated from subjects with early MS and healthy controls (HC) following activation with IL-12 and IL-15.
Methods: NK cells were negatively isolated from buffy coats of five healthy donors or peripheral blood of five subjects with early MS. NK cells were sorted based on the lack of expression of CD3 and expression of CD56 into $CD56^{bright}$ and $CD56^{dim}$ populations. Isolated cell populations from HC and MS subjects were cultured without activation or under activation with IL-12 and IL-15. Gene expression and cytokine production were assessed at various time-points. Viable cells were then re-sorted and co-cultured with autologous CD3+ T cells to assess suppressor function of NK cells.
Results: No differences in $CD56^{bright}/CD56^{dim}$ proportion were observed between MS subjects and HC. Culture with IL-12 plus IL-15 induced proliferation of $CD56^{bright}$ and $CD56^{dim}$ NK cells from MS subjects and HC. $CD56^{bright}$ NK cells from HC, but not MS patients, were able to suppress autologous T cell proliferation. Interestingly, also $CD56^{dim}$ NK cells displayed some suppressor function in HC. The expression of granzyme K or other cytotoxic enzymes was not impaired in NK cells from HC nor in NK cells from MS subjects.
Conclusions: Our data provide evidence that the suppressor function of $CD56^{bright}$ NK cells is impaired in early MS under multiple

activation conditions. Functional studies are required to dissect the mechanism of such suppressor function in HC and the altered response observed in MS. Interestingly, also $CD56^{dim}$ NK cells could be able to suppress autologous T cell proliferation under selected stimuli in HC.

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Scavenger receptor A expression on antigen presenting cells is important for CD4+ T-cell proliferation in EAE mouse model

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Objective: Multiple sclerosis (MS) is an autoimmune disease of the central nervous system (CNS) characterized by damage to the neuronal myelin sheath. One of the key effectors for inflammatory injury is the antigen-presenting cell (APC). The class A scavenger receptor (SRA), constitutively expressed by APCs in peripheral tissues and the CNS, was shown to play a role in the phagocytosis of myelin; however, the role of SRA in the development of experimental autoimmune encephalomyelitis (EAE) and autoimmune reaction in the periphery has not yet been studied;
Methods: We measured the clinical score of EAE induced C57BL/6 and $SRA^{-/-}$ mice characterized CNS pathology using immunohistology staining. Furthermore we assessed pro-inflammatory cytokine response in cell cultures;
Results: We discovered that EAE progression and CNS demyelination were significantly reduced in $SRA^{-/-}$ mice compared to WT mice. In addition, there was a reduction in infiltrating peripheral immune cells into the CNS lesion of $SRA^{-/-}$ mice, which was associated with reduced astrogliosis. Immunological assessment showed that SRA deficiency resulted in significant reduction of pro-inflammatory cytokines, such as IL-2, IFN-gamma, IL-17 and IL-6. Furthermore, we discovered that $SRA^{-/-}$ APCs showed impairment in activation and in their ability to induce $CD4^{+}$ T cell proliferation. Recently, we have shown development of brain lesions in EAE induced NOD mice (Levy et al., 2010). Here we discovered an increase in SRA expression in infiltrated cells surrounding the brain lesion in those mice.
Conclusions: Expression of SRA on APCs is important for $CD4^{+}$ T-cells proliferation in EAE mouse model. Further studies of SRA-mediated cellular pathways in APCs may offer useful insights into the development of MS and other autoimmune diseases, providing future avenues for therapeutic intervention.

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Ageing impairs the phagocytic capacity of macrophages: Implications for central nervous system repair

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Objective: Macrophages and microglia play important roles in the repair of myelin sheaths (remyelination). These potent phagocytic cells remove myelin debris from lesions which is essential for repair processes that follow. Many studies report that macrophage function is altered as a result of ageing. Interestingly, the efficiency of remyelination also decreases with ageing. We hypothesise that ageing impairs the ability of macrophages to phagocytose myelin debris.
Methods: We investigated the ability of macrophages from young and aged mice to phagocytose myelin debris and fluorescent particles. Microglia, peritoneal, splenic and bone-marrow derived macrophages from young and aged mice were prepared. Fluorescently labelled myelin debris or fluorescent particles were added to these cultures and phagocytosis was analysed by flow cytometry and immunofluorescence microscopy. In addition, *in vivo* phagocytosis was measured by intraperitoneal injection of fluorescent particles in young and aged mice.
Results: Phagocytosis was significantly reduced in microglia, peritoneal and splenic macrophages from aged mice compared to macrophages from young mice. This reduction in phagocytosis was also demonstrated *in vivo*. Interestingly, ageing did not alter phagocytosis in bone marrow-derived macrophages generated from young or aged mice in our system.
Conclusions: In conclusion, myelin debris phagocytosis is impaired in macrophages from aged mice. Therefore, decreased remyelination efficiency with ageing may be caused by dysfunctional aged macrophages. These findings potentially have important therapeutic implications in the development of remyelination enhancing treatments for demyelinating diseases such as Multiple Sclerosis.

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Macrophages, myelin debris and the efficiency of CNS remyelination: The problems of growing old

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Objective: Remyelination of demyelinated lesions can occur through the formation of new myelin sheaths, which are recruited as oligodendrocyte precursor cells (OPCs) after demyelination. This process appears to become less effective with age, and one of the reasons that OPCs do not remyelinate is due to the accumulation of myelin debris. The failure of macrophages to remove debris may be an important process behind the inhibition of remyelination and recovery. A role for the nuclear retinoid X receptor (RXR) in remyelination has been shown in OPCs, but its role in macrophages and myelin debris clearance is still unknown. The goal of my project is to characterize the role of nuclear receptors in the activation of myelin-clearing macrophages and determine the factors in young macrophages that allow them to effectively phagocytose debris to allow for remyelination.
Methods: Myelin debris clearance was tested by comparing young and old bone-marrow derived macrophages (BMDMs) from female C57bl/6 mice. BMDMs were isolated from 2 month old and 32 month old mice. Immunocytochemistry was performed by fixing cells in 4% paraformaldehyde and staining using standard protocols. Antibodies against Iba-1 and MBP were used to indicate myelin debris uptake by BMDMs. mRNA will be isolated from young and old BMDMs using the Qiagen RNeasy Mini kit, and the transcriptomes will be sequenced on an Illumina platform to determine differences in gene expression between the young and old macrophages in response to myelin.
Results: Nearly 55% (+/−11%) of young BMDMs were able to effectively clear myelin, while only 10% (+/−5%) of old BMDMs were able to clear debris *in vitro*. In order to study whether the role of

RXR in remyelination is macrophage-mediated, 1 μM 9-cis retinoic acid (9cRA), an RXR agonist, was added along with myelin debris to BMDMs. 9cRA significantly increased the amount of myelin cleared by 32 month old BMDMs to 34% (+/−9%) and creates a more youthful state. 9cRA has no significant effect on young BMDMs, indicating that RXR may play an important role in the difference between young and old myelin-clearing macrophages.
Conclusions: These results indicate that studying the role of macrophages in remyelination is important and that nuclear receptors may play a vital role in the cause of neurological symptoms in some MS patients. Repairing myelin damage using macrophages may be the next step to discovering effective treatments for prominent demyelinating disorders.

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Sex-related differences in cAMP specific PDE4B3 splice variant, cytokine mRNAs and ICER expression following LPS induced neuroinflammation

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Objective: Sex-related differences have been observed in the incidence and severity of several neurological diseases and in sepsis in humans. cAMP has been shown to play an important role in modulating the inflammatory environment during neuroinflammation and importantly in protecting myelin from excitotoxic cell death. cAMP is hydrolyzed by cyclic nucleotide phosphodiesterase (PDE).

In the 1970s a specific PDE4 inhibitor, rolipram, was developed in the aim to treat depression, as it was shown that augmented cAMP levels enhance the noradrenergic neurotransmission in CNS and altered peripheral immune system with increase in several pro-inflammatory cytokines has been reported in depressed patients and in animal models of depression.

Considering the sexual dimorphism in the functional properties of oligodendrocytes and the importance of a systemic inflammation in the progression and relapses of multiple sclerosis together with the relation reported for depression to both these aspects, we focused on identifying possible sex-related differences in the alterations previously reported for the two PDE4B splice-variants (PDE4B2 and PDE4B3) mRNA expression during innate neuroinflammation.
Methods: We analyzed the influence of an intra-peritoneal lipopolysaccharide injection on the distribution pattern and expression levels of the four PDE4B mRNA splicing variants and of COX-2, IL1-β and TNF-α mRNAs in both male and female mice brains by semi-quantitative analyses of *in situ* hybridized brain sections. Double ISHH was used for characterization of the cell populations involved in the PDE alterations. Furthermore, western blot analyses of the expression of inducible cAMP early repressor (ICER), phosphor-CREB and PDE4B together with cAMP assays was used to explore the sex-related differences observed.
Results: Clear differences were observed in PDE4B2 and PDE4B3 mRNA expression levels in males compared to females in a time-dependent manner. Furthermore, we observed that the clear downregulation of PDE4B3 mRNA was reflected in a lower percentage of oligodendrocytes positive for this transcript which correlated with a decrease in inducible cAMP early repressor (ICER) expression in female corpus callosum.
Conclusions: Knowledge about PDE4B mRNAs expression in mouse brain in both sexes and the alterations provoked by LPS administration might help us to clarify sex-related differences in psychiatric disorders and not to mention in the susceptibility to autoimmune diseases.

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The multiple sclerosis susceptibility MAPK1 allele is associated with decreased degranulation by bulk NK cells and CD56dim NK cells in response to MHC class I-deficient B cell line

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Objective: Natural killer (NK) cells are capable of killing target cells and secreting cytokines that regulate immune response. They have been implicated in different aspects of multiple sclerosis (MS), including susceptibility and acute inflammatory events. Here, we assessed whether the function of NK cells is influenced by genetic variants associated with MS susceptibility and could mediate some of the functional consequences of the risk-associated alleles.
Methods: We investigated the role of mitogen-activated protein kinase 1 (*MAPK1*) allele given that this protein is implicated in NK cell mediated cytotoxicity. The dataset consists of phenotypic and functional NK cell profiles of 96 healthy subjects using flow cytometry. The results were replicated in the cohort of 65 subjects.
Results: We observed a strong association of the *MAPK1* risk allele with decreased degranulation (as measured by CD107α) by $CD56^{dim}$ NK cells in response to HLA class I-deficient B cells in both the discovery ($p=0.02$) and replication samples ($p=0.005$). This association remained significant for total NK cells ($p=0.01$). These data indicate that the *MAPK1* MS susceptibility allele alters the degranulation response of NK cells; an effect that is mainly driven by $CD56^{dim}$ subset.
Conclusions: MAPK1 has previously been shown to play an important role in NK cells effector function, possibly by its control of mobilization of secreted components such as perforin upon target cell engagement. We hypothesize that the *MAPK1* MS susceptibility allele may predispose to decreased cytotoxicity of NK cells that may result in insufficient clearance of activated immune cells. Modulation of the MAPK1 pathway could therefore provide an interesting avenue to modulate dysfunctional immune responses in MS.

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Host factors driving the detrimental response to Campylobacter causing the Guillain–Barré syndrome

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Objective: *Campylobacter jejuni* is the predominant preceding infection in the Guillain-Barré syndrome (GBS) and is associated with severe weakness and poor outcome. Molecular mimicry between *C. jejuni* lipo-oligosaccharides (LOS) and gangliosides induces cross-reactive antibody responses precipitating peripheral nerve damage. This detrimental

immune response after *C. jejuni* infection only occurs in a small minority of susceptible persons: probably in less than 1 in 1000 *C. jejuni* infections. The recurrence rate of GBS is approximately 5%, suggesting a role for host factors in GBS susceptibility. In pilot studies we previously found that dendritic cell (DC) and subsequent B-cell responses to *C. jejuni* LOS differ significantly between persons. We hypothesized that high responders to *C. jejuni* LOS may especially be at risk to develop GBS after *C. jejuni* infection. We investigated the constitutional difference in the DC/B-cell response to *C. jejuni* LOS in 20 healthy controls at 3 time points during a six-month follow-up. In addition we compared the DC/B-cell response between *C. jejuni* related ex-GBS patients ($n=27$) and control subjects ($n=28$).
Methods: Peripheral blood monocyte-derived DC were stimulated with *C. jejuni* LOS. The expression of maturation markers was determined using flow cytometry and cytokines were measured in the supernatant. Additionally, DC supernatants were assessed for their ability to promote B-cell proliferation.
Results: The results indeed confirm our earlier observation that the DC/B-cell response was highly variable between healthy donors. The differential responsiveness to *C. jejuni* LOS was confirmed at 3 and 6-months follow-up, suggesting a constitutional factor that determines the extent of the response. When comparing *C. jejuni*-related ex-GBS patients and control subjects, we found significant differences in the DC maturation markers CD38 and CD40, but not in other markers including CD80, CD83, CD86 and HLA-DR. Although the B-cell proliferative capacity of the DC supernatant as well as the production of tumour necrosis factor-α was not significantly different, there was a trend towards increased type I interferon production by DC of ex-GBS patients ($p=0.09$).
Conclusions: These results indicate that, using an in vitro DC/B-cell model, high and low responding healthy individuals to *C. jejuni* LOS can be identified. Ongoing research will reveal whether the subtle differences in the DC response to *C. jejuni* LOS play a role in GBS susceptibility.

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Immunomodulatory effects of Type II macrophages and microglia in experimental autoimmune encephalomyelitis

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Objective: Microglia (MG), the macrophage (MΦ)-like cell type of the central nervous system (CNS) are important in the inflammatory responses that occur in the brain and spinal cord. Studies also suggest that MG may also have a protective role in neuroinflammation. MΦ can be induced into several activation states. Type II MΦ have a regulatory phenotype, producing increased IL-10 and decreased IL-12. Type II activated MΦ and Type II inducing treatments are protective against experimental autoimmune encephalomyelitis, however the effects these have on MG is unknown. The aims of this project were to determine if MG are capable of type II activation and to determine the effects of treating mice with type II inducing substances has on microglial activation, in both normal conditions and in experimental autoimmune encephalomyelitis (EAE).
Methods: In order to investigate whether MG can also be type II activated, MG were isolated from the CNS of adult mice and maintained with M-CSF for 4 weeks before stimulation with lipopolysaccharide LPS or LPS, which induce classical or type II activation in MΦ respectively. To determine whether MG activation is altered *in vivo* by Type II inducing treatments, bone marrow chimeras were created using mice congenic for CD45 to allow MG to be analysed separately from invading cells. In addition we used immunohistochemistry to look at the expression of several markers associated with MG function and inflammation.
Results: Under type II activating conditions, where MG are stimulated with LPS and immune complexes, MG produced less IL-12 compared to those under classical activating conditions (LPS alone). Treatment with immune complexes is protective in mice with EAE, we found that MG from IC treated mice exhibit a less inflammatory phenotype than those that are isolated from untreated mice.
Conclusions: MG appear to be capable of achieving type II activation which is a regulatory phenotype. MG activated to a regulatory phenotype should be capable of altering the immune microenvironment in the CNS which would potentially protect against neuroinflammation, such as that seen in Multiple Sclerosis.

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Lack of TGFβ signaling in myeloid cells prevents entry into the remission phase of experimental autoimmune encephalomyelitis

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Objective: Mechanisms to control autoimmunity are of immense importance for the organism. TGF-beta is a potent regulatory cytokine with diverse effects on hematopoietic cells. In experimental autoimmune encephalomyelitis (EAE), a model for multiple sclerosis, it has been shown that the expression level of TGF-beta is increased during the remission phase. However, the role of TGF-beta in regulating myeloid cells during autoimmune responses is not thoroughly studied.
Methods: In order to dissect the role of TGF-β in the innate immune system during autoimmunity, we induced EAE in conditional knockout mice with the specific deletion of the TGFβRII gene in activated phagocytosing myeloid cells (phag-TGFbRII −/−).
Results: Our study shows that in contrast to control animals MOG-immunized phag-TGFbRII −/− mice develop stronger EAE and are not able to remit from the disease. Histological analysis of these mice revealed significantly stronger demyelination and accumulation of inflammatory dendritic cells (infDCs). Interestingly, we observed up-regulated expression of the Nox-2 gene both in total lysates and in infDCs. Nox-2 encodes for a subunit of NADPH oxidase, responsible for the reactive oxygen species (ROS) production. Furthermore, we found enhanced phagocytic activity in infDCs derived from the CNS of EAE-diseased phag-TGFbRII −/− mice.
Conclusions: Our data suggest that TGF-β signaling in myeloid cells has a crucial regulatory role in the amelioration phase of EAE and in protection from damage by myeloid cells. TGFb may exert its regulatory function via regulation of ROS production and phagocytosis.

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GM-CSF induces dendritic-cell like microglia through classical PKC/AP-1-dependent pathway

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Objective: Microglia are resident macrophage-like antigen presenting cells in the central nervous system (CNS), which play both inflammatory and neuroprotective roles in CNS inflammation including multiple sclerosis (MS), Alzheimer's disease, and amyotrophic lateral sclerosis. We and others showed that GM-CSF induced expression of CD11c, MHC class II, CD86, and PD-L1 in microglia to be dendritic-cell (DC) like phenotype. However, it is still unclear how GM-CSF induces DC-like microglia. We have examined how GM-CSF induces microglial differentiation into DC like cells.
Methods: Microglia were isolated from mixed glial cell cultures from newborn C57BL/6 mice by shaking off method. After pretreatment with each inhibitor including Gö6976 (PKC inhibitor), Wortmannin (PI3K inhibitor), Rapamycin (mTOR inhibitor), and Akt inhibitor, for 30 min, cells were stimulated with GM-CSF. Total RNA was extracted and RT-PCR was performed to assess CD11c and GAPDH mRNA expression. Flow cytometry was performed to analyze surface expression of CD11c and MHC classs II and costimulatory molecules including CD80, CD86, and PD-L1. For detection of PKC-α, c-Fos, and c-Jun, microglia were stimulated with GM-CSF. After washing twice, cells were lysed with TNES buffer and Western blotting was performed. Gel shift assay and Chip assay were also performed to detect nuclear translocation of transcription factor and binding of transcription factor to DNA.
Results: Both Gö6976 and Wortmannin, but not Rapamycin, and Akt inhibitor, inhibited microglial CD11c expression. GM-CSF induced both PKC-α and PI3Kp55 phosphorylation. PI3Kp55 phosphorylation was suppressed by Gö6976, suggesting that classical PKC regulates PI3Kp55 phosphorylation. Gel shift assay and Chip assay showed that GM-CSF-induced CD11c expression was mediated by AP-1 family transcription factor, c-Fos and c-Jun. Both c-Fos and c-Jun phosphorylation was inhibited by Gö6976, suggesting that classical PKC was upstream of c-Fos and c-Jun.
Conclusions: These results suggest that GM-CSF induces microglia to differentiate to CD11c+ cell differentiation through classical PKC-PI3K-AP-1 (c-Jun and c-Fos) dependent pathway in microglia. It is reported that local antigen presentation in the CNS by CD11c+ DCs is important to drive progression of experimental autoimmune encephalomyelitis, an animal model of MS. Thus, classical PKC/AP-1 (c-Jun and c-Fos)-targeting therapy in microglia may be useful strategy to treat MS.

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Granulocytes and their growth/ mobilizing factors as candidate therapeutic targets in autoimmune demyelinating disease

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Experimental autoimmune encephalomyelitis (EAE) is a murine model of autoimmune demyelination that is frequently used as a model for multiple sclerosis (MS). We have previously shown that granulocytes are critical for blood–brain-barrier (BBB) breakdown and clinical disease in mice actively immunized with myelin antigens. In the current study we investigated the pathways by which granulocytes are regulated during EAE. We found that granulocyte-colony stimulating factor (G-CSF) and CXCL1 were upregulated in the serum shortly after immunization with myelin peptide in CFA. The induction of these mobilizing factors correlated with granulocyte expansion in the bone marrow and their accumulation in the circulation. Sustained expression of serum G-CSF was dependent on the administration of *Bordetella pertussis* toxin, which is required for the clinical manifestation of EAE in our model. Induction of systemic G-CSF and mobilization of granulocytes were both dependent on IL-1R1 signaling, which was previously implicated in the pathogenesis of autoimmune demyelinating disease. Transfer of IL-23-polarized, myelin-specific CD4 T cells into naïve syngeneic hosts was sufficient to upregulate G-CSF and to mobilize granulocytes into the circulation. Clinical EAE was attenuated in the absence of the receptor for G-CSF. Interestingly, individuals with MS have been reported to have experienced severe exacerbations when given recombinant G-CSF following bone marrow transplant. Collectively, these data demonstrate the importance of G-CSF-driven granulocyte mobilization in the development of autoimmune demyelinating lesions and implicate granulocytes and their growth/ mobilizing factors as novel therapeutic targets in MS.

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Adherence enables monocytes to efficiently polarize memory CD4 T-cells upon stimulation with LPS

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Objective: Human monocytes have the ability to polarize memory CD4 T-cells in non-lymphoid tissues. This has been linked to the pathophysiology of autoimmune diseases characterized by a skewing of CD4 T-cell responses to the TH17 phenotype. We studied this effect in an environment which does not facilitate adherence of monocytes, comparable to the bloodstream compartment.
Methods: We used culture plates coated with a hydrogel layer that minimizes adherence for the culture of monocytes and compared it to monocytes grown in normal tissue culture plates. Monocytes were co-cultured with memory CD4 T-cells and activating αCD3 antibody with or without LPS. Percentage of IL-17 and IFNγ producing memory CD4 T-cells was measured by intracellular cytokine staining, cytokine release by monocytes was measured by ELISA and surface expression of co-stimulatory molecules was measured by flow cytometry.
Results: We found that upon stimulation with LPS non-adherent monocytes were not able to polarize memory CD4 T-cells as efficiently as adherent monocytes. This was not related to increased spontaneous apoptosis of non-adherent monocytes or difference in proliferation. CD4 T-cell polarizing cytokine release upon LPS-stimulation was impaired in non-adherent monocytes and expression of co-stimulatory molecules by non-adherent monocytes was altered upon stimulation with LPS as compared to adherent cells. However neither addition of recombinant cytokines nor exogenous stimulation of co-stimulatory molecules led to a reversal of reduced T-cell polarization by non-adherent monocytes. Activation of the β2-integrin pathway partly rescued the impaired LPS-induced cytokine release.
Conclusions: Monocytes encountering LPS in an environment that does not facilitate adherence have an impaired ability to polarize

memory CD4 T-cells. They secrete less T-cell polarizing cytokines, which is partly reversed by activation of the β2-integrin pathway, and have an altered surface expression of co-stimulatory molecules.

Models of disease

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CB2 signaling is required for susceptibility to cerebral malaria

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The endocannabinoid system (eCBs) is suggested to control neuroinflammatory responses and therefore harbors potential for the treatment of parasite-induced CNS infections. In this study we investigated the role of the cannabinoid receptor 2 (CB2) in a mouse model of cerebral malaria (CM). CB2 knockout ($Cnr2^{-/-}$) mice inoculated with *Plasmodium berghei* ANKA-infected erythrocytes (PbA-RBC) were shown to exhibit enhanced survival, while all wild type (WT) controls succumbed to disease. In addition, therapeutic application of the CB2 antagonist SR122458 conferred enhanced ECM resistance in C57BL/6 mice. PbA-RBC infected $Cnr2^{-/-}$ mice showed a reduced brain vessel sequestration of parasitized erythrocytes, a less compromised blood brain barrier and diminished neuroinflammation. Although a general reduction of infiltrating hematopoietic cells was observed in $Cnr2^{-/-}$ mice, $CD11b^{+}$ macrophage ratios within the brain infiltrate were increased. Higher numbers of $CD11b^{+}$ cells expressed markers of alternatively activated macrophages, suggesting that they mediate beneficial responses during parasite infection. Thus, targeting CB2 during parasite infection is a promising approach for the development of alternative treatment regimes for controlling parasite-induced neuroinflammatory responses.

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Interaction of inflammation and hyperoxia in a rat model of neonatal white matter damage

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Objective: Intrauterine infection and inflammation are major reasons for preterm birth. The switch from placenta-mediated to lung-mediated oxygen supply during birth is associated with a sudden rise of tissue oxygen tension that in preterm infants amounts to relative hyperoxia. Both, infection/ inflammation as well as hyperoxia have been shown to be involved in brain injury of preterm infants. Since it is unclear how the combination of these two insults might contribute to tissue injury, we investigated the influence of a systemic lipopolysaccharide (LPS) application on hyperoxia-induced white matter damage (WMD) in newborn rats.
Methods: Three-day-old Wistar rat pups received 0.25 mg/kg LPS i.p. and were subjected to 80 % oxygen on P6 for 24 hours. The extent of WMD was assessed by immunohistochemistry and western blot analysis. In addition, the effects of LPS and hyperoxia were studied in an *in vitro* co-culture system of primary rat oligodendrocytes and microglia cells.
Results: Both noxious stimuli, hyperoxia and LPS, induced a significant increase in apoptotic cell death as revealed by elevated cleaved caspase-3 levels and TUNEL-positive cells. Furthermore, both hyperoxia and LPS caused hypomyelination as revealed by Western blot and immunohistochemistry. However, single hit and two hit treated pups evoked the same degree of hypomyelination *in vivo*. Interestingly, LPS pre-incubation reduced premyelinating-oligodendrocyte susceptibility towards hyperoxia *in vitro* which also involved a modulation of the inflammatory response regarding cytokine expression.
Conclusions: In summary, our data suggest that inflammation as well as hyperoxia strongly attenuate oligodendrocyte cellular dynamics involving apoptotic pathways. Besides, our *in vitro* data indicate that neuroinflammatory mechanisms contribute to vulnerability of immature oligodendrocytes. This study demonstrates that the interactions among inflammation and hyperoxia in neonatal brains might contribute to WMD in premature born infants and are of major importance to the field of perinatal neurology.

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Characterization of a relapsing remitting type of EAN induced in Lewis rats

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Objective: Guillain–Barré syndrome (GBS) and chronic inflammatory demyelinating polyradiculoneuropathy (CIDP) are autoimmune-mediated inflammatory diseases of the PNS. Valuable insights in the immunopathogenic mechanism of GBS has been gained from the animal model, experimental autoimmune neuritis (EAN) that can be actively induced in Lewis rats by immunization with peripheral myelin homogenates, myelin proteins or derived peptides. But this model really only mimics the acute form of GBS (AIDP) and, at the moment, there is no useful animal model for CIDP, the chronic form.

In a previous study, we have shown that thiopalmitolation of encephalitogenic T-cell epitopes of CNS myelin proteolipid protein, enhanced immune responses as well as the development and chronicity of EAE, an animal model of the human demyelinating disease, multiple sclerosis. We hypothesize that the injection of Lewis rats with the thiopalmitoylated immunodominant P0 neuritogenic epitope would induce a chronic type of EAN.

The objective of this study is to characterize the EAN model we have induced in Lewis rats by injection of the thiopalmitoylated peptide palmP0(180–199).
Methods: We have synthesized palmitoylated and non-palmitoylated P0(180–199) to study and compare their immunogenic and neuritogenic properties after injection in Lewis rats. The disease course was followed by clinical assessment and histopathology of

the sciatic nerve. Demyelination and infiltration of inflammatory cells were assessed at different stages of the disease model and the cellular immune response was studied in detail.
Results: The acute course, clinical score and profile of EAN induced after injection of P0(180–199) as well as the histopathological characterization of the sciatic nerve were in complete accordance with the literature. In contrast, palmP0(180–199) induced a relapsing-remitting type of disease with 2 relapses (31, 42 dpi) after the first attack (12 dpi) with an important demyelination at 21 and 31 dpi associated with macrophages and T cells infiltration assessed by immunohistology. Complete remyelination was observed at 62 dpi when the animals had fully recovered. Preliminary results of proliferation assays show that there is an increase in the proliferation of LNC when palmP0(180–199) is used to either inject the rats or as a stimulating antigen.
Conclusions: From these data we concluded that palmP0(180–199) is able to induce a "CIDP-like relapsing" T-cell mediated disease in the Lewis rats.

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Gastrointestinal inflammation in a valproic acid-induced murine model for autism

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Objective: The gut–brain axis has been implicated in various psychiatric disorders, including autism. Although it has been reported that autistic patients suffer from intestinal problems, there is still much debate on the role of the gastrointestinal (GI) tract in autism. Therefore, we used a valproic acid (VPA)-induced murine model of autism to investigate whether VPA offspring exerted disturbances in the GI tract.
Methods: Pregnant mice were subcutaneously injected with 600 mg/kg sodium valproate or PBS at day 11 of gestation. After birth, the offspring was housed with the mother until weaning at postnatal day 21. At day 28–30 a social behavior test was performed, whereupon mice were sacrificed and tissue was collected for histology and protein analyses. For histology, paraffin embedded sections of proximal and distal ileum and colon were stained using standard H&E staining. Tissue samples of jejunum, middle ileum, distal ileum and colon were homogenized and neutrophil infiltration was quantified by measuring myeloperoxidase (MPO) levels.
Results: Only male offspring of VPA-treated mothers showed reduced social behavior compared to the offspring of PBS-treated mice. In the GI tract of the male offspring of VPA mothers, profound cellular inflammation and a disturbed epithelial layer was observed predominantly in the distal ileum. A significant increase in MPO, a marker for neutrophil infiltration, was observed in the distal ileum of male offspring of VPA mothers, confirming the histological observations. Tissue samples of female offspring are currently under investigation, but first results indicate that no inflammation is present in the GI tract of female offspring of VPA mothers.
Conclusions: These findings demonstrate that VPA maternal challenge in mice not only leads to behavioral impairments in the male offspring, as reported by various groups, but prenatal exposure to VPA also induces gastrointestinal neutrophilic inflammation, predominantly in the distal ileum. Since the use of VPA during pregnancy increases the risk of autism in offspring in humans as well, these data indicate that autism might indeed be associated with GI inflammation. Future research is focused on the pathway in which prenatal exposure to VPA induces GI inflammation to gain more knowledge on potential targets for treatment.

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Gimap5 deficiency in Lewis rats causes lymphopenia and exacerbates Experimental Autoimmune Encephalomyelitis

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Objective: Deficiency of the *GTPase of the immunity associated protein 5* (Gimap5) causes severe T cell lymphopenia and is responsible for the development of autoimmune Type 1 Diabetes in BB rats. Gimap5 in these rats carries a single point mutation, resulting in the expression of a truncated, non-functional protein. Consequently, peripheral CD8 + and CD4 + T lymphocyte numbers are dramatically diminished due to increased death of recent thymic emigrants. This appears to explain the selective enrichment of autoreactive T cells as indicated by an oligoclonal expansion of the remaining T cells. Interestingly, Gimap5 deficiency in PVG rats also leads to T-lymphopenia and autoimmunity, although Gimap5 deficient rats on this genetic background develop eosinophilic bowel disease rather than Type 1 diabetes. Moreover, Gimap5 polymorphisms in humans are linked to systemic lupus erythematosus and these patients are also lymphopenic.
Methods: To study Gimap5 function in neuroinflammatotory diseases, we have backcrossed the mutant Gimap5 allele to Lewis rats, a strain known to be particularly prone to develop experimental autoimmune encephalomyelitis (EAE). By using speed congenics we were able to obtain a 99% pure Lewis background within 5 generations.
Results: Gimap5 deficient Lewis rats develop T lymphopenia in the periphery and show an altered TCR repertoire. Whereas the V beta chain usage in the thymus is unchanged, some V beta chains are overrepresented on peripheral T cells of Gimap5 deficient Lewis rats as compared to controls. However, the affected V beta chains are not identical to the ones altered in BB rats. Although up to now we did not observe any spontaneous disease development, Gimap5 deficient rats suffer from a fulminant disease course after active EAE induction. This would be consistent with the observation in MS patients that an altered frequency of certain V beta chains is linked to the development of MS.
Conclusions: Collectively, our data suggest that a skewed TCR repertoire, as caused by Gimap5 deficiency, can aggravate neuroinflammatory diseases such as EAE. This might contribute to a better understanding of the etiology of MS.

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Neurocognitive and Immunologic Alterations Associated with Self Injurious Behavior in a Rhesus Macaque Model

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Objectives: Self Injurious Behavior (SIB) is a common comorbidity of psychiatric disorders and is emerging as a unique syndrome. A dearth of information about the neuroimmunologic mechanisms underlying this behavior is available, and few representative animal models exist. Previous work has demonstrated alterations in HPA axis and cortisol function, which are known regulators of the immune system. We describe a cohort of rhesus macaques, with 100% incidence of SIB post puberty, in which changes within the central nervous system (CNS) and immune system were associated with the SIB.
Methods: Wounding behavior was documented using a wound score (WS) scheme to measure severity and area affected by self-inflicted wounding. To evaluate the predictive value of cognitive deficits for the later development of wounding behavior, standardized touchscreen cognitive function tests were used to assess impulsivity (Stop Signal Response Time SSRT) and attentional set shifting (Intradimensional/Extradimensional shift ID/ED). These data were correlated to WS in SIB macaques at 2.5, 4, and 4.5 years of age (YoA). To investigate whether perturbations of the dopaminergic nervous system contribute to wounding, PET-MRI [11C]raclopride imaging of D2 dopamine receptor expression within the striatum was performed at 5 YoA and correlated with adolescent WS. CNS inflammation has been reported with disorders comorbid with SIB and we quantified cerebrospinal fluid levels of the inflammatory cytokine IL-6 using a commercially available ELISA. Given that platelet activation has been previously noted in conjunction with SIB, we also examined markers of platelet activation using FACS.
Results: SIB macaques had increased SSRT (Spearman $r = 0.563$ $p = 0.045$) and increased ID/ED errors (Spearman $r = 0.692$ $p = 0.009$) correlating with WS at preadolescent ages preceding the development of observable wounding. D2 receptor expression within the striatum was elevated in animals with increased severity of wounding (Spearman $r = 0.662$ $p = 0.014$). Macaques with SIB also had increased levels of CSF IL-6 compared to normal animals (Mann Whitney $p = 0.043$). Platelets had increased surface expression of PAC-1 and pselectin (MW $p = 0.023$, 0.065).
Conclusions: Alterations in neurocognitive and immunologic functions associated with the severity of SIB were found in these macaques. These animals may provide a model to study the complex interactions of the nervous and immune systems in psychiatric disease.

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Inflammatory monocytes exacerbate injury following intracerebral hemorrhage

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Objective: Neuroinflammation is a major component of secondary injury following intracerebral hemorrhage (ICH). However, little is known about which of the many invading leukocyte populations mediate secondary injury. Inflammatory $Ly6C^{hi}$ monocytes have been shown to exacerbate injury in other models of sterile inflammation. In this study we examined the role of $Ly6C^{hi}$ monocytes on inflammation and injury in a mouse model of ICH. The purpose of this study was to determine how inflammatory monocytes influence inflammation and secondary injury following ICH.
Methods: The autologous blood injection ICH model was performed in C57BL/6J (WT) or B6.129S4-*Ccr2*^{tm1Ifc}/J ($CCR2^{-/-}$) mice. Briefly, 20 μl blood were stereotaxically injected 2.5 mm right and 3 mm deep, relative to bregma. Injecting blood into this location causes a right basal ganglia ICH, resulting in a left-sided motor deficit. Motor function was assessed using the cylinder test. Mice were placed into a glass cylinder and observed for 20 rears by a blinded scorer. For each rear, the initial forelimb placed on the wall of the cylinder was recorded. A laterality index (L.I.) was calculated as (right placements − left placements) ÷ (lefts + rights + boths), where zero indicates no forelimb preference and 1 indicates that only the right forelimb was used. Flow cytometry was performed on ipsilateral brain hemispheres following mechanical and enzymatic digestions and centrifugation on a 30%/70% percoll gradient. Bone marrow transplants were performed by injecting 1×10^6 WT or $CCR2^{-/-}$ bone marrow cells into lethally irradiated CD45.1 mice.
Results: $CCR2^{-/-}$ mice have significantly fewer $Ly6C^{hi}$ monocytes, $Ly6C^{low}$ monocytes, and dendritic cells in the brain at post-ICH days 1 and 3. A reduction in these leukocyte populations coincides with significantly improved motor function in $CCR2^{-/-}$ mice at day 1 (L.I. $= 0.13 \pm 0.20$ $CCR2^{-/-}$ versus 0.71 ± 0.34 WT; $p < 0.05$ by t-test). Both groups of mice perform similarly on the cylinder test beyond day 1. Irradiated CD45.1 mice with $CCR2^{-/-}$ circulating leukocytes had a significantly abrogated motor deficit compared to CD45.1 mice reconstituted with WT bone marrow cells (L.I. $= 0.30 \pm 0.21$ $CCR2^{-/-}$ versus 0.55 ± 0.19 WT; $p < 0.05$ by t-test).
Conclusions: Together, these experiments show that bone marrow-derived CCR2-expressing monocytes contribute to early injury following ICH. In the absence of these monocytes, mice exhibit less severe motor deficits and have a decreased inflammatory response.

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Gastrointestinal symptoms and probiotic treatment in a mouse model of an autism risk factor

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Objective: While autism is a neurodevelopmental disorder characterized by language and social deficits, recent studies have highlighted striking dysregulation in the neural, peripheral, and enteric immune systems of autistic individuals. There are also reports that subsets of children with autism spectrum disorder (ASD) display gastrointestinal (GI) abnormalities, including chronic inflammation of the colon, increased intestinal permeability and altered composition of GI microbiota. Moreover, antibiotic treatment and restricted diet are reported to provide behavioral improvements for some ASD children. We use a mouse model of an ASD risk factor, maternal immune activation (MIA), to assess whether offspring, which display core behavioral and neuropathological features of autism, also display ASD-associated GI symptoms. To explore the potential connections between GI problems and the brain and behavior, we test whether postnatal administration of a probiotic influences GI and ASD-related behaviors.
Methods: Pregnant mice are injected with poly(I:C) (to evoke a maternal inflammatory response) or saline on E12.5. Adult poly(I:C) offspring are confirmed to exhibit autism-related behavioral abnormalities and neuropathology. Offspring are fed three doses of probiotic bacteria at weaning. Young and adult offspring are assessed for a) intestinal barrier integrity by measuring leakage of FITC-dextran through the intestinal epithelium and tight junction expression, b) enteric immune abnormalities by assessing profiles and function of leukocytes derived from the mesenteric lymph nodes, c) GI inflammation by cytokine Luminex array and histology, d) serum metabolome profiles by GC-MS and LC-MS.
Results: MIA offspring display decreased intestinal barrier integrity and corresponding changes in levels of tight junction proteins. These symptoms are associated with altered expression of colon cytokines and changes in serum metabolite levels. Postnatal probiotic treatment ameliorates these GI abnormalities, and normalizes certain serum metabolites and several ASD-related behaviors.

Conclusions: These studies highlight the importance of the gut-brain axis, where primary manipulations of the intestinal microbiome can influence GI physiology and behavioral performance. The results raise the possibility of testing a probiotic therapy in individuals with autism and co-morbid GI problems. Also, findings of altered serum metabolite profiles in the MIA mouse model raise the possibility of testing particular metabolites as candidate biomarkers for subsets of human ASD.

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Neuroimmune changes in a mouse model of the maternal infection risk factor for schizophrenia and autism

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Objective: Infection in pregnant women is associated with increased risk for autism and schizophrenia in the offspring. In a mouse model of this risk factor, activation of the maternal immune system by injection of the viral mimic poly(I:C) sets in motion a cascade of molecular events that ultimately results in autism- and schizophrenia-related behaviors in offspring. The finding that interleukin-6 (IL-6) is a crucial mediator of these effects led us to examine the mechanism by which this cytokine influences fetal development *in vivo*. Here we examine the placenta and fetal brain as sites of early IL-6 action. We further assess whether MIA offspring display altered postnatal immune profiles and function as a potential later-life consequence of maternal immune activation (MIA).
Methods: Pregnant mice are injected with saline, poly(I:C), or IL-6 on E12.5, or given an intranasal influenza infusion on E9.5, and the placentas, fetuses, and postnatal offspring are examined by immunohistochemistry, microarray, Luminex cytokine array, ELISA, flow cytometry, quantitative real-time PCR, hematopoietic stem cell (HSC) differentiation and behavioral testing.
Results: The cytokine IL-6 is a key mediator of the effects of MIA on the fetus, as shown by blocking or genetically inactivating IL-6 in the MIA dam, or conversely, by a single i.p. IL-6 injection in the control dam. MIA activates downstream IL-6 signaling pathways in the placenta, altering its endocrine functions. These actions at the materno-fetal interface are associated with altered cytokine profiles directly in the fetal brain. Transcriptome profiling of the fetal brain response to poly(I:C), IL-6 or flu infection reveals an acute, transient up-regulation of crystallin family genes. These early changes are associated with altered immune profiles in young and adult offspring, characterized by deficits in T regulatory cells and increased levels of neutrophilic and monocytic Gr-1 + cells. Interestingly, behaviorally-abnormal MIA offspring that have been irradiated and transplanted with immunologically normal bone marrow no longer exhibit several autism- and schizophrenia-related behaviors.
Conclusions: These results support a key role for IL-6 in the placenta and fetal brain in shaping neural development and behavior in MIA offspring, and suggest that altered peripheral immunity can contribute to the development of abnormal behaviors.

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Hydroxyl radical formation in peripheral nerves of rats with experimental autoimmune neuritis

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Objective: To elucidate hydroxyl radical (HR) formation in the peripheral nerves of rats with experimental autoimmune neuritis (EAN), and if any, to indicate the cellular sources of HR.
Methods: Synthetic peptide of bovine P2 protein was injected into the hind limb of female Lewis rats with complete Freund's adjuvant. Clinical signs were assessed daily. The rats were sacrificed at 7, 11, 14, 25 days after immunization ($n = 5$), respectively. Fifteen minutes before sacrifice, 300 mg/kg of sodium salicylate (SS) were given intraperitoneally. The cauda equina (CE) were removed and stored in −80 °C until biochemical analysis. CE were then homogenized, trapped HR by SS was measured as a value of 2,5-dihydroxybenzoic acids (DHBA) by high-performance liquid chromatography using an electrochemical detector (ESA-model 5500: ESA, Bedford, MA). Formalin-fixed, paraffin embedded CE at L4 level were employed for the immunohistological analysis. Immunoreactivity for malondialdehyde (MDA), produced by HR from lipid, were assessed by monoclonal antibody, 1F83 (JaICA, NIKKEN SEIL, Shizuoka, JAPAN) after standard antigen retrieval protocol.
Results: EAN group developed flaccid tail paralysis on 11 days after immunization (11 d.p.i) and worsened gradually. At 14 d.p.i, severe flaccid paraplegia was developed and then recovered spontaneously. After 25 d.p.i., tail tip paralysis remained. Motor paralysis did not develop throughout the experimental period in control group. 2,5-DHBA in EAN group was significantly increased compared to the control group on 11 and 14 d.p.i. Immunohistological examination revealed patchy distribution of MDA in CE which correlates with the foci of CD45 + but not always of CD3 + infiltrating cells.
Conclusions: HR is produced especially within infiltrating cell foci in CE during active demyelination/inflammation. The cellular source of the HR is postulated as macrophages rather than lymphocytes. Further investigation is warranted to elucidate precise cell source, significance and function of HR.

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HHV-6A infection enhances EAE severity in the common marmoset

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Objective: The ubiquitous human herpesvirus HHV6A is associated with several neurologic diseases including multiple sclerosis (MS).The biology of HHV6A infection is largely unknown, though it is considered neurotropic. We investigated HHV6A infection in common marmosets (*C. jacchus*), which unlike rodents express the receptor for viral entry. Experimental autoimmune encephalomyelitis (EAE) is an inflammatory demyelinating disease of the CNS induced by immunization with CNS tissue. Marmoset EAE recapitulates specific aspects of MS pathology and enables tracking of lesion development by MRI. As viruses may act as triggers in MS, we asked if marmosets previously inoculated with HHV6A exhibit an altered EAE disease course compared to previously naïve marmosets.
Methods: We inoculated four marmosets with HHV6A intranasally, in light of our recent work demonstrating the olfactory pathway as a route of HHV6 entry. Subsequently, two of these marmosets were immunized with human white matter homogenate (WMH) in CFA to induce EAE (WMH + virus) along with two previously naïve control marmosets (WMH alone). Disease was monitored clinically, immunologically and by MRI through to humane endpoints.
Results: WMH + virus marmosets exhibited clinical symptoms earlier than WMH alone marmosets.WMH + virus marmosets also exhibited more severe disease by MRI, with increased white matter lesion load and leukocortical lesions in the brain. Seropositivity for an immunogenic

myelin protein, MOG, often correlates with overt clinical EAE. We detected increased levels of serum anti-MOG IgG in WMH + virus marmosets compared to WMH alone marmosets. Following HHV6A intranasal infection, virus-specific antibodies were not detected but upon EAE induction, virus-specific IgM was detected in one WMH + virus marmoset. Ongoing studies may elucidate the mechanism(s) of accelerated disease including immunohistochemical analyses of CNS tissues, detection of viral DNA from CNS tissues and flow cytometry-based analyses of cellular responses to HHV6 and MOG.
Conclusions: We observed accelerated clinical EAE disease in marmosets previously inoculated with HHV6A.These preliminary observations support a role for viruses in exacerbating CNS immunopathology. Viruses may act as triggers in autoimmune conditions such as MS through molecular mimicry, bystander activation or unknown mechanisms that compromise or activate the blood brain barrier. This animal model may further our understanding of the longstanding association between viruses and MS.

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Toll-like receptor 4 deficiency ameliorates experimental autoimmune neuritis by downregulating classically activated macrophages (M1)

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Objective: Toll-like receptors (TLRs) are a group of pattern recognition receptors sensing exogenous and endogenous deleterious signals. Activation of TLRs through the MyD88-dependent or MyD88-independent pathway triggers T helper (Th)1-oriented autoimmune responses by inducing production of IL-12 and tumor necrosis factor (TNF)-alpha. TLR4 is one of the most extensively studied members of the TLR family and has been associated with many autoimmune disorders. We aimed to study the specific role of TLR4 in the pathogenesis of experimental autoimmune neuritis (EAN), an animal of human Guillain–Barré syndrome.
Methods: EAN was induced in TLR4 knockout (KO) mice and in C57BL/6 mice as wild type (WT) controls by immunization with P0 peptide 180–199 emulsified in Freund's complete adjuvant. The effects of TLR4 deficiency on the clinical course of EAN and the functions of T cell and macrophages were studied.
Results: TLR4 deficiency remarkably ameliorated EAN. From day 15 post immunization, the clinical signs of TLR4 KO mice were significantly less severe than those of their WT counterparts. Upregulated expression of TLR4 was detected on the infiltrating cells in cauda equina of EAN WT mice and was correlated with the clinical scores. The proliferation of lymphocytes in response to either antigenic or mitogenic stimulation was attenuated in the KO mice. Nevertheless, TLR-4 deficiency downregulated the production of alternatively activated macrophage (M1), characterized by reduced production of interleukin (IL)-12 and nitric oxide (NO), and enhanced production of IL-10, both in vivo and in vitro.
Conclusions: Toll-like receptor 4 deficiency ameliorates EAN by downregulating classically activated macrophages (M1).

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Encephalitogenic potential of discrete T cell epitopes of aquaporin-4 (AQP4)

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Background/objective: Neuromyelitis optica (NMO) is associated with AQP4-specific IgG1 antibodies, a T cell-dependent antibody subclass, indicating that AQP4-specific T cells are involved in NMO pathogenesis. AQP4 is a multideterminant autoantigen. Previously, we identified immunodominant and subdominant determinants of AQP4 in SJL and C57BL/6 mice. In adoptive transfer studies, donor SJL/JT cells specific for the immunodominant AQP4 determinant elicited inflammatory responses in recipient SJL/J mice, whereas donor C57BL/6T cells did not elicit CNS inflammation in recipient C57BL/6 mice. Here, we examined whether donor SJL/JT cells that recognized subdominant AQP4 determinants elicited CNS inflammation.
Methods: SJL/JT cells specific for AQP4 peptide (p) 16–30, p23-35, p101-119 or p126-140 were tested for induction of CNS inflammation in syngeneic SJL/J recipient mice by adoptive transfer of peptide-specific CD4+ T cells polarized to Th1 and Th17 phenotypes.
Results: Donor SJL Th1 or Th17 cells specific for AQP4 p16-30, p23-35 and p101-119 induced CNS inflammation in brain, spinal cord, and, often, the optic nerves of irradiated SJL mice. More lesions were detected after injection of anti-AQP4 antibodies with the T cells. These mice did not show physical symptoms of CNS disease. In contrast, AQP4 p126-140-specific T cells did not induce CNS inflammation.
Conclusions: The murine AQP4-specific encephalitogenic response is not only strain-dependent, but also epitope-specific. In this regard, T cells specific for some, but not all, subdominant determinants induced CNS inflammation, findings that may be relevant to AQP4-specific T cell responses in NMO.

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Lack of adiponectin leads to increased immune activation and a worse MS model

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Objective: Multiple sclerosis (MS) is a presumed autoimmune disease directed against central nervous system (CNS) myelin, for which there are currently only partially effective treatments. The main animal model for MS is experimental autoimmune encephalomyelitis (EAE), in which activated myelin-specific T cells enter the CNS leading to inflammation, demyelination and axonal damage. It is now known that immune responses can be influenced by endogenous metabolic molecules produced mainly by fat cells, called adipokines. One such adipokine is adiponectin, which has anti-inflammatory effects and which levels are decreased in obesity. Here, we tested the hypothesis that adiponectin has a protective role in EAE and MS.
Methods: EAE was compared in adiponectin deficient versus wild type mice. Clinical, histopathological and immunological assessments

were performed. Effects of administration of exogenous adiponectin during EAE were also tested.
Results: ADP KO mice developed more severe EAE with greater CNS inflammation, demyelination and axon injury. Lymphocytes from immunized ADP KO mice proliferated more and produced higher amounts of inflammatory cytokines in response to antigen than WT lymphocytes. These findings were associated with fewer T regulatory cells with reduced functional capacity in ADP KO compared to WT mice. Treatment with globular adiponectin ameliorated EAE, and led to an increase in T regulatory cell numbers.
Conclusions: Adiponectin is immunomodulatory during EAE, suggesting a new avenue of investigation for development of MS therapies.

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Astrocytes affect neuroprotection: Glatiramer acetate mediates neuroprotection in an animal model of Huntington's Disease

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Objective: To investigate neuroprotective effects and therapeutic efficacy of glatiramer acetate (GA) in a transgenic animal model of Huntington's disease.
Background: Huntington's disease (HD) is a neurodegenerative disorder with a progressive disease course for which there is no cure available so far. Although the exact mechanisms that lead to neurodegeneration are not fully understood, impaired transcription and transport of brain-derived neurotrophic factor finally resulting in decreased brain levels of BDNF may play a considerable role in the pathogenesis of HD. GA, composed of a synthetic polypeptide mixture mimicking myelin basic protein, is an approved drug already used for treatment of multiple sclerosis. Besides its immunomodulatory effects, GA seems to have a bystander effect on CNS resident glia cells. GA exerts neuroprotective effects via increased BDNF upregulation in astrocytes and neurons rather than in immune cells of the periphery.
Methods: R6/2 transgenic mice were immunized subcutaneously with GA or with ovalbumin as control. The role of astrocytes in GA mediated neuroprotection was analyzed by in vitro stimulation of primary astrocyte cultures with GA and astrocytes viability assay. Coronal brain sections were immune- and histochemically processed for Cresyl violet-, Ubiquitin-, NeuN-, GFAP- and BDNF-staining. Quantitative real time PCR was performed to investigate potential differences in BDNF expression in the brain within the different groups. Concerning clinical course motor impairment was evaluated by accelerating Rotarod and Clasping score and survival was analyzed.
Results: Neurodegeneration and astrogliosis were reduced after GA treatment. Furthermore, GA treatment restored BDNF levels in the brain, quantified by real time PCR and immunofluorescence. First results indicate a possible bystander effect of astrocytes in GA mediated neuroprotection in R6/2 mice being underlined by immunofluorescence double staining with GFAP and BDNF where number of BDNF positive astrocytes was raised. Motor function revealed an attenuated motor impairment and dyskinesia was reduced after GA treatment as well.
Conclusions: Our results provide evidence for neuroprotection in an animal model of Huntington's disease mediated by GA. This neuroprotection may be linked to an upregulation of BDNF, described to be downregulated in Huntington's disease. Based on our findings, we propose a direct action of GA on astrocytes with beneficial effects on neurons.

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Characterizing demyelination and remyelination by flow cytometry and its application in models of demyelination

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Objective: Characterizing cells of the oligodendrocyte lineage is crucial for understanding de- and remyelination in the CNS. Here we present a novel method for the rapid, unbiased analysis of oligodendrocyte lineage cells (OLCs) using flow cytometry. Amassing cellular data we produced a global profile of oligodendrocyte populations from immature oligodendrocyte progenitor cells (OPCs) to mature myelin-forming oligodendrocytes (OLs) and from that characterized de- and remyelination in the CNS.
Methods: To effectively characterize OLCs in the CNS, we optimized OLC antibodies, dissociation enzymes, and purification methods from mouse CNS. Then using this technique we successfully characterized OLCs in mouse early postnatal development. We next applied our method to two murine models of demyelination: cuprizone-induced demyelination and experimental autoimmune encephalomyelitis (EAE).
Results: SJL/J mice were fed cuprizone for five weeks to induce widespread demyelination and then normal chow for a sixth week to allow for remyelination and were sacrificed weekly throughout. Flow analysis of OLCs revealed a robust loss of mature OLs by week five with considerable recovery at week six. OPCs expanded early in the disease course but decreased dramatically during remyelination suggesting a gradual loss of mature OLs and an early, robust expansion of OPCs that failed to fully differentiate until cuprizone withdrawal. In SJL/J mice immunized with $PLP_{139–151}$, EAE produced a relapsing–remitting disease course similar to types of multiple sclerosis. Mice were sacrificed at onset, peak, remission, and relapse of clinical disease, and brains and spinal cords were analyzed by flow cytometry. At onset of disease there was a dramatic loss of mature OLs with a concomitant increase in early and late stage OPCs. By disease peak pre-myelinating OLs were increased suggesting a maturation of the previously expanded OPC pool. The mature pool never fully expanded to pre-disease levels, suggesting an arrest in OLC differentiation before functional maturation to myelinating OLs.
Conclusions: We present the first evidence of quantification of OLCs and global analysis of the myelin-producing cells in postnatal and adult mouse CNS by flow cytometry. Characterization in models of human CNS disease confirmed the sensitivity of the assay and supports its use in rapidly evaluating potential therapeutic interventions for direct and indirect effects on demyelination and myelin regeneration.

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Mice lacking microglial CX3CR1 exhibit greater neurological disability after intracerebral hemorrhage

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Objective: Intracerebral hemorrhage (ICH) results in an inflammatory response promoted by chemokine-mediated leukocyte recruitment to the brain. Leukocyte populations infiltrate in the initial hours after ICH, peak at 72 h, and diminish by 14 days. Spleen weights are enlarged at the 72-hour peak of inflammation. The chemokine

CX_3CL1, expressed by neurons, selectively binds to its receptor CX_3CR1 found on T cells; resident monocytes ($Ly6C^{lo}$), and microglia. CX_3CR1- null mice have been shown to have less injury in ischemic stroke and greater injury after spinal cord injury. Our objective is to identify the effect of CX_3CR1 activation on neurological outcome and the inflammatory response after ICH.
Methods: ICH was modeled by injection of 20 μl of blood into the right striatum. Neurological deficit was quantified using digital gait analysis (DigiGait™) and then mice were sacrificed at 72 h. C57/BL6 (WT) and B6.129P-Cx3cr1tm1Litt/J ($CX_3CR1^{GFP/GFP}$) mice as well as bone marrow chimeras consisting of WT and $CX_3CR1^{GFP/GFP}$ hosts with reconstituted leukocytes from a B6.SJL-Ptprca Pep3b/BoyJ (CD45.1) donor (CD45.1➔WT or CD45.1➔$CX_3CR1^{GFP/GFP}$) were tested.
Results: Digital gait analysis showed $CX_3CR1^{GFP/GFP}$ mice had greater external rotation of the left hind paw (WT $-9.2°\pm4.6°$ vs. $CX_3CR1^{GFP/GFP}$ $-15.9°\pm2.1°$, $p<0.05$) and shorter left hindlimb stride length (WT 7.2 cm ± 0.4 cm vs. $CX_3CR1^{GFP/GFP}$ 6.7 cm ± 0.2 cm, $p<0.05$) at 72 h. This indicated a persistent left hemiparesis in the mice lacking functional CX_3CR1. In order to determine the contributions of leukocyte vs. microglial CX_3CR1, experiments were conducted in chimeras with host CX_3CR1 vs. WT cells. The chimeras with $CX_3CR1^{GFP/GFP}$ host were too weak to run on the treadmill at 72 h, while the WT host chimeras could easily complete the testing. There were no differences in overall body weight loss between the chimeras. The $CX_3CR1^{GFP/GFP}$ chimeras showed an exaggerated spleen enlargement with higher percentages of spleen weight relative to body weight compared to WT chimeras (WT 0.32 g ± 0.02 g vs. $CX_3CR1^{GFP/GFP}$ 0.44 g ± 0.03 g, $p<0.05$).
Conclusions: $CX_3CR1^{GFP/GFP}$ mice showed greater neurological disability after experimental ICH. Bone marrow chimeras with $CX_3CR1^{GFP/GFP}$ hosts demonstrated greater disability and enlarged spleens at 72 h. The data suggests an enhanced inflammatory response in mice lacking microglial CX_3CR1. Ongoing work is aimed at determining mechanisms and long-term outcomes.

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Detection of neurological sequalae in a post-meningitic mouse model using the automated IntelliCage system

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Objective: Behavioural alterations, learning difficulties, hearing loss and motor impairments are common long-term sequelae in survivors of pneumococcal meningitis (PM). Immunological profiles of PM patients and animal models reveal the up-regulation of pro-inflammatory cytokines, e.g. IL-1β, IL-6 and TNF. The elevation of these cytokines in the brain recently has been shown to be associated with sickness behaviour and cognitive alterations. Histopathology studies have revealed neuronal damage, including cortical necrosis and hippocampal apoptosis, in PM.

The current study aimed to characterise alterations in behaviour, learning and memory in a post-meningitic C57BL/6J mouse model using the IntelliCage, an automated behavioural testing system.
Methods: Female mice were given 5×10^5 cfu of *Streptococcus pneumoniae* serotype 3 strain WU2 via intracerebroventricular injection. Controls received saline in the same way. Ceftriaxone antibiotic (100 mg/kg i.p.) treatment was initiated 20 h later and was repeated once each day for three days. Ten days post-infection, 12 to 16 C57BL/6J mice, both uninfected controls and surviving post-meningitic mice, were socially housed and simultaneously tested in the IntelliCage. Mice were exposed sequentially to the following modules: free adaptation; nosepoke adaptation; drinking session adaptation; LED stimulus test; patrolling; spatial and discrimination reversal.
Results: Deficits in spatial learning and working memory were found in the PM mouse model subjected to automated patrolling tasks. In addition, inhibition of visiting, nosepoking, and drinking behaviours was found in these mice when they were first exposed to the novel, social IntelliCage environment, suggesting the presence of sickness behaviours, which has not been reported before in PM rodents. Likewise, impaired behavioural flexibility in post-meningitic mice was demonstrated in the early spatial reversal task, which has been consistently reported in hippocampal-lesioned mice. Furthermore, aversive responses to a constant LED-lit corner as well as impairments in the discrimination reversal task were observed in our study.
Conclusions: The study revealed several aspects of long-term neurological sequelae in a PM mouse model. The results suggest that the series of behavioural tests may allow fast and standardised screening of neurological alterations in post-meningitic mice modified genetically or treated pharmacologically, which may encourage therapeutic advances.

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Skeletal muscle pathology in the experimental animal model of muscle-specific receptor tyrosine kinase (MuSK) related myasthenia gravis

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Objective: Myasthenia gravis (MG) is characterized with muscle weakness mostly induced by acetylcholine receptor (AChR) or muscle-specific receptor tyrosine kinase (MuSK) antibodies (Ab). MG patients with MuSK-Ab often present with a severe disease course and muscle atrophy. To investigate the influence of MuSK autoimmunity on muscle pathology, MuSK associated experimental autoimmune MG (MuSK-EAMG) was induced in C57BL/6 (B6) mice.
Methods: The immunopathological findings of B6 mice immunized with recombinant human MuSK in complete Freund's adjuvant (CFA) (n = 14) were compared with those of control B6 mice immunized with only CFA (n = 11). Muscle weakness was assessed by clinical observation and inverted mesh hanging test. Serum levels of anti-MuSK Ig isotypes and complement C3 were measured by ELISA. Neuromuscular junction (NMJ) Ig and complement deposits were evaluated by immunofluorescence. NMJ AChR content was estimated by α-bungarotoxin (α-BTx)-binding sites, whereas NMJ MuSK content was estimated by immunofluorescence and western blot using an anti-mouse MuSK-Ab. Muscle histology was evaluated with hematoxylin-eosin (H&E), succinate dehydrogenase (SDH) and cytochrome oxidase (COX) staining.
Results: Mild to severe clinical muscle weakness was observed in 13 of 14 (93%) B6 mice immunized with MuSK-CFA. Ten (71%) mice had to be terminated due to severe muscle weakness. MuSK-immunized mice showed significantly increased serum anti-MuSK IgG, IgG1, IgG2b and C3 levels and an abundance of NMJ IgG, IgG1, IgG2b, C3 and membrane attack complex (MAC) deposits. While MuSK-

immunized mice had significantly reduced α-BTx binding sites, their muscle MuSK content was found to be unaffected by immunofluorescence and Western blot. H&E staining yielded type 1 muscle fiber atrophy in MuSK-CFA immunized mice, whereas muscular SDH and COX expressions were normal.
Conclusions: MuSK immunization induces EAMG with very high incidence and clinical severity. Although most MuSK-Ab positive MG patients do not exhibit complement-fixing serum IgGs, MuSK-EAMG is characterized with NMJ deposits of C3, MAC and complement-fixing IgG2b antibodies, suggesting that complement-mediated NMJ damage might contribute to muscle weakness in severe MuSK-MG patients. Muscle weakness in MuSK-EAMG is associated with NMJ AChR loss and muscle atrophy. Normal expression of mitochondrial enzymes suggests that muscle atrophy is not associated with mitochondrial dysfunction.

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Omics

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Gene expression for mouse BV-2 cells activated by lipopolysaccharide

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Microglia are resident immune cells within the central nervous system and regulates innate and adaptive immune system. Lipopolysaccharide (LPS) induces a strong inflammation in macrophage-like cells by regulating gene expression. To identify genes up- and down-regulated in mouse BV-2 microglia cells during LPS-induced inflammation, we performed whole RNA shotgun sequencing (RNA-seq) with 10 ng/ml LPS for 2 and 4 hours. Among statistically significant 775 genes (for 2 hr) and 1542 genes (for 4 hr) obtained from RNA-seq, 210 genes (for 2 h), 310 genes (for 4 h) were increased by LPS treatment and most of them were transcription factors, histone modifier such as *Irf1*, *Klf7* and *Jmjd3*, zinc finger family proteins and non-coding RNAs including miR5109. *Irf-1* is an interferon-gamma (IFN-γ)-induced transcription factor related with regulation of infection and inflammation, and miR5109 influences the expression of target genes associated with anti-inflammatory mechanisms. These results suggest that LPS affects inflammation-related transcription regulators in BV-2 cells. This study may provide the potential to identify regulators associated with inflammation mechanisms in activated BV-2 cells with LPS treatment.

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Systems biology of gender-based blood transcriptomes unravels a role for SP1 dependent gene transcription in multiple sclerosis

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Objective: Although affecting the central nervous system, multiple sclerosis (MS) is characterized by alterations in peripheral immunity. Our objective was to investigate the effect of gender on global blood gene expression in healthy and diseased subjects and to identify novel pathological processes associated with MS.
Methods: We investigated global gene expression by Illumina microarrays in peripheral blood mononuclear cells (PBMC) of healthy and relapsing-remitting MS individuals and performed gender-based analyses. Moreover, we reconstructed and analyzed the interactomes linked to the differentially expressed genes in women and men with MS via systems biology tools. Finally, we validated the contribution of one "in silico" predicted interactor to the generation of T cell responses by in vitro and ex vivo immunological assays and by in vivo experiments in the animal model of MS.
Results: First of all, we monitored sex-related gene expression differences in PBMC from healthy or MS subjects. Using relaxed statistics to maximize the number of differences between genders, about 10% of the filtered genes resulted sex-specific in the healthy population, while only 2% in MS subjects. Moreover, only a minor part of the MS sex-specific genes were natural, pre-existing sex-specific genes. These data demonstrate that MS pathology is associated with a dramatic change both in the quantity and in the quality of the genes that are normally differentially expressed between genders. Further, the application of stringent statistics on the female or male populations led to the identification of distinct gene signatures associated with MS, which were characterized by the same biological content. In fact, bioinformatical analyses of the transcriptional signatures and of the relative interactomes highlighted ontology terms related to transcription, DNA binding and chromatin modification, suggesting that epigenetics may be the underlying pathogenic mechanism in MS. We focused the attention on the transcription factor SP1 which appeared among the epigenetic interactors. Specific inhibition of SP1 reduced encephalitogenic T cell responses, incidence and clinical expression of EAE, demonstrating that SP1 dependent transcription modulates autoimmune responses and that its blockade may represent a novel target for MS treatment.
Conclusions: In light of the results, we propose gender-based systems biology as a novel tool to acquire novel information on disease associated functional processes.

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Myeloid shedding vesicles OMICS

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Objective: Microparticles (MPs) are released by most cell types into the microenvironment in physiological conditions and are emerging as a novel way of cell-to-cell communication. Composition and biological activity of MPs vary depending on the state of donor cells and on their stimulation. In the recent years, a series of studies has indicated relevant physiological and pathological functions for extracellular vesicles within the brain. Microglia and perivascular macrophages, both derived from myeloid progenitors, can become

polarized to pro- or anti-inflammatory states called M1 and M2, respectively, by the local microenvironment.

In order to identify the content of myeloid MPs we performed *in vitro* studies on murine macrophages.

Methods: We treated macrophages obtained from peritoneum (Pec) with IFNγ/LPS and IL4 to differentiate them into to M1 and M2. We then induced MPs shedding from Pec by ATP administration. We analysed MPs by flow cytometry for IB4, AnnexinV and CD9 to investigate which macrophage phenotype is more involved in MPs release. In addition, to isolate shedding vesicles, MPs were subjected to different ultra-centrifugations and we considered P2P3 fraction corresponding to shedding vesicles. We studied mRNA expression of shedding vesicles and of their donor cells via qRT-PCR and microarray analysis. We investigated also protein expression through mass spectrometry.

Results: We observed that IFNγ/LPS and IL4, after ATP administration, all increased the release of shedding vesicles when compared to the control.

We found that iNOS, TNFα and IL1β mRNA are expressed in IFNγ/LPS treated Pec. On the contrary, IL4 treated Pec expressed M2 markers mRNA such as Ym1, Arg1, Mrc1 and CCL22. This was confirmed also in M1 and M2 shedding vesicles.

Microarray analysis proved an up-regulation of M1 and M2 shedding vesicles genes involved in inflammatory and anti-inflammatory pathways and they also allowed to detect shedding vesicles potential housekeeping genes.

Proteomic studies demonstrated a specific protein expression reduction in M1 and M2 shedding vesicles compared to the untreated.

Conclusions: Our results suggest that the content of MPs is affected by the microenvironment. In particular, in neurodegenerative disease, myeloid shedding vesicles may be a solid marker for disease status and response to therapies. Thus, the characterization of shedding vesicles in human monocytes that we are performing is important.

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Allelic combinations of immune response genes as possible composite markers of optimal interferon-beta efficacy in Russian multiple sclerosis patients

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Objective: Interferon-beta (IFNb) is widely used as a first-line disease-modifying treatment for multiple sclerosis (MS). However, a significant share of MS patients experiences modest benefit from IFNb treatment. Carriage of certain genetic variants affecting the clinical response to IFNb may allow predicting optimal response in MS patients prior to treatment initiation. The study aimed to analyze an association of the immune response genes polymorphisms, which mainly code proteins mediating IFNb action, with IFNb treatment response. This study emphasizes the exploration of gene variants that jointly provide better and more accurate prediction on IFNb response than individual genes do.

Methods: Unrelated 253 Russian MS patients treated with IFNb were studied. Event-free patients who were receiving IFNb treatment no less than 2 years were considered as optimal responders, other patients were considered as non-optimal responders. We have performed association analyze of polymorphisms in the functionally significant regions of the following candidate genes: *IFNB1* (153T>C, rs1051922), *IFNAR1* (16725G>C, rs1012335), *IFNG* (874T>A, rs2430561), *TNF* (−308G>A, rs1800629), *TGFB1* (−509C>T, rs1800469), *IL7RA* (C>T in the exon 1, rs6897932), *CCR5* (w>del32, rs333), *CTLA4* (49A>G, rs231775) and *DRB1* HLA class II. The allelic combinations, in which carriage was associated with optimal IFNb treatment response, were identified using APSampler algorithm with following independent validation by means of the exact Fisher's test and the permutation test.

Results: The carriage of *TGFB1**-509C and *CCR5**d alleles was associated with optimal IFNb response by itself. Carriage of triallelic combinations (*CCR5**d + *IFNAR1**G + *IFNB1**T/T) or (*CCR5**d + *IFNAR1**G + *IFNG**T) is beneficial for IFNb treatment efficacy ($p_{perm} = 0.017$ and 0.035, correspondingly) and increases the odds of favorable outcome to 14.3- and 2.8-fold times, correspondingly. The strongest indication for epistasis is observed in the interaction of *CCR5**d, *IFNAR1**G and *IFNB1**T/T in above triallelic combination, whereas the additive mechanism was observed for *CCR5**d, *IFNAR1**G and *IFNG**T allelic set.

Conclusions: The results of the present study suggest that the influence of immune response genes on individual IFNb response has a cumulative nature. Indicated triallelic combinations may be considered as composite markers, which carriage is beneficial for IFNb treatment response in Russian MS patients.

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Comprehensive identification of soluble factors involved in mesenchymal stem cells-mediated neuroprotection utilizing the SILAC approach

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Objective: Mesenchymal stem cells (MSCs) display remarkable neuroprotective properties during the autoimmune attack to the central nervous system characterizing the experimental autoimmune encephalomyelitis. Their mechanism of action is believed to depend mainly on the release of soluble factors, the identities of which is still mainly unknown. This study aims at identifying the molecules of the MSCs secretome mediating the neuroprotective effect utilising a proteomic technique named Stable Isotope Labelling with Amino acids in cell Culture (SILAC).

Methods: The complete spectra of proteins secreted by MSCs in presence or absence of the inflammatory stimulus interferon-γ (IFN-γ), were identified through the SILAC, based on the metabolic incorporation of isotopic "heavy" $^{13}C_6$-Lysine and $^{13}C_6$-Arginine or "light" $^{12}C_6$-Lysine and $^{12}C_6$-Arginine into the proteins expressed by IFN-γ primed or unprimed MSCs, respectively. The spent medium of each condition was collected and the proteins identified and quantified by nLC-ESI-Q-TOF MS/MS mass spectrometry. The signaling pathways and the biological processes mostly represented in the two conditions were dissected utilizing the Ingenuity Pathways Analysis software.

Results: Analysis of the IFN-γ primed MSCs in comparison to unprimed MSCs highlighted an increased expression of proteins directly involved in the neurotrophic, anti-apoptotic and anti-proliferative effects. Moreover, proteins belonging to the IFN-γ signaling, protein synthesis and cell metabolism were up-regulated. Analysis of unprimed MSCs spent medium identified proteins that belong not only to the extracellular compartment but also to intracellular compartments.

Conclusions: Utilizing a proteomic technique, we are elucidating in a single step multiple molecular pathways induced in MSCs under inflammatory conditions. We found an enhanced expression of proteins involved in the neuroprotective and anti-inflammatory effects and of other proteins not directly related to these effects. The presence of proteins belonging to the intracellular compartments in media from MSCs cultures suggests that the mechanism of action of MSCs is, at least partially, based on the release of membrane vesicles of endocytic origin. Further functional assays and a comparative analysis with the spent medium from a control cell line are ongoing to define in detail the molecular mechanism of MSCs involved in their neuroprotective effect.

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Gene expression analysis implicates monocytes in the pathogenesis of all clinical subtypes of multiple sclerosis

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Objective: Multiple sclerosis (MS) is a heterogeneous, chronic, immune-mediated disease of the central nervous system. Previous studies have indicated differences in the pathogenesis in relapsing-remitting (RRMS), secondary progressive (SPMS) and primary progressive (PPMS) disease, but it is controversial if all subtypes are associated with systemic immune activation. The aim of our study was to compare gene expression in peripheral blood mononuclear cells (PBMCs) in RRMS, SPMS, PPMS and healthy controls (HC).
Methods: RNA was extracted from PBMCs from 18 HCs, 18 RRMS patients, 18 PPMS patients and 17 SPMS patients. In addition, gene expression was studied in six immunomagnetically purified PBMC subsets. For analysis on Affymetrix Human Gene 1.0 ST arrays with 28,869 genes and 764,885 distinct probes, RNA was amplified, labeled and hybridized on the microarray. The arrays were scanned on the Affymetrix GeneArray 3000 scanner. Gene expression values were extracted from CEL-files by one step probe set summarization in the Partek Genomics Suite 6.5.
Results: We found 406 genes that were differentially expressed with a false discovery rate (FDR) $<5\%$ in RRMS, PPMS, SPMS and HC. 197 of the genes were upregulated, and 209 where downregulated compared to HC. There were no statistically significant differences in gene expression when comparing SPMS, PPMS and RRMS (Bonferroni-corrected p-values >0.05). Primary component analysis showed that HCs and MS patients clustered separately, but there was no clustering of the MS subtypes along the first two principal components.

Ingenuity Pathway Analysis (IPA) of differentially expressed genes revealed that: RRMS patients had activation of the canonical pathways: "T helper cell differentiation" and "ICOS-ICOS-L Signaling in T helper cells"; SPMS patients had activation of "TREM1 Signalling" and "LXR/RXR Activation"; PPMS patients had activation of "IL-6 Signaling", "MIF Regulation of Innate Immunity and "IL6 Signaling in Gastric Cells". Finally, bout onset patients (RRMS and SPMS) had activation of "Altered T Cell and B Cell Signaling in Rheumatoid Arthritis", whereas patients with PPMS or SPMS had activation of "Communication between Innate and Adaptive Immune cells". The majority of differentially expressed genes were mainly expressed in monocytes, and we found differential gene also in purified monocytes in MS.
Conclusions: Changes in gene expression in PBMCs exist in all subtypes of MS. We confirm that RRMS is primarily associated with activation of the adaptive immune system, whereas PPMS is associated with innate immune activation. SPMS appears to be an intermediate phenotype with features of both innate and adaptive immune activation. Most differentially expressed genes were mainly expressed in monocytes, suggesting a pivotal role of moncytes in all subtypes of MS.

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Microarray analysis of the astrocyte transcriptome in normal appearing white matter in multiple sclerosis

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Objective: Multiple sclerosis (MS) is a chronic, inflammatory demyelinating disease of the central nervous system (CNS). Typical white matter lesions in MS are surrounded by areas of non-demyelinated normal appearing white matter (NAWM) with complex pathology, including blood brain barrier (BBB) dysfunction, axonal damage and glial activation. Astrocytes, the most abundant cell type within the CNS, may respond and/or contribute to several neurodegenerative pathologies including MS. We aimed to investigate the transcriptomic profile of astrocytes in NAWM to determine whether specific glial changes exist in the NAWM compared with control white matter, which may contribute to lesion development or prevention of disease progression.
Methods: Laser capture microdissection (LCM) in combination with immunohistochemistry (IHC) was used to isolate an enriched population of $GFAP^+$ astrocytes from snap frozen, post-mortem control WM (5 cases) and NAWM (5 cases), obtained from the MS Society Tissue Bank. RNA was extracted from approximately 1000 $GFAP^+$ cells and target labelled anti-sense RNA was amplified and hybridised to Affymetrix Human Genome U133 Plus 2.0 gene microarrays. The chips were scanned using a GC30007G scanner and the data processed using the GCOS and GeneSpring 11x software (Agilent). The Database for Annotation Visualisation and Integrated Discovery (DAVID) was used to group genes according to their function and identify functional pathways. Validation was carried out on selected candidate genes using polymerase chain reaction (qPCR) and IHC on 10 additional control and MS cases.
Results: $GFAP^+$ astrocytes isolated from MS NAWM showed a significant upregulation of a number of genes involved in oxidative stress and iron metabolism, including metallothionein I–II, ferritin and transferrin, compared to astrocytes from control cases.
Conclusions: Altered regulation of iron homeostasis and response to oxidative stress in NAWM may reflect adaptive changes associated with the pathogenesis of multiple sclerosis white matter lesions.

Pathology

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Decreased astrocytic response in streptozotocin-induced diabetes after gliotoxic lesion in the rat brainstem

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Objective: It is widely described that ethidium bromide (EB) injection into the CNS induces local oligodendroglial and astrocytic death, resulting in demyelination, blood–brain barrier disruption and Schwann cell invasion. Surviving astrocytes present a vigorous reaction around the injury site with increased immunoreactivity to glial fibrillary acidic protein (GFAP). Hyperglycemia in diabetes mellitus causes morphological and functional changes in peripheral neurons and Schwann cells. Delay on oligodendrocyte remyelination was pointed in streptozotocin-diabetic rats after EB injection in the CNS, but astrocytic response was not investigated. Thus, the aim of this study was to evaluate the effect of diabetic hyperglycemia on the expected astrocyte repairing behaviour (estimated through GFAP expression) after EB injection.
Methods: Adult male Wistar rats were used and some received a single injection of streptozotocin (50 mg/kg) in 0.01 M citrate buffer into the tail vein to induce diabetes. Ten microlitres of 0.1% EB were locally injected into the cisterna pontis of diabetic (group I) and non-diabetic (group III) animals. The same was done using 0.9% saline solution (group II, with diabetic rats, and IV, with non-diabetic rats). Groups V (diabetic) and VI (non-diabetic) did not receive any intracisternal injection and were used as control groups. All rats were perfused by the heart with buffered 10% formaldehyde at 15 and 31 days after intracisternal injection and GFAP immunohistochemical staining was done by the avidin-biotin complex method (rabbit anti-cow GFAP antibody, ZO334, Dako, 1:1000). Astrocytic evaluation was done using a computerized image analysis system, measuring by colorimetry GFAP-stained areas. Mean areas of each group were compared.
Results: In both diabetic and non-diabetic groups GFAP-stained areas were significantly greater in EB-injected rats than in saline-injected or control animals, but were smaller in the diabetic rats (group I, $41669{,}63 \pm 7204{,}08$) in comparison to the non-diabetic ones (III, $55354{,}38 \pm 5825{,}37$; $p = 0.001$). As for diabetic rats compared to non-diabetic, no difference was found between control groups (V and VI) and saline injected groups (II and IV) nor between diabetic groups II and V and non-diabetic groups IV and VI.
Conclusions: Results show that diabetes hindered the astrocytic increase of GFAP expression in the EB-injected group compared to the non-diabetic one, but did not affect GFAP response in the saline-injected group or in diabetic control animals.

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Dexamethasone decreases normal expression of GFAP in astrocytes of dogs

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Objective: Expression of glial fibrillary acidic protein (GFAP) has become a prototypical marker for immunohistochemical identification of astrocytes, the most numerous glial cells in the nervous tissue and that exhibit receptors to several steroid hormones, which are possibly capable of affecting astrocyte genic expression. Corticosteroids are widely used in clinical practice as anti-inflammatory and immunosuppressive drugs under different therapeutic protocols and apparently exert unclear effects on central nervous system (CNS) glial cells. The aim of this investigation was to evaluate if dexamethasone (DX), a potent synthetic member of the glucocorticoid class of steroid drugs largely administered to humans and animals, would be capable of influencing the astrocytic expression of GFAP.
Methods: Samples from the pons and from the spinal cord (first thoracic segment - T1) of dogs, treated or not with DX, were submitted to GFAP immunohistochemical staining and astrocytic reactivity was determined by colorimetry in a computer system for image analysis. The dogs used in the study died by different causes and belong to distinct breeds, ages and sexes. Tissue samples were collected and used according to previously established criteria related to antigenic preservation. Some dogs had received DX in their last 15 days of life (with doses ranging from 1 to 3 mg/kg, by intramuscular route, at regular administration intervals of 4 to 6 hours, during a maximum period of 7 days) and some were clinically healthy dogs, without any macroscopic or microscopic detectable lesion in the CNS and no history of drug administration 15 days prior to death.
Results: Difference statistically significant ($p \leq 0.05$) was noted for the mean areas stained with GFAP in the pons of dogs treated (6222.4 ± 1399.7) and non-treated (8752.6 ± 566.61) with DX, as well as in the spinal cord of dogs that previously received the corticoid (7738.5 ± 1153.7) or not (11785 ± 1901.9), showing a strong tendency of drug-induced reduction in the astrocyte expression of GFAP. No difference was seen between the two regions (pons versus spinal cord) within the groups treated with DX and not treated.
Conclusions: Thus, results demonstrate that DX administration decreases normal GFAP expression in astrocytes, suggesting that corticosteroids may affect in a negative manner astrocytic repairing behaviour and may prevent or reduce glial scar development following injury.

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Inclusion body myositis: Nitric oxide mediates inflammatory cell stress in skeletal muscle

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Objective: Sporadic inclusion body myositis (sIBM) is the most common acquired myopathy in older patients and no treatment is available to halt the continuous decline in muscle strength. Its pathology consists of a complex network of inflammatory and degenerative pathomechanisms and recently a specific interrelationship between IL-1β and accumulation of β-amyloid has been demonstrated. In the brain of experimental dementia, blockade of nitric oxide (NO) has been shown to reduce protein aggregation and ameliorate the disease course.
Methods/results: Quantitative PCR revealed a significant overexpression of inducible nitric oxide synthase (iNOS) in muscle biopsy samples from patients with sIBM compared to disease controls including dermatomyositis, polymyositis, muscular dystrophy and non-myopathic muscle. By immunohistochemistry, protein expression of iNOS was confirmed in sIBM muscle, whereas the signal remained much lower in the disease controls. Staining for nitration of tyrosine residues identified intrafiber production of NO in sIBM muscle and its signal significantly correlated and co-localized with accumulations of β-amyloid. *In vitro* exposure of human myotubes or myoblasts to interleukin (IL)-1β plus interferon (IFN)-γ induced a several-fold upregulation of iNOS at the mRNA- and protein level. Flow cytometry of myoblasts demonstrated an elevated intracellular production of NO upon IL-1β + IFN-γ as evidenced by dichlorofluorescein and diaminofluorescein, fluorescent detectors of reactive oxygen species and NO. By immunocytochemistry and fluorescent microscopy, intracellular nitration of tyrosine in muscle cells co-localized with amyloid precursor protein, but not with desmin. Subsequent necrotic cell death of muscle cells upon IL-1β + IFN-γ was evidenced by flow cytometry and cytochemistry; a similar morphology and magnitude of cell death was induced by exogenous NO, which was

released from chemical donors such as DETA. Specific inhibition of iNOS by 1400 W reduced the cytokine-induced production of NO as well as the nitration of tyrosine and prevented accumulation of β-amyloid and cell death.
Conclusion: Taken together the data suggest that iNOS is an important molecule within the network of pathomechanisms in sIBM and may be a key mediator of IL-1β-induced accumulation of β-amyloid in muscle cells. The results may help to design alternative treatment strategies in chronic myositis.

Psychoimmunomodulation

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Neuropsychiatric and cognitive dysfunctions are associated with early stages of experimental allergic encephalomyelitis (EAE), a mouse model of multiple sclerosis

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Objective: Multiple sclerosis (MS) is often associated with co-morbid neuropsychiatric and cognitive impairments, affecting around 50% of MS patients. Herein, we investigated these abnormalities in an animal model of MS, called EAE, during the presymptomatic stage of the disease.
Methods: EAE was induced in 8–10 week old C57BL/6 mice by injecting myelin oligodendrocyte glycoprotein peptide (MOG_{35-55}) with complete Freund's adjuvant (CFA) subcutaneously and pertussis toxin (PTX) intraperitonially. Mice injected with saline only or, CFA and PTX were used as naïve or sham controls, respectively. Animals were scored daily for assessment of motor deficits associated with EAE after disease induction and showed no motor deficits until d9. A series of neurobehavioral tests, namely, open field test, elevated plus maze (EPM), forced swim test (FST), tail suspension test (TST), fear conditioning and Morris water maze test (MWM), were performed between d6–d8 after EAE induction. Plasma cytokine levels were mesured by luminex multiplex array, while brain cytokine transcripts were measured by semi-quantitative RT-PCR. Corticosterone levels were measured by ELISA. Luxol-fast blue staining was used to study the extent of demyelination.
Results: EAE mice spent more time in the outer zone of open-field and in the closed arms of EPM, and showed decreased latency for immobility in TST and FST compared with sham and naïve controls. All these results were indicative of anxiety-like behavior. Further, EAE mice spent less time in the target quadrant compared to sham controls during probe trial in MWM indicative of memory impairment, while displaying faster memory extinction in the fear conditioning test. Plasma levels of IL-5 increased and MIP-2 decreased in EAE mice compared to sham, whereas both sham and EAE mice displayed elevated G-CSF, IFN-ϒ, IL-6 and IL-17. Plasma corticosterone level was also significantly upregulated in the EAE mice compared to naïve and sham controls. Transcript analysis by RT-PCR from the brain revealed up-regulation of interleukin (IL)-1β in the hippocampus of both sham and EAE mice compared to naïve mice without any change in TNF-α, Arginase-1 and IL-5. MIP-2 transcript level was decreased in the hippocampus of EAE mice compared to sham. No apparent demyelination or microglial activation was observed in EAE mice at this stage.
Conclusions: In conclusion, emotional and cognitive deficits observed in the early stages of EAE (and possibly MS) may be due to neurological changes associated with altered corticosterone levels, leading to a disbalance in M1/M2 macrophage activation in the periphery and CNS, in absence of demyelination.

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Impaired spatial learning in mononeuropathic B7H1 knockout mice

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Objective: Chronic pain is a complex, multidimensional experience frequently associated with deficits in cognitive functions including memory, attention, and verbal performance. However with the practical challenge in modelling these facets in a rodent model of chronic pain, the underlying pathophysiological mechanisms remain unexplored. Cytokines have an established function in the initiation and maintenance of pain and an emerging role in cognitive processes in several pathological disorders. B7-H1 (PD-L1, CD274), a co-inhibitory molecule, attenuates T cell proliferation and cytokine production and protects from inflammatory tissue damage. Hence, mice lacking this gene provide an opportunity to study the role of cytokines in pain induced cognitive impairment.
Methods: Chronic neuropathic pain was induced in B7H1 −/− and C57Bl/6 wild-type (WT) littermates by chronic constriction injury (CCI) of the right sciatic nerve. Mechanical allodynia and thermal hyperalgesia was assessed on days 7, 14 and 20 post CCI/sham. Forced swim test (FST) to assess depression like behavior and the Morris water maze (MWM) to evaluate spatial memory and learning were performed between post-operative days 22–27. Serum, nerve and brain tissues were harvested on 28 for PCR and ELISA analysis.
Results: Mechanical withdrawal thresholds (von Frey test) were lowered in both B7H1 −/− and WT mice beginning day 7 post CCI with no significant intergroup difference. However, in the acquisition task performed in the Morris water maze, the escape latency was significantly increased in B7H1 −/− compared to WT nerve injured mice (Trail day 1, 2: $P<0.05$; trail day 3,4: $P<0.01$). A significant day by genotype interaction was also seen in the sham operated B7H1 −/− ($P<0.05$). The contribution of pro- and anti-inflammatory cytokines is being investigated.
Conclusions: The present findings demonstrate that B7H1 −/− CCI mice are impaired in their spatial cognitive abilities. The altered cytokine profile observed in B7-H1 knockouts following nerve injury, with an increase in the pro-inflammatory and reduction in anti-inflammatory cytokines makes this an ideal model to elucidate the link between cytokine dysregulation and pain induced cognitive impairment.

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The sympathetic catecholamine norepinephrine increases Th17 responses in a β2-adrenergic receptor-dependent manner

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Objective: To determine the impact of the sympathetic catecholamine norepinephrine (NE, noradrenaline) on human Th17 cell responses. The sympathetic nervous system influences peripheral immune responses by triggering catecholamine release from the adrenal medulla, and from adrenergic nerve terminals that innervate secondary lymphoid tissues. Th17 cells are helper T cells (Th) implicated in pathogenesis of multiple sclerosis (MS). How catecholamines affect Th17 responses is not known. In T cells, NE signals through the

β2-adrenergic receptor (β2-AR), a G-protein coupled receptor that induces the second messenger cAMP.
Methods: Purified, primary human memory T cells were differentiated into Th17 cells using a cocktail of factors including anti-CD3, anti-CD28, anti-IFNγ, anti-IL-4, and recombinant IL-23, in the absence or presence of NE, or the specific β2-AR agonist terbutaline. As a control, forskolin was used, a drug that directly activates cAMP production. Th17 responses were measured by intracellular cytokine staining and enzyme-linked immunosorbent assay.
Results: The proportion of Th17 cells increased 6 fold in the presence of terbutaline ($p = 0.014$), as did the proportion of a hybrid subset expressing IL-17A and IFNγ (denoted Th1/17). The level of soluble IL-17A cytokines showed a corresponding increase in the supernatants of T cells exposed to NE or terbutaline ($p = 0.034$). In contrast, forskolin potently inhibited Th17 responses by over 90% ($p = 0.030$). Th1 cells were not significantly affected by the β2-AR agonists.
Conclusions: Surprisingly, human Th17 cell responses were increased by the β2-AR agonists NE and terbutaline. β2-AR-signalling is generally thought to be inhibitory towards T cells, suggesting that Th17 cells have differences in downstream signal transduction pathways that produce stimulatory signals. This data has implications when designing rehabilitation programs for patients suffering from Th17-mediated autoimmune conditions such as MS. Stress or intense exercise can acutely elevate catecholamine levels, thus, these situations may promote Th17-bias responses.

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Dietary intervention with omega-3 polyunsaturated fatty acids restores decreased dopaminergic activity in the prefrontal cortex of cow's milk allergic mice

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Objective: The dopaminergic system in the prefrontal cortex (PFC) is known to modulate attentional skills, organization of thought and perception and social behavior. Supporting these findings, decreased levels of dopamine in the PFC are implicated to be involved in schizophrenia, ADHD and autism. Besides an altered dopaminergic system, disturbed levels of polyunsaturated fatty acid (PUFA) in blood have also been observed in these psychiatric disorders. Beneficial effects of PUFA on behavior of schizophrenia, ADHD and autism have been described, in both human and animal studies. However, larger studies are required to confirm these effects and little is known about the pathway in which PUFA can modulate behavior.

We set up a mouse model for cow's milk allergy (CMA) that exerts aberrant social behavior and altered dopaminergic activation in the PFC. The aim of this study was to investigate whether dietary intervention with PUFA can modulate altered dopamine levels in the PFC, which are associated with behavior in these allergic mice.
Methods: Male C3H/HeOuJ mice were orally sensitized with whey protein and cholera toxin (CT) or CT alone, once a week for 5 weeks and subsequently orally challenged with whey protein once. Starting two weeks before first sensitization, mice were kept on an omega-3 PUFA diet or control diet. The morning after challenge, mice were sacrificed and brains were collected. Prefrontal cortex (PFC) was isolated and dopamine levels were determined by using HPLC.
Results: Dopamine levels were significantly decreased in PFC of CMA mice compared to controls. A dietary intervention with omega-3 PUFA completely restored the levels to those found in control (non-allergic) mice. Levels of three metabolites of dopamine (HVA, 3-MT and DOPAC) were also significantly decreased in the PFC of CMA mice. The omega-3 PUFA diet also restored these metabolites to control levels.
Conclusions: Cow's milk allergy in mice caused a significant decrease in dopamine and its metabolite levels in the PFC and a dietary intervention with omega-3 PUFA restored the levels to those found in control mice. Therefore, PUFA possibly exerts its beneficial effect on normalizing behavior via modulation of the dopaminergic system in the PFC and might offer a dietary intervention in some psychiatric disorders.

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Neuropsychological changes in a mouse model for cow's milk allergy

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Objective: The gut-brain axis has been implicated in various psychiatric disorders and a role for food allergy in autism has been suggested. However, little is known about the effects of food allergy on behavior and brain functioning. The aim of this study was to investigate in a murine model whether cow's milk allergy (CMA) affects behavior and neuronal activation in the brain.
Methods: Male C3H/HeOuJ mice were orally sensitized with whey protein and cholera toxin (CT) or CT alone, once a week for 5 weeks and subsequently orally challenged with whey protein once. The morning after challenge a social behavior test was performed whereupon mice were perfused and brains were collected. Brains were sectioned into 40 μm coronal slices and stained for c-Fos to determine neuronal activation.

A murine model for colitis was used as a control. Male C57BL/6 mice received 2% DSS in their drinking water for 5 consecutive days, followed by 2 days of normal drinking water. At day 7 a social behavior test was performed.
Results: Reduced social behavior was observed in whey-sensitized mice compared to sham-sensitized mice. Altered social behavior was not observed in DSS-colitis mice. Moreover, in the brain of CMA mice, a different pattern of neuronal activation was found. Neurons in the orbital prefrontal cortex (oPFC) were almost ten times more activated in CMA mice compared to control mice, while regions in the medial prefrontal cortex were unaffected. Neuronal activation in the paraventricular nucleus (PVN) of the hypothalamus of CMA mice was significantly decreased compared to control mice.
Conclusions: Cow's milk allergy in mice caused reduced social behavior. Alteration in social behavior was not observed in a murine model for DSS-induced colitis, indicating that this observation does not result from sickness behavior induced by gastrointestinal discomfort. Furthermore, neuronal activation in the oPFC and PVN of CMA mice was altered compared to control mice. Behavioral alterations in CMA mice are possibly induced by aberrant neuronal activation in the oPFC and PVN. Knowledge on how food allergy can cause neuropsychological changes might offer possibilities for treatment of psychiatric disorders such as autism, where the gut-brain axis potentially plays a role.

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Serotonin reuptake inhibitors as immunomodulators: A proof-of-concept

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Objective: Although serotonin reuptake inhibitors (SRIs) are generally regarded as safe drugs with few side effects, data suggest that they may compromise cellular immune responses. The objective of this study was to prospectively investigate the T cell immunosuppressive effects of SRIs in obsessive compulsive disorder (OCD) patients treated with high doses of SRIs and to analyze *in vitro* whether the observed results are due to a direct effect of SRIs on T cell proliferation and viability. In addition, we used a murine model of graft-versus-host disease (GvHD) to determine if SRIs can inhibit in vivo allo-antigen induced T cell responses.
Methods: We took blood samples of twenty patients suffering from OCD before and after a twelve-week treatment with high doses of paroxetine or venlafaxine and analyzed the *ex vivo* proliferative response to T cell mitogens. We analyzed the direct effects of six different SRIs (paroxetine, fluoxetine, sertraline, citalopram, fluvoxamine and venlafaxine) on *in vitro* viability and proliferative responses of peripheral blood T cells from healthy volunteers ($n=6$). The effect of fluoxetine on murine GvHD was determined in a MHC-matched, minor HA-mismatched bone marrow transplantation (BMT) model ($n=13$ from two experiments).
Results: While all patients showed normal T cell proliferative responses before SRI treatment, we found a completely absent response after treatment in 5 out of 20 patients. After discontinuation of the treatment, aberrant proliferative responses returned to normal. *In vitro* results revealed an anti-proliferative effect of all compounds tested and a pro-apoptotic effect of paroxetine, fluoxetine, sertraline, fluvoxamine and citalopram. Interestingly, we found that the pro-apoptotic effect was significantly stronger in activated than in naïve T cells, indicating a higher sensitivity of activated T cells to SRI-induced apoptosis. In the murine BMT model, fluoxetine significantly reduced the clinical severity of GvHD relative to vehicle-treated control animals.
Conclusions: These results show that SRIs can suppress T cell responsiveness in OCD patients and that this effect is due to a direct anti-proliferative and pro-apoptotic effect on T cells. In addition, this study provides evidence for a beneficial effect of SRIs on the severity of murine GvHD. Given their favorable and well characterized safety profile, we conclude that SRIs may hold therapeutic potential as a novel class of T cell immunosuppressants.

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Prior restraint stress induced different effects on the inflammatory response to LPS in the hippocampus and the amygdala

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Objective: Stress prior to a bacterial challenge can enhance the production and secretion of proinflammatory cytokines in different brain areas (e.g., hippocampus, frontal cortex). However, several studies have revealed that stress effects on the brain are not homogeneous. Recent findings on stress-induced structural plasticity in rodents have identified opposite effects between the hippocampus and amygdala. The following study was designed to test the hypothesis that stress effects on pro- and anti-inflammatory cytokines are different between the amygdala and hippocampus.
Methods: Male Sprague–Dawley rats were exposed to 4 sessions of restraint stress, 4 h each (versus homecage controls), and inoculated with 100 mg/kg of LPS 24 h after the last stress session. Animals were sacrificed and perfused with PBS 2 h after the LPS inoculation. Hippocampus and amygdala were collected and assayed for relative IL-1β, IL-6, TNFα, BDNF, IL-4, IL-1ra, IL-10 and C-fos mRNA expression using PCR.
Results: In the hippocampus, prior restraint stress increased gene expression of the proinflammatory cytokines IL-1β and TNFα with no changes in IL-6. The anti-inflammatory cytokine IL-1ra was decreased, while IL-4 and IL-10 showed no change. BDNF and c-fos were reduced. Similarly, in the amygdala, we observed a reduction in IL-1ra and c-fos and no changes in IL-10. On the contrary, we observed in the amygdala a decrease of the pro-inflammatory cytokines IL-1β and IL-6, and the anti-inflammatory IL-4. As opposed to the hippocampus, TNFα and BDNF gene expression did not change in the amygdala.
Conclusions: Prior restraint stress induced opposing effects on the proinflammatory response to LPS in the hippocampus and the amygdala but similar effects on the anti-inflammatory response in the two areas. The neuronal activation, measured by c-fos mRNA expression, showed no regional differences, suggesting that the neuronal activation is not altered or contributing to the stress-induced differences in proinflammatory effects. Finally, the reduced gene expression of BDNF in the hippocampus, but not in the amygdala following stress points to a possible divergent role of stress-induced structural plasticity when followed by infection.

These findings warrant further examination in order to determine 1) if stress alters the immune response differently in the hippocampus and the amygdala and 2) if those alterations are relevant for cognitive and affective symptoms associated with stress.

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Rajbangshi tribe group in Bangladesh: Their knowledge on central nervous systems and possible role in primary health care

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Objective: Traditional medicines are used to treat central nervous systems, which have often been viewed as culturally specific problems, and so these treatments have been seen as effective only in the specific context. Periodic field investigations were organized in connection to different seasons during the year 2010–2012 in different parts of Bangladesh.
Methods: The information were collected through the dialogue, discussion, and arranged meetings with the Rajbangshi tribe group, who have sufficient knowledgeable of the plants. The plants were identified with the help of floras. Information was collected through interview with old people aged among 40–80, who had the traditional knowledge of plants.

To determine the authenticity of information collected during field work, the data were cross-checked from different informants. Thus, only the specific and reliable information cross-checked with at least 12 informants have been incorporated. The information provided by

the Rajbangshi tribe group has been compared with the published literature.
Results: Information on 39 plants was obtained. These plants included *Abrus precatorius* L., *Achyranthes aspera* L., *Adenanthera pavonina* L., *Alangium salviifolium* (L.f.) Wangerin, *Aloe vera* (L.) Burm.f., *Amorphophallus konjac* K.Koch, *Argyreia nervosa* (Burm.f.) Bojer, *Azadirachta indica* A.Juss., *Bacopa monnieri* (L.) Wettst., *Bixa orellana* L., *Calotropis procera* (Aiton) Dryand., *Camellia sinensis* (L.) Kuntze, *Cannabis sativa* L., *Cheilocostus speciosus* (J.König) C.Specht, *Crataeva tapia* L., *Datura metel* L., *Euphorbia royleana* Boiss., *Flacourtia indica* (Burm. f.) Merr., *Ficus benghalensis* L., *Hemidesmus indicus* (L.) R.Br. ex Schult., *Ipomoea aquatica* Forssk., *Lawsonia inermis* L., *Linum usitatissimum* L., *Luffa acutangula* (L.) Roxb., *Mimosa diplotricha* Sauvalle, *Nerium oleander* L., *Nicotiana tabacum* L., *Nigella sativa* L., *Ocimum gratissimum* L., *Oroxylum indicum* (L.) Kurz, *Orthosiphon aristatus* (Blume) Miq., *Passiflora coccinea* Aubl., *Piper cubeba* L.f., *Pterygota alata* (Roxb.) R.Br., *Ricinus communis* L., *Semecarpus anacardium* L.f., *Solanum virginianum* L., *Wedelia chinensis* (Osbeck) Merr., and *Withania somnifera* (L.) Dunal.
Conclusions: The investigation is new of its kind with the aim to document and widespread the hidden knowledge of the Rajbangshi tribe group and their practices towards central nervous systems of Bangladesh, is of very much significant for the biochemists and pharmacologists for further scientific research to develop new pharmaceutical preparations.

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Sleep influences the immune response: Evidence from a skin allograft

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Objective: Sleep impacts immune parameters that are directly involved in the allograft rejection process. We chose a skin allograft as an immune challenge to assess whether the rejection process could modulate the sleep pattern of transplanted mice because of the bidirectional communication between the nervous and immune systems. Conversely, we also investigated whether paradoxical sleep deprivation (SD) or sleep restriction (SR) would affect the allograft rejection.
Methods: Adult (25–30 g) BALB/c and C57BL/6 J mice were used as donors and recipients, respectively. The recipients were underwent SD for 72 h before and after the transplantation or to SR for 15 days by the modified multiple-platform method or transplanted and maintained in their home-cage. The SR protocol consisted of 21 hours of SD and 3 h of sleep. The skin allograft was inspected daily to determine the survival time and the sleep pattern was determined during all the rejection process. Spleen, blood and skin grafts were harvested on the 5th day after transplantation for cell counting and flow cytometry analysis of T cells, IL-2 and sIL-2R (Luminex), corticosterone (Radioimmunoassay) and skin grafts histopathological analysis and global gene expression. A general linear model (GLM) was conducted for analysis, using the group as the main factor.
Results: We observed a reduction in the paradoxical sleep throughout the entire allograft rejection process. Both SD and SR prolonged allograft survival compared to the non-sleep-deprived group due to reductions in $CD4^+$ and $CD8^+$ T cells subpopulations, graft-infiltrating $CD4^+$ T cells, graft global gene expression, MHC class II expression and sIL-2R levels. Moreover, there was an increase of spleen $CD4^+Foxp3^+$ T cells in SD groups compared with TX group. No significant differences were observed regarding the corticosterone levels.
Conclusions: These evidences reinforce the existing hypothesis of the importance of sleep in immune response integrity and development since sleep deprivation induced significant effects on immune parameters such as cellular immune response, cytokine receptor and local inflammatory response. Sources of research support: FAPESP (#07/55445-6), CNPq and Associação Fundo de Incentivo à Psicofarmacologia (AFIP).

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Are anxiety behaviour and oxidative stress interrelated in the early phase of experimental arthritis in rats?

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Objective: The impact of stress and depression in rheumatoid arthritis (RA) has been confirmed. Yet, anxiety is a relevant area to examine in arthritis as it increases rheumatic pain sensations, and predisposes to stress reactions. Unpredictable disease course, chronic pain, and movement limitations can all be a cause of anxiety in arthritis. Recent knowledge however points at crucial role of oxidative stress. Anxious rheumatic patients express enhanced amounts of blood free radicals and lower activity of antioxidant systems. They also tend to develop neuro-inflammation. The aim of this study was to examine anxiety behaviour, and oxidative stress alterations in hippocampus on animal model of RA, adjuvant arthritis (AA), during its early phase what enables to avoid influence of pain, and movement impairments on anxiety behaviour.
Methods: AA was induced to Lewis rats at 7 weeks of age by a single subcutaneous injection of 100 μl of complete Freund´s adjuvant. Two intervals of early arthritis were studied (day 2 and 4). Anxiety behaviour was tested in the elevated plus maze apparatus, and in the open field. Oxidative stress changes in the hippocampus were evaluated by quantitative real-time PCR. A two-way ANOVA with repeated measures on factor day was used for statistical calculations.
Results: In arthritic rats, open arm entries, and time were lower on both days ($F_{(1,25)}=9.14$ $p<0.01$ $F_{(1,25)}=4.63$ $p<0.05$). In the same disease intervals, open arm entries and time related on their total values decreased in arthritic rats ($F_{(1,25)}=7.14$ $p<0.02$ $F_{(1,25)}=5.21$ $p<0.05$) indicating higher anxiety index in these group. Furthermore, arthritic rats spent less time in the central area of the open field, and ambulated less in the central circle on both days ($F_{(1,11)}=5.87$ $p<0.05$ $F_{(1,11)}=4.05$ $p<0.05$). Hippocampal nicotine amide adenine dinucleotide phosphate (NADPH) oxidase 1 mRNA was up-regulated on both arthritis days (AA 2, and AA 4 vs. controls: $p<0.05$). Hippocampal mRNA for IL-1β showed a mild up-regulation on day 2 with more pronounced increase on day 4 of AA (AA day 2 vs. controls: $p<0.05$ AA day 4 vs. controls: $p<0.001$).
Conclusions: In the early phase of arthritis, anxiety behaviour was accompanied with oxidative stress alterations in the hippocampus. Interestingly, mRNA expression of IL-1β and of NADPH oxidase 1 correlated what indicates that neuro-inflammation, and oxidative stress are interrelated, and both cohere with anxiety.
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Genetics

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Parent-of-origin effects affect inheritance of multiple sclerosis-like neuroinflammation in rats and implicate novel risk genes

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Objective: Parent-of-origin effects in multiple sclerosis (MS) suggest an epigenetic component in disease etiology. Epigenetic mechanisms refer to heritable changes in gene expression that do not require changes in the DNA sequence. We sought to establish the magnitude of contribution of parent-of-origin effects to an MS-like disease, experimental autoimmune encephalomyelitis (EAE), and to identify novel risk genes that are under epigenetic regulation.
Methods: We investigated the impact of parent-of-origin on EAE susceptibility using a reciprocal backcross strategy designed to identify the parental origin of disease-predisposing alleles. Genome-wide expression in reciprocal backcross rats and allele-specific expression in reciprocal hybrids were used to facilitate identification of genes that mediate parent-of-origin effects. The impact of candidate genes on the immune system and EAE development was investigated in reciprocal congenic rats and transgenic mice with flow cytometry and quantitative PCR.
Results: More than 30% of all detected disease-predisposing loci depended on parental transmission. Accounting for parental origin enabled identification of novel risk factors and increased up to 4-fold disease variance explained by identified factors, revealing an important source of '*missing heritability*'. The majority of loci displayed imprinting-like pattern, whereby a gene expressed only from the maternal or paternal copy exerts an effect. Accordingly, several identified loci, comprise well-known clusters of imprinted genes. Paternally inherited alleles at *Eae9* conferred increased EAE risk and lower expression of *DLK1*, which agrees with known paternal expression of genes in the *Dlk1-Dio3* locus. $Tg^{Dlk1\text{-}70}$ BAC mice (da Rocha et al., 2009) suggested involvement of *Dlk1* in the immune system development and EAE severity.
Conclusion: We demonstrated a significant contribution of parent-of-origin in the etiology of MS-like disease, suggestion involvement of epigenetic mechanisms. Moreover, we defined locations in the genome, including *Dlk1*, which are regulated by epigenetic mechanisms and might provide molecular clues to gene-environment interactions in etiology of neuroinflammation.

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High resolution genetic mapping of the cytokine response in the rat heterogeneous stock

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Objective: Inflammation is by definition orchestrated by cytokines. Therefore, understanding their genetic regulation is important for the understanding of diseases where cytokines are key mediators, such as multiple sclerosis and its' animal model experimental autoimmune encephalomyelitis (EAE). We use a heterogeneous stock (HS) of rats, that resembles a human population, in order to identify quantitative trait loci (QTL) and genes that regulate cytokine response, and further dissect the pathogenic mechanisms underlying neuroinflammation.
Methods: HS stems from a standard outbreeding scheme of eight different rat strains bred for more than 60 generations, which leads to a high recombination density. We have collected, within the EURATools consortium, phenotypic EAE information on 2000 rats that have been SNP mapped across their genome with 500,000 SNPs. Besides the clinical evaluation of the disease, we measured 23 pro-inflammatory and anti-inflammatory cytokines using a multiplex assay based on luminex technology in blood of naïve rats after stimulation with lipopolysaccharide (LPS). In addition, other phenotypes, such as serum antibody levels will be determined by ELISA.
Results: Our preliminary genome-wide association scan identified QTLs with high resolution and narrow confidence intervals, around 1-3Megabase (Mb) containing few-to-50 genes. Furthermore, the QTLs that regulate cytokine response and overlap with QTLs for EAE phenotypes imply shared genetic variant among phenotypes. For example, we identified a QTL on chromosome 13 that regulates the pro-inflammatory cytokines TNF and IL-1β, which overlaps previously known EAE QTL. A region on chromosome 11 regulates IL-1α and IL-1β response and overlaps with QTLs regulating incidence and onset of EAE, implying that potentially there is a gene in the QTL that confers susceptibility to the disease through IL-1α/β regulation. In addition, by combining genomic sequence data from the founder strains with mapping data we can further infer causative candidate polymorphisms.
Conclusions: The rat HS is a new resource suitable for fine-mapping of QTLs due to high recombination density, and potentiates single gene identification and further dissection of the underlying autoimmune neuroinflammation pathways.

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RBP-Jk SNP is associated with differential expression of RBP-JK isoforms and induction of IL-9 in memory T cells

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Objective: RBP-Jk is the transcriptional effector of the Notch pathway and plays a crucial role in T-cell development and differentiation. Recently, meta-analysis of genome wide association studies of type 1 diabetes (T1D) and rheumatoid arthritis (RA) identified a susceptibility locus, rs10517086, which implicated the RBPJ gene. Our group and others have demonstrated that Notch/RBP-Jk plays an important role in the differentiation of naive CD4 + T-cells into regulatory and effector T cells (Elyaman et al., 2007; Bassil et al., 2011; and Elyaman et al., 2012). Here we examined the biologic phenotypes of healthy subjects selected based on being homozygous for the risk or protective allele in order to examine the pathway from genotype to phenotype.
Methods: CD4+CD45RO+memory T-cells were isolated by MACS from PBMCs of healthy donors using ficoll density centrifugation. Cells were activated with anti-CD3/CD28 (1 μg/ml) for 5 days in the presence or absence of TGF-β1 (5 ng/ml).

Cytokine expression was measured in live cells (7AAD-Negative) by flow cytometry using antibodies against IL-9 (Biolegend) on LSRII and analyzed using FlowJo software.

Gene expression was carried out on a Vii 7.0 using SybrGreen. Primers were designed to cover exon boundaries of each isoform using Primer3.
Results: We found that healthy donors homozygous for the rs10517086 risk allele have altered expression of RBP-Jk isoforms in CD4+CD45RO+ memory, but not naïve T-cells relative to control subjects homozygous for the protective allele as determined by qPCR. Indeed, isoform specific qPCR showed a 4-fold decrease in isoform NM_202283 and a 5-fold increase in isoform NM_203284 in subjects with the risk allele. As a consequence, the proliferation of memory T-cells homozygous for the protective allele increased and this was associated with an alteration of their cytokine profile marked by a specific increase in IL-9 production as measured by flow cytometry five days following polyclonal stimulation in the presence of TGF-β1. These findings are in agreement with our recent study

demonstrating high levels of IL-9 in memory T-cells from T1D subjects polarized under Th17 cell conditions.
Conclusions: We have found that CD4+CD45RO+ memory T-cells show differential expression of RBP-Jk isoforms based upon allele status and this is also associated with a specific increase in IL-9 in risk allele homozygotes. These data demonstrate that IL-9-producing CD4 cells may play a role in human autoimmune diseases.

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To investigate the functional role of ZFP36L1 in multiple sclerosis: Possible connection between brain and stomach

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Objective: Genetic and environmental factors contribute to the onset of multiple sclerosis (MS). The goal of this project is to explore the functional consequences of the MS risk variant rs4902647 in the *ZFP36L1* locus ($p = 9.3 \times 10^{-12}$) that was found in a recent large genome scan by the International MS Genetics Consortium. 8 different isoforms can be transcribed from *ZFP36L1*, which encodes for an RNA binding protein that controls the degradation of other transcripts. Inflammatory cytokines are its predicted molecular targets along with many oncogenes, such as *FOS*, *MYC*, *BCL2* and *PTGS2*. *ZFP36L1* is also known as butyrate response factor 1 (BRF1) and can be modulated by sodium butyrate (NaB), a fermentation product of the gut flora and a known histone deacetylase inhibitor (HDACi).
Methods: *Expression analysis* was conducted in pre-genotyped healthy control and MS individuals to measure the level of different RNA isoforms. *Flow cytometry* data were obtained to investigate genotypic correlation with the proliferation of $CD4^+$ T cells.
Results: In peripheral mononuclear cells (PBMC) from MS subjects ($n = 225$) and purified monocytes from healthy individuals ($n = 26$), the short isoform of the gene has a greater relative expression compared to the long isoform in the presence of the risk allele ($p = 1.8 \times 10^{-11}$; $p = 0.08$ respectively). Further, the frequency of the risk allele correlates with increased proliferation of $CD4^+$ T cell ($n = 23$, $p = 0.05$).
Conclusions: Little is known of the role of *ZFP36L1* in immune function. Our data suggest that the disease-associated variant alters the distribution of RNA isoforms, increasing the proportion of the shorter ZFP36L1 molecules that might lack a critical phosphorylation site involved in the regulation of the zinc finger binding to the 14-3-3 protein and hence in its activity. Further studies are under way to validate our observations in additional cell types and to investigate direct and indirect ways in which this alteration in transcript population could affect $CD4^+$ T cell proliferation.

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Genome-wide association of autoimmune neuroinflammation in the heterogeneous stock of rats

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Objective: To elucidate mechanisms involved in multiple sclerosis (MS), we investigated inheritance of experimental autoimmune encephalomyelitis (EAE). With the objective to capture the complexity of human disease while benefitting from the advantages of animal models, we use the Heterogeneous Stock of rats (HS). Considerable advantages of the HS include the genetic resolution, which enables identification of risk genes, and the capacity to study parent-of-origin effects, epistatic interactions and gene–environment interactions in a complex setting.
Methods: The HS is a genetic mosaic established from eight inbred strains: ACI/N, BN/SsN, BUF/N, F344/N, M520/N, MR/N, WKY/N and WN/N. Rats in the European HS colony have been bred for more than 50 generations to maximize recombination density and reduce the size of inherited haplotypes. EAE was induced by myelin oligodendrocyte glycoprotein (MOG) in 2006 HS rats. Additionally, the stress response, something that has been debated as a possible trigger of MS, and other physiological phenotypes were measured and environmental information collected. The 8 founder strains were sequenced and a targeted genotyping panel with 500,000 SNPs that enable imputation of the sequence have been completed in 1200 HS rats. Additionally, 200 of the breeding couples have been genotyped to study parent-of-origin effects to identify important routes of disease transmission. The association studies in the HS involve novel analytical methods and statistical packages developed specifically for this population. A "multiple QTL model" is fitted using a model averaging method, meaning that the association between phenotype and genotype at any one locus is corrected by the pattern of associations over the rest of the genome.
Results: We confirmed a previously identified quantitative trait loci (QTL) on rat chromosome 10 associated to EAE incidence and reduced the confidence interval from 60 Mb to 5 genes. Two additional previously known QTLs on rat chromosomes 10 and 18 were confirmed, and the HS provided a short list of candidate genes. Targeted investigations are ongoing to identify the causative genes/variants.
Conclusions: The mapping resolution provided by the HS enables rapid identification of risk genes for EAE. Further, the HS provides the unique opportunity to study genetic, epigenetic, environmental and behavioral factors that contribute to inheritance of autoimmune neuroinflammation, and by extrapolation to MS.

miRNA

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miRNA signature from five subsets of CD4+ T lymphocytes from multiple sclerosis patients in the remission phase

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Objective: To determinate the miRNA signature from nine T lymphocyte subpopulations of multiple sclerosis (MS) patients in the remission phase.
Methods: We have determined the expression level of 866 human miRNAs, using Agilent miRNA microarray technology, of Naive, Central memory, Effector memory, Effector RA memory and regulatory T Cells from CD4+ lymphocyte populations, obtained from five multiple sclerosis patients in the remission phase and five healthy donors matching for age and gender. Taqman qRT-PCR was used to validate

miRNA expression; we also made a bioinformatics approach to infer miRNA targets and their role in T cell biology.
Results: The analysis of miRNA expression by microarray revealed significant changes in all five subsets of T lymphocytes in the MS patients. Moreover, qRT-PCR confirmed ($p<0.05$) the significant up-regulation of six miRNAs (miR-517b, miR-519e-5p, miR-575, miR-1225-5p, miR-326 e miR-Let7f1) and the down regulation of four miRNAs (miR-19b, miR-24, miR-484, and miR-1295) in the MS patients. Bioinformatics prediction of the differentially expressed miRNA target genes revealed an enrichment of immune response related genes. The influence of these miRNAs on their predicted target genes appears to favors the Th17 response in all analyzed cell subsets.
Conclusions: By using this strategy we obtained a molecular characterization of miRNA expression of five subsets of T lymphocytes and revealed a possible role of these miRNAs in the immune response of MS.

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Role of miR-223 in multiple sclerosis and its animal model

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Objective: Multiple sclerosis (MS) is a complex autoimmune disease of unknown etiology involving central nervous system (CNS) inflammation, demyelination, and axonal damage. Experimental autoimmune encephalomyelitis (EAE) is the prime animal model for MS; work using this model has led to four approved therapies for MS thus far. MicroRNAs (miRNAs) are a novel class of small, non-coding RNAs discovered to regulate gene expression post-transcriptionally. They represent potential therapeutic targets as they have been implicated in the pathogenesis of several human diseases, including autoimmune diseases. MiR-223 was found to be up-regulated by microarray analysis in whole blood and active brain lesions in MS patients compared to controls. MiR-223 is expressed in myeloid cells and it modulates the NF-Kb pathway, with effects on inflammatory and immune responses. The present work will further characterize the potential role of miR-223 in the EAE model and MS.
Methods: The expression levels of miR-223 were analyzed in the peripheral blood mononuclear cells (PBMCs) of two different populations by Real Time PCR. The first cohort consisting of 20 samples from MS patients and 20 from healthy subjects and the second of 15 sample from MS patients and 12 from healthy subjects. In active EAE experiment 8 male miR-223 knock out (miR-223 KO) mice and 4 littermate control mice were immunized with the myelin oligodendrocyte glycoprotein $(MOG)_{35-55}$ peptide. In this experiment, EAE clinical course and spleenocytes cell phenotyping were compared in miR-223 KO vs control mice.
Results: We found an upregulation of miR-233 in the PBMC of MS samples vs controls (fold change over controls 1.64 ± 1.25 vs. 1.20 ± 0.95, $P = 0.018$). This result was confirmed in a different cohort of subjects; miR-233 was upregulated in MS vs control subjects (fold change over controls 0.81 ± 0.65 vs. 0.40 ± 0.26, $P = 0.010$). In active EAE experiment MiR-223 KO mice developed a significantly less severe disease ($P<0.0001$ by two-way ANOVA) with a significantly higher percentage of granulocytes/neutrophils (Ly6C/Ly6G positive cells) and eosinophils (Siglec F positive cells) compared to control mice.
Conclusions: These results demonstrate altered levels of miR 223 in the PBMC of MS patients and suggest that miR-223 plays a role in EAE. This may lead to the identification of new disease biomarkers of therapeutic targets.

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The microRNAs miR-183 and miR-9-2 target Nurr1 and SOCS2 genes, establishing a negative feedback loop that inhibits inflammation in multiple sclerosis

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Objective: Pregnancy provides an original model to investigate the regulatory mechanisms of inflammation, given that during gestation the maternal immune system strengths its anti-inflammatory component and several autoimmune diseases, including multiple sclerosis (MS), frequently go into remission. In a recent genome-wide transcriptional analysis, we identified a gene transcription signature for MS, mostly encoding negative regulators of inflammation, and reverting back to normal during gestation. Among the others, transcripts *SOCS2*, *TNFAIP3* and *Nurr1* were particularly interesting since they were reported to be associated with several autoimmune diseases and encode negative regulators of inflammatory responses.

Because miRNAs are believed to play an important role in gene expression regulation, we ought to understand the role of miRNAs in this deregulated gene pathway.
Methods: We conducted a genome-wide miRNA-seq transcriptional analysis of peripheral blood mononuclear cells (PBMCs) obtained from 5 women with MS and 5 healthy controls before and at 9th month of gestation. Results were confirmed by real time PCR in 19 women with MS and 8 healthy controls followed during pregnancy. After, we performed an integrative analysis of miRNA/mRNA expression, overlaying the present miRNA signature onto our published gene transcription signature. Expression levels were tested in PBMCs from 45 untreated MS patients.
Results: first, we identified 15 miRNAs that were up-regulated in MS patients respect to healthy controls and whose impaired expression reverted to normal during gestation. Secondly, miRNA target-gene prediction analysis revealed relationships between miR183 and miR9-2 and *Nurr1* and *SOCS2* genes, both included in our previous MS gene signature. The functional relationship of miRNA/mRNA pairs, was confirmed by the presence of a strong anti-correlation in clinical specimens, comparing expression levels of the mir183/*Nurr1* and mir9-2/*SOCS2* pairs. miRNA/mRNA target interactions for both genes were also experimentally confirmed by luciferase assay.
Conclusions: data highlight a regulatory role of miR183 and miR9-2 on *Nurr1* and *SOCS2*, two critical players in the negative feedback regulation of inflammation in MS.

This specific pattern of gene/miRNA expression might be associated to the pregnancy-related decrease in disease activity. Modulating the miR183/*Nurr1* and miR9-2/*SOCS2* pairs as during pregnancy, may thus represent a new approach to treating MS and autoimmunity overall.

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micro-RNAs and abnormal regulation of pro-inflammatory B cell cytokines in MS patients: The novel miR-132-surtuin-1 axis

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Objective: B cell effector cytokine profiles are dysregulated in patients with MS and may contribute to disease propagation both in the periphery and within the CNS. However, the mechanisms underlying B cell effector cytokine regulation are unknown. We considered micro (mi)RNAs as potential regulators of cytokine production by B cells in both the healthy state and in patients with MS.
Methods: Expression of 102 candidate miRNA was quantified (qPCR) in circulating B cells of MS patients and age/sex matched healthy subjects (HS) under different modes of activation. miRNA expression was correlated with secreted levels of interleukin (IL)-10, lymphotoxin (LT), and tumor necrotizing factor alpha (TNFα). The functional capacity of implicated miRNAs to regulate particular cytokines was confirmed by transfection of B cells with miRNA mimics and by pharmacological manipulation of downstream molecules.
Results: miRNA expression-profiles in normal B cells were modulated under different modes of activation, in association with distinct B cell cytokine secretion profiles. Compared with activated B cells of HS ($n=13$), activated MS B cells ($n=15$) expressed higher levels of miR-132 (3.71 ± 0.82 vs 8.13 ± 1.38; HS vs MS; $p<0.01$) in association with enhanced LT and TNFα production. Transfection of miR-132 mimic selectively enhanced LT and TNFα but not IL-10 production from B cells. We further discovered that Sirtuin-1 (SIRT1), a miR-132 target, was expressed at significantly lower levels in MS B cells compared to those of HS and that pharmacological inhibition of SIRT1 enhanced B cell production of LT and TNFα, while activation of SIRT1 suppressed production of these cytokines by MS B cells.
Conclusions: We report expression of distinct miRNAs as a novel mechanism underlying effector cytokine regulation by human B cells. Levels of miR132 are abnormally increased in B cells of MS patients, resulting in suppression of SIRT1 and over expression of B cell LT and TNFα. These discoveries establish a basis for the aberrant pro-inflammatory B cell cytokine responses recently implicated in MS relapses and provide novel targets for therapeutic intervention.

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miR-684 regulation of neurexophilin-1 and alpha-neurexin expression

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Objective: Using the Theiler's murine encephalomyelitis virus (TMEV) model of multiple sclerosis, candidate miRNAs are involved in demyelination and clinical deficits were identified. The studies described herein focus on miR-684, hypothesized to have binding sites in the 3′ untranslated region (UTR) of neurexophilin-1 and the receptor for neurexophilin-1, α-neurexin.
Methods: These studies utilized real time RT-PCR, indirect immunostaining, flow cytometry, in situ hybridization, and luciferase reporter gene assays. Clinical function was assessed using the Catwalk Gait Analysis system.
Results: We demonstrated that TMEV-infected mice treated with an adenovirus vector expressing glial growth factor (Ad-GGF) have improved clinical function compared to Ad-Ctl treated mice as determined by the Catwalk Gait Analysis system. Histological studies demonstrate that mice treated with glial growth factor experienced reduced inflammation in the brain and spinal cord compared to control-treated mice. Ad-GGF treated mice have lower levels of miR-684 than Ad-Ctl treated mice. *In silico* analysis identified α-neurexin and its ligand, neurexophilin-1 as having potential miR-684 binding sites in their 3′-UTRs. Examination of the effect of endogenous miR-684 as well as precursors and antisense-miR-684 RNA on 3′-UTR constructs validated the prediction that neurexophilin-1 is regulated by miR-684. miR-684 binds neurexiphilin-1 and α-neurexin mRNA transcripts at positions 136–140 and 1624–1626 of the 3′UTRs respectively. Both α-neurexin and neurexophilin-1 are expressed in areas of lesion formation in the spinal cord white matter of mice infected with TMEV. Flow cytometry data demonstrate that CD5+B220+ cells are positive for α-neurexin and neurexophilin-1.
Conclusions: Together, these data demonstrate that miR-684 binding to the 3′UTR of either neurexophilin-1 or α-neurexin decreases expression of these proteins, thereby functioning as a means of post-transcriptional control. Because of the difference in the levels of miR-684 between animals with differing levels of clinical function, neurexophilin-1:α-neurexin interactions may be involved in appropriate functioning of the mature CNS, and provide a target for clinical intervention in models of CNS disease.

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Modulation of inflammatory monocytes with a unique microRNA-gene signature ameliorates ALS mice

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Objective: To determine if there are unique microRNA signatures in peripheral $Ly6C^{Hi}$ monocytes in SOD1 mice and whether these signatures are present in ALS subjects.
Methods: Peripheral blood monocyte subsets from 18 sALS, 4 fALS, 8 MS-RR, 13 AD and 33 age-matched controls were studied using quantitative Nanostring profiling of 800 miRNAs and 180 inflammation-related genes.
Results: Nanostring analysis revealed that the top 10 deregulated miRNAs targets (including let-7b, miR-155, miR-146a, miR-223 and miR-27a) in $Ly6C^{Hi}$ splenic monocytes in SOD1 mice were associated with an M1 phenotype two months prior to disease onset and during disease progression. Treatment with anti-Ly6C mAb modulated the $Ly6C^{Hi}$ monocyte cytokine profile, reduced their recruitment to the spinal cord, diminished neuronal loss and extended survival. Genetic ablation of miR-155 in SOD1 mice attenuates recruitment of inflammatory monocytes into the spinal cord, polarizes M1 to M2 miRNA-genetic signature in $Ly6C^{Hi}$ monocyte and extends survival by 51 days. In ALS subjects, $CD14^{+}/CD16^{-}$ monocytes (the human analogue of $Ly6C^{Hi}$ monocytes) exhibited a disease specific microRNA and genetic pro-inflammatory signatures identical to that observed in the SOD1 mouse.
Conclusions: 1) Recruitment of pro-inflammatory monocytes plays an important amplifying role in disease progression and modulation of these cells is a potential therapeutic approach in ALS; 2) identification of microRNA abnormalities in peripheral inflammatory monocytes of ALS as was found in SOD1 mice provide a direct link between the animal model and the human disease; 3) this unique monocyte microRNA profile in ALS provides the basis for the development of miRNA-based therapeutic targets and a blood biomarker to monitor disease progression and response to therapy.

Neuroinflammation

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T cell killing of neurons is promoted by microglia

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Objective: Multiple sclerosis (MS) is a neurodegenerative disorder in which axonal loss has been correlated with accumulation of pathogenic T cells and microglia/macrophages. Both T cells and microglia/macrophages can kill neurons in culture, however no demonstration of a co-operative role has been reported thus far. This study therefore aims to investigate whether close contact between T cells and microglia can exacerbate neuron loss and the mechanisms behind this.
Methods: Human fetal brain samples were processed and neurons as well as microglia were isolated. T cells from syngeneic brain donors or from healthy adult human peripheral blood were co-cultured in combination with neurons and/or microglia and blocking antibodies were used to determine the mechanism of interactions between these cell types.
Results: We observed that neurons that were subjected to culture with both microglia and anti-CD3/anti-CD28 activated PBMCs had a significantly lower survival percentage when compared to neurons incubated with microglia or T cells alone. This co-culture induced a significant increase in the level of many cytokines, of both the 'pro-' and 'anti-' inflammatory types, as well as granzyme B. When an antibody that blocks the CD18 (β2 integrin) receptor was introduced the neuron killing was markedly reduced to baseline levels.
Conclusions: These findings indicate that killing of neurons at lesion sites in the CNS is likely exacerbated when both activated T cells and microglia are in contact with each other. Blockade of CD18 dramatically reduces this killing correspondent with the attenuation of the up-regulation of cytokines and granzyme B. Thus, this study suggests that therapeutic blockade of CD18 may not only reduce the migration of encephalitogenic T cells to the CNS during MS, but may also reduce the capacity of T cells and microglia to cooperatively cause damage to neurons.

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Immune-mediated mechanisms in the pathogenesis of cerebral amyloid angiopathy-related inflammation and Alzheimer's disease: Role of anti-Aβ auto-antibodies

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Objective: Cerebral amyloid angiopathy (CAA) is characterized by the progressive deposition of amyloid-β (Aβ) protein in the walls of small/medium sized arteries of cerebral cortex and leptomeninges, representing an important cause of spontaneous intracerebral haemorrhage and cognitive impairment.

A subgroup of CAA patients develop perivascular inflammation linked to the Aβ laden vessels, associated with vasogenic edema (VE) and to a rapid cognitive decline, leading to a condition known as CAA-related inflammation (CAA-ri). This syndrome has parallels with what observed in about 10% of patients affected by Alzheimer's disease (AD) who developed reversible VE after immunization with the anti-Aβ antibody bapineuzumab, where postmortem examination revealed inflammation and/or vasculitis associated with CAA, implying the discontinuation of therapeutic protocols. Recent MRI data have also shown that up to 17% of the treated patients have signs of VE directly related to the drug dose, even if in the absence of clinical correlates.
Methods: thanks to a novel technique for the ultra sensitive evaluation (patent application pending), we followed the concentration of anti-Aβ antibodies in the CSF of 10 CAA-ri patient during the acute phase (acCAA-ri) and after the remission phase (rpCAA-ri), compared to 8 non-inflammatory CAA, 10 AD, 10 MS and 20 healthy control subjects.
Results: we demonstrated that the concentration of anti-Aβ antibodies is specifically increased in the CSF of acCAA-ri patients, followed by a progressive reduction of their concentration after steroid treatment, accordingly to clinical-radiological improvements. Moreover, we observed a spontaneous decrease of these autoantibodies in rpCAA-ri patients without any immunosuppressant treatment, finally proving that the event is not secondary to an unspecific effect of treatment, but strictly related to disease progression.
Conclusions: our data support the hypothesis that the pathogenesis of CAA-ri is caused by a specific autoimmune reaction against Aβ, directly mediated by anti-Aβ autoantibodies. Since an invasive procedure such as brain biopsy is still needed for a definite diagnosis of CAA-ri, the outcomes implied by anti-Aβ dosage in CSF may be proposed to support future targets for early diagnosis and follow-up, in association with clinical and radiological features, and as a surrogate biomarker for clinical trials of disease modifying therapies.

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Role of CD8 + T cells in myelin-induced neuroinflammation

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Objective: The detailed role of CD8 + T cells in neuroinflammation is yet not fully elucidated. In the animal model there are contradictory reports concerning a beneficial or detrimental contribution to the pathology. The aim of this study was to characterize different CD8 + phenotypes and investigate their activity in the course of neuroinflammation. We used two-photon laser scanning microscopy (TPLSM) to track CD8 + T cells in the inflamed central nervous system (CNS) and elucidate their behavior.

Methods: Active experimental autoimmune encephalomyelitis (EAE) was induced by immunization with myelin oligodendrocyte glycoprotein $_{35–55}$ (MOG). For TPSLM analysis red fluorescent protein (RFP)-expressing CD8 + T cells and enhanced green fluorescent protein (EGFP)-expressing CD4 + T cells were transferred. To transfer the results into the human system, $MBP_{85–99}$-specific CD4 + Th17 cells were co-cultured with *in vitro* expanded CD8 + T cells from the same donor.
Results: After induction of active EAE in C57Bl/6 mice we were able to detect CD8 + T cells in the CNS, although in smaller amounts compared to CD4 + T cells. Visualizing their behavior by TPLSM, we found that the presence of CD8 + T cells did not influence the behavior of CD4 + T cells, as there were no specific long lasting interactions between both cell types detectable. Furthermore, in this condition, we could not detect any damage on the neurons subsequent to contacts between CD8 + T cells and neuronal structures. Flow cytometry analysis of CNS CD8 + T cells revealed a high production of Interferon (IFN)-gamma, no markers so far known to characterize CD4 + T regulatory cells were detectable in the CD8 + T cell population. On the other hand, when we isolated and *in vitro* expanded CD8 + T cells from the spleens of immunized mice in remission, those CD8 + T cells showed regulatory capacities leading to a massively reduced proliferation of co-cultured CD4+2d2Th17 T cells and a significantly increased cell death of CD4 + T cells. Transferring those results into the human system, human CD8 + suppressor T cells were also found to kill autologous myelin-specific CD4 + target cells.
Conclusions: CD8 T cells, indicated to play a role in multiple sclerosis, are present in the CNS in a model of myelin-induced neuroinflammation, but do not seem to aggravate disease course. Furthermore, our findings indicate a subgroup of CD8 + T cells which apparently exhibit suppressive capacities in the murine as well as in the human system.

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Strawberry notch 2, a novel IL-6/gp130 family cytokine-regulated target gene is differentially induced in astrocytes and microglia

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Objective: The pleiotropic cytokine Interleukin 6 (IL-6) is a key participant in the response to injury and infection in the CNS, where astrocytes and microglia are its major targets. By DNA microarray analysis we identified strawberry notch 2 (Sbno2) as a potentially novel IL-6-stimulated gene in astrocytes and microglia. Sbno2 is a mouse homolog of the Sno gene in Drosophila that was shown to modulate Notch signaling. However, the function of Sbno2 in mammalian cells is ill defined. Here we determined the regulation of the Sbno2 gene in cultured glial cells *in vitro* and in the murine brain.
Methods: The regulation of Sbno2 mRNA was examined by RPA or qPCR in: (1) primary murine and human astrocyte and murine microglial cultures as well as in murine C8-B4 and EOC13 microglial cell lines treated with hyper-IL-6 or different cytokines, or (2) in brain from GFAP-IL6, GFAP-IL12 and GFAP-IFNα transgenic mice.
Results: In murine astrocytes and microglial cells, hyper-IL-6 stimulated a dose- and time-dependent increase in Sbno2 mRNA. Sbno2 mRNA was also increased in human astrocytes in response to hyper-IL-6. In glial cells from mutant mice that lack one or the other of the two main IL-6 signaling pathways, hyper-IL-6 induced up-regulation of Sbno2 was mainly mediated via the gp130 Jak/STAT signaling pathway. However, Sbno2 was also constitutively expressed in glial cells and this basal expression was not altered by a lack of STAT signaling. In mouse astrocytes, Sbno2 mRNA was highly up-regulated by OSM and to a lesser degree by LIF and IL-11 as well as by IL-1β and TNFα, whereas in microglia these cytokines had no effect. Finally, Sbno2 mRNA was highly up-regulated in the brain of GFAP-IL6 mice, to a lesser degree in GFAP-IL12 mice and not at all in GFAP-IFNα mice compared with wild type mice.
Conclusions: (1) IL-6 is a potent stimulus for Sbno2 gene expression in microglia and astrocytes via IL-6/gp130 mediated JAK/STAT signaling, (2) the *in vivo* relevance of these findings was reflected by elevated Sbno2 mRNA levels in GFAP-IL6 and -IL12 transgenic mice, and (3) the regulation of Sbno2 gene expression by other gp130 cytokines and by other pro-inflammatory cytokines is distinct and differs between the two glial cell types suggesting divergent roles for Sbno2 in the action of these cytokines on astrocytes and microglia.
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Immunological mechanisms

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EMMPRIN, A Multi-Faceted Modulator Of Immune Cell Functions In MS

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Objectives and background: In multiple sclerosis (MS), immune cells transmigrate the blood–brain barrier (BBB) to invade the CNS parenchyma resulting in demyelination and axonal destruction. Proteases such as matrix metalloproteinases (MMPs) are highly upregulated in CNS regions in MS and in its animal model experimental autoimmune encephalomyelitis (EAE). Immune cells are shown to utilize MMPs to cleave components of the BBB to enter the CNS. Extracellular matrix metalloproteinase inducer (EMMPRIN, CD147, basigin) is an upstream inducer of the expression of several MMPs. We reported previously that EMMPRIN is upregulated in EAE and that blocking EMMPRIN activity reduced neuroinflammation and clinical EAE severity (Agrawal et. al., J Neurosci 2011). Here, we analysed EMMPRIN expression in MS brain specimens and used anti-EMMPRIN function blocking antibodies in several experimental models to interrogate the roles of EMMPRIN in immune cells.
Methods: MS brain sections were stained for EMMPRIN expression. Human T cell and monocyte cultures were used to test the effect of anti-EMMPRIN antibody (clone 10) (Agrawal et al., J Neuroinflammation, 2012) versus isotype control.
Results: In normal human brain, EMMPRIN is localized to endothelial cells. In MS specimens, EMMPRIN is significantly upregulated around inflammatory perivascular cuffs, on leukocytes and astrocytes. EMMPRIN upregulation was not observed in non-inflammed brain areas of MS. In culture, activated human T cells elevate their expression of EMMPRIN. Treatment of activated T cells with anti-EMMPRIN function blocking antibody decreased MMP production, prevented their ability to progress through the cell cycle without inducing apoptosis, and reduced their adhesion to endothelial cells. Monocytes also decreased their production of MMPs whilst elevating their levels of the physiological MMP inhibitors, TIMPs, and were prevented from transmigrating a model of the BBB when exposed to anti-EMMPRIN. Finally, anti-EMMPRIN exposed T cells were attenuated in their capacity to kill human neurons.
Conclusions: We describe EMMPRIN as a novel regulator of inflammation in MS that is significantly upregulated in inflamed lesions of MS

brain specimens. Activities of EMMPRIN beyond the induction of MMPs include the control of several important T cell functions such as proliferation, activation, adhesion and cytotoxicity.

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Microglial cells during forebrain development

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Objective: Microglial (MG) cells are the resident macrophages of the central nervous system. Their progenitor cells develop in the yolk sac at E7.5 and infiltrate the early forebrain at E9.5, through blood circulation. The entry of MG cells into the brain just before the onset of the neurogenesis could presume an important role of microglia in this process and the existence of a possible functional interplay between MG cells and embryonic neural stem cells that support the entire neurogenic process. The aim of this project is to study MG recycling in the developing and the post-natal forebrain-i.e. cell proliferation/differentiation/immigration- and to identify a cross-talk between MG cells and neural cells during forebrain development.
Methods: We analyzed MG cell distributions at E14.5 and E18.5 stages, by using specific MG cell markers. Furthermore, by using stereology, we also reconstructed the spatial distribution of MG cells in the developing forebrain. We also evaluated MG cell proliferation in vivo by using timidine analogs and in a transgenic mouse line that expresses the GFP in MG cells we attempted to study the ultrastructure of MG cell by electron microscopy. Finally, we also purified MG cells from the forebrain by using flow cytometry-mediated cell sorting.
Results: Although the absolute MG cells number increases along neurogenesis, MG cell density is maintained constant. MG cells are preferentially positioned into germinal niches of the forebrain and into the plexus, while very few of them are located into the cortical plate. A relative small percentage of MG cells proliferate within the developing forebrain.

MG cells collected from the developing forebrain express members of the Delta/Notch molecular machinery. Interestingly, the over expression of Delta, in neural stem cells of the developing forebrain, induced a significant accumulation of MG cells into both the cortical parenchyma and the choroid plexus. These results suggest that the neurogenesis and the MG influx are correlated processes and the Delta/Notch machinery might control the dynamic accumulation of MG cells in the developing forebrain.
Conclusions: MG cells are preferentially placed within neurogenic niches. Their relative abundance is maintained constant during forebrain development. MG cells express genes of the Notch/Delta molecular machinery. The over-expression of Dll1 increased MG cells in both CNS parenchyma and ventricular space.

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Cross-immunoreactivity between bacterial and human aquaporins: A possible trigger of neuromyelitis optica

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Neuromyelitis optica (NMO) is a chronic inflammatory disease of the central nervous system of unknown etiology. Astrocyte aquaporin 4 has been proposed as the primary autoimmune target in NMO based upon the presence of self-reactive antibodies in patient's sera. In this study, we examined whether an anti-Aqp4 antibody response may arise through a mechanism of cross-immunoreactivity. We analyzed bacterial (*E. coli*) AqpZ and human Aqp4 proteins for structural homology and cross-immunoreactivity using proteomics software and a number of immune-based assays. Recombinant proteins, synthetic peptides, sera from patients with NMO, immune mouse serum raised against AqpZ, anti-Aqp4 and anti-AqpZ antibodies were used in these experiments. Analysis of the amino acid sequence of AqpZ and Aqp4 identified several regions of significant homology, some of which localized within the protein domain targeted by the self reactive anti-Aqp4 antibody in NMO. Cross-reactivity between AqpZ and Aqp4 was observed in multiple independent assays, including ELISA, latex agglutination, western blot, immunoprecipitation, immunohistochemistry and antibody-mediated cytotoxicity. Finally, immunization of mice with either AqpZ or its homologous peptides resulted in CNS disease. Based on our experiments we concluded that cross-immunoreactivity between bacterial AqpZ and human Aqp4 may give rise to a self-reactive antibody response against Aqp4. Our findings are novel and relevant to the etiology and pathogenesis of NMO.

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Inhibitory effects of CD25 blockade on development of pro-inflammatory innate lymphoid cells is associated with decreased intrathecal inflammation in MS

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Objective: The IL-2 system is genetically linked to autoimmune diseases such as multiple sclerosis (MS). Blockade of the IL-2Rα chain (CD25) by the humanized monoclonal antibody daclizumab decreases MS-associated inflammation. Although daclizumab was designed to selectively inhibit activated T cells, investigation of its mechanisms of action (MOA) has revealed unexpectedly broad effects on the innate immune system. In search for full MOA of daclizumab in MS, we compared development of immune responses associated with yearly influenza immunization in daclizumab-treated MS patients and controls. One of the major observations from this experiment was decrease in the number of circulating innate lymphoid cells (ILCs) observed in the daclizumab cohort. Thus, we investigated phenotype and function of ILCs in MS and the mechanisms of their modulation by daclizumab therapy.
Methods: We used combination of ex-vivo immunophenotyping of patients enrolled in NIH clinical trials of daclizumab in MS with extensive in-vitro functional and mechanistic studies.
Results: The number of circulating pro-inflammatory ILCs, including c-kit+, RORgt-expressing lymphoid tissue inducer (LTi) cells is significantly increased in untreated MS subjects as compared to healthy controls. Daclizumab therapy decreases the number of LTi cells and modifies their phenotype toward a natural killer (NK) cell lineage. Although LTi cells express CD25, daclizumab inhibits their development paradoxically in IL-2-dependent manner; by

steering differentiation of common ILC precursor derived from CD34+ hematopoietic stem cells towards immunoregulatory $CD56^{bright}$ NK cells, via enhanced intermediate affinity IL-2 signaling. Because adult LTi cells may retain their ability to induce or sustain formation of lymphoid follicles and associated adaptive immunity, we indirectly measured meningeal inflammation by quantifying intrathecal CXCL13 and immunoglobulin G (IgG) index. Both of these MS-associated inflammatory biomarkers were significantly inhibited by daclizumab treatment.
Conclusions: Daclizumab therapy reveals a novel link between IL-2 and ILCs, providing a plausible explanation for a broad genetic association of IL-2 system to autoimmunity in humans. Future studies should investigate the role of ILCs, especially pro-inflammatory LTi cells in the initiation and maintenance of compartmentalized adaptive immune response associated with chronic inflammation and end-organ damage.

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Reduction of dendritic cell in the long term than in early injection drug users

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Objective: To quantify the dendritic cell subsets in injecting drug users (IDUs), HIV-1 infected patients and healthy HIV-1 seronegative individuals and correlate with virological, immunological and clinical parameters.
Methods: Blood samples from 30 IDUs, 30 healthy HIV-1 seronegative individuals, and 30 HIV-1 positive patients were collected after obtaining written informed consent. The distribution of the peripheral blood dendritic cells and its subsets was analyzed in these samples by four color flowcytometry. The study was approved by the institutional ethics committee.
Results: The frequency of DCs and its subsets were significantly lower in HIV-1 infected patients as compared to (IDUs) and healthy individuals. We also observed a positive correlation of the DCs and their subsets with $CD4^{+}$T cells and a negative correlation with HIV-1 viral load. A salient of this study was the lower frequency of DCs and its subsets in the long term IDUs (>5 years of injection drug abuse) as compared to the early IDUs (<5 years of injection drug abuse).
Conclusions: This is the first study to determine the frequency of dendritic cells subpopulations in IDUs who are at high risk for HIV-1 infection. The lower level of dendritic cells and it's subsets in long term IDUs may be one of the factors plays a crucial role in resistance or in protection from HIV-1 infection in IDUs, which needs to be further evaluated by performing longitudinal studies.

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Modulation of Plasmacytoid Dendritic Cells by TLR9 agoinists in EAE

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Objective: In this study, we aim at developing a better understanding of the effects of TLR7 and TLR9 agonists on plasmacytoid dendritic cells (pDCs) in experimental autoimmune encephalomyelitis (EAE). Toll-like receptors (TLRs) were initially described as stimulatory molecules that activate early defence mechanisms against invading pathogens. However, they can also exert regulatory functions and thus represent valuable targets in the prevention/therapy of inflammatory diseases including multiple sclerosis (MS). pDCs are the principal cellular targets of TLR7 and TLR9 agonists, such as viral ssDNA and microbial CpG-containing DNA sequences, respectively.
Methods: Female C57BL/6 mice were immunized with MOG(35–55) and scored daily for clinical signs of disease. After humane killing, spleens were obtained and a variety of methods used to acquire relevant data from splenocytes including flow cytometry, RT-PCR and Western blotting.
Results: Data compiled so far indicate that TLR9 stimulation can significantly reduce the severity of EAE. Specifically we found that TLR9 agonist CpG-A and to a lesser extent CpG-B delay the onset of disease and limit disease severity. Splenocyte IL-17 production was significantly reduced by CpG-A as compared with PBS treated mice. In vitro experiments showed that stimulation of spleen plasmacytoid dendritic cells by TLR9 agonists induced the expression of indoleamine dioxygenase (IDO) and the secretion of kynurenines with distinct dose–response profiles and that was associated with in vitro expression of FoxP3 in CD4+ T cells.
Conclusions: These results are encouraging as they suggest point towards the occurrence of a potentially protective TLR9/IDO axis, probably involving pDCs, which could be exploited for the induction of immune tolerance.

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In vivo anti-LAP monoclonal antibody treatment exacerbates disease in the EAE model

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Objective: Regulatory T cells (Tregs) play a critical role in the maintenance of immunological tolerance and the development of strategies to manipulate these cells for the treatment of autoimmune conditions is a major goal. Previous results from our lab have shown that oral administration of anti-CD3 monoclonal antibody suppresses experimental autoimmune encephalomyelitis (EAE) in mice and the suppression induced by this treatment was mediated by the induction of a subpopulation of Tregs that expresses latency-associated peptide (LAP) on the cell surface. We and others have also found similar results in different disease models (lupus, diabetes, arthritis, atherosclerosis). In the present study, we investigated the effect of *in vivo* treatment with an anti-LAP monoclonal antibody generated in our lab on the development of EAE.
Methods: C57BL/6 or Foxp3-GFP knock in mice received i.p. injections of anti-mouse LAP monoclonal antibody and spleen and lymph node cells were harvested and analyzed for their immunological profile (FACS and in vitro culture). In some experiments, mice were immunized with MOG/CFA one day after last anti-LAP antibody injection to test the effect of this treatment on EAE induction. We also studied the effect of anti-LAP antibody treatment in anti-CD3 induced oral tolerance in the EAE model.
Results: Treatment with anti-LAP antibody led to a marked decrease in the number of $CD4^{+}LAP^{+}$ Tregs in spleen and lymph nodes without affecting the number of $CD4^{+}Foxp3^{+}$ Tregs. Spleen and lymph node cells from anti-mouse LAP treated mice were studied *in vitro*. Spleen cells from anti-mouse LAP treated mice proliferated more vigorously *in vitro* and produced more IL-2, IL-17 and IFN-gamma. Moreover, injection of anti-mouse LAP antibody abrogated the protective

effect afforded by oral anti-CD3. Surprisingly, *in vivo* anti-LAP treatment before MOG immunization led to development of severe EAE in animals not given pertussis toxin, which is usually required for EAE induction. These mice treated with anti-LAP presented higher Th1 and Th17 infiltrates in CNS after MOG/CFA immunization.
Conclusions: Our results demonstrate a central role for LAP^+ Tregs in the control of EAE and that suppression of EAE by the oral anti-CD3 is dependent on LAP^+ Tregs. Furthermore, LAP expression appears to be a key marker of Th3 type Treg cells induced by oral antigen/anti-CD3.

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Effects of pubertal transition on autoimmune risk in female mice

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Objective: For reasons that remain unclear, the incidence of multiple sclerosis (MS) is higher in women than in men. Epidemiological studies suggest that the increased disease risk in women develops post-puberty and that early menarche is associated with an earlier age of onset and incidence of MS in women. The objective of this study was to characterize the changes that occur in the T cell adaptive immune response with pubertal transition in female mice via studies in the experimental autoimmune encephalomyelitis (EAE) model.
Methods: Female SJL/J mice were either ovariectomized or provided a sham surgery prior to pubertal onset. EAE was induced in these female mice during adulthood (>8 weeks of age) via immunization of mice with proteolipid protein (PLP) p139-151 in Complete Freund's Adjuvant. The recall proliferation and cytokine production by PLP p139-151-specific cells in spleen and draining lymph nodes was examined. Adoptive transfer studies and in vitro immunological assays were conducted to pinpoint the relevant immune populations involved in the puberty-associated increase in autoimmune risk in female mice.
Results: Ovariectomy was associated with a reduced incidence in EAE in female mice that correlated with a decreased expansion of PLP p139–151-reactive T cells. Adoptive transfer studies indicated that ovariectomy of recipients as opposed to T cell donor mice was associated with reduced autoimmunity. Further studies revealed that the less robust immune response with ovariectomy mapped to the dendritic cell compartment. Splenic $CD11c^+$ dendritic cells (DCs) isolated from ovariectomized mice were less efficient at priming myelin-specific TCR transgenic Th1 and Th17 responses *ex vivo*. Ovariectomized DCs also exhibited reduced expression of maturation markers and production of innate cytokines as compared to sham counterparts.
Conclusions: These data indicate that puberty is associated with an increase in the maturation of DCs, thus possibly explaining why women become more susceptible to develop autoimmunity when they enter childbearing years.

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The role of neuronal-derived TNF in protective immunity against central nervous system tuberculosis

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Objective: Central nervous system (CNS) tuberculosis is a severe complication form of extrapulmonary tuberculosis, characterized by tuberculosis meningitis (rupture of rich foci into the ventricle and subarachnoid space) or tuberculoma (rupture of rich foci into the brain or spinal cord). The purpose of this study was to investigate the role of cell type-specific tumor necrosis factor (TNF) derived from brain neuronal cells during CNS tuberculosis.
Methods: We generated a novel neuron specific TNF knockout mice ($NsTNF^{-/-}$) by breeding a synapsin-1 Cre recombinant mouse (Cre recombinase is expressed only in the neurons of the brain and spinal cord) with a TNF floxed mouse strain using Cre/loxP recombination system. TNF has been found to play a crucial role in host defence against bacterial infections. Mice from three strains (TNF floxed as wild type, $NsTNF^{-/-}$ as cell type-specific, and global $TNF^{-/-}$) were intracerebrally injected with $1x10^5$ CFU of *Mycobacterium tuberculosis* (H37Rv).
Results: All mice, 6–8 weeks old, were on a C57BL/6J genetic background. Genotypes of mice were confirmed by polymerase chain reaction (PCR) analysis. Our preliminary data show that cell type-specific mice ($NsTNF^{-/-}$) could survive for more than 18 weeks along with the control TNF floxed mice, however the global $TNF^{-/-}$ succumbed by day 21. Also the cerebral bacterial burden and inflammation were, however, slightly increase in $NsTNF^{-/-}$ mice when compared to the TNF floxed mice after 3 weeks post infection, but most importantly, they did not succumb to infection in which the global $TNF^{-/-}$ succumbed.
Conclusions: These findings show that $NsTNF^{-/-}$ mice are not susceptible to *Mycobacterium tuberculosis* infection.

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IFN-γ limits Th9 mediated autoimmune inflammation through dendritic cell modulation of IL-27

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Objective: IL-9 producing Th9 cells have been associated with autoimmune diseases, such as experimental autoimmune encephalitis. However, the factors that negatively regulate Th9 cells during autoimmune inflammation are unclear.
Methods: A, Induction and evaluation of EAE. Mice were injected subcutaneously in both flanks with 100 μg of MOG35–55 peptide dissolved in PBS emulsified in an equal volume complete Freund's adjuvant — CFA supplemented with 5 mg/ml *Mycobacterium tuberculosis* H37Ra and injected twice intravenously with 200 ng of pertussis toxin administered on the day of immunization and 48 h later. Clinical assessment of EAE was performed daily after disease induction according to the following criteria: 0, no disease; 1, tail paralysis; 2, hindlimb weakness or partial paralysis; 3, complete hindlimb paralysis; 4, forelimb and hindlimb paralysis; 5, moribund state. B, CD4 + T Cell culture. For in vitro Th9 cell differentiation naïve $CD4^+$ $CD62L^{hi}$

$CD44^{lo}$ T cells are sorted by flow cytometry and activated with plate bound anti-CD3 and anti-CD28 (2 μg/ml) in the presence of TGF-β (3 ng/ml), IL-4 (20 ng/ml) with or without IL-27 (100 ng/ml). C, Adoptive T cell transfer. 2D2 $CD4^+$ T cells were stimulated under Th9 differentiation condition in the presence of absence of IL-27. Restimulated cells were collected and extensively washed with PBS. 5×10^6 cells were injected i.v. into Rag-$1^{-/-}$ mice. Recipient mice were injected i.p. with 200 ng of pertussis toxin (PT) (List Biological Laboratories) on day 0 and day 2 after T cell transfer.
Results: We found that IFN-γ inhibits Th9 differentiation both *in vitro* and *in vivo*. This suppressive activity was dependent on the transcription factor STAT-1. In addition to its direct inhibitory effect on Th9 differentiation, IFN-γ suppressed Th9 cells through the induction of IL-27 from dendritic cells. *In vitro*, treatment of naive $CD4^+$ T cells with IL-27 suppressed the development of Th9 cells, which was partially dependent on the transcription factors STAT-1 and T-bet. Moreover, IL-27-treatment completely abrogated the encephalitogenicity of Th9 cells in the EAE model.
Conclusions: Our results identify a previously unknown mechanism by which IFN-γ limits Th9 mediated autoimmune inflammation through dendritic cell modulation of IL-27.

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VAV1 controls inflammation in experimental autoimmune encephalomyelitis (EAE)

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Objective: VAV1, a member of the family of guanine nucleotide exchange factors (GEF) for the Rho family of GTPases, plays an important role in T-cell and B-cell development, activation, tissue homing and effector functions. *Vav1* was found within Eae4, a rat quantitative trait locus (QTL) that regulates experimental autoimmune encephalomyelitis (EAE), a model for MS, and overlaps with several QTLs for inflammatory diseases. Analysis of a human cohort showed association of certain haplotypes of the gene with MS, and increased levels of *VAV1* mRNA were found in peripheral blood and CSF of MS patients as compared to controls. In this work we attempt to further characterize the function of VAV1 in the development of autoimmunity.
Methods: Interval-specific congenic rat lines carrying the disease predisposing or protective Vav1 variants were used to dissect disease mechanisms. EAE was achieved by immunization of rats with recombinant MOG in IFA. Immunopathology was analyzed by clinical scoring of disease severity, ex-vivo analysis of cells and tissues by flow cytometry, qPCR and ELISA, and in vitro assays with cultured T cells, B cells and APCs.
Results: Analysis of naïve animals showed that the strain carrying the protective *Vav1* variant, which leads to a lower level of VAV1 protein expression, presented with increased CD4+ Treg numbers. While in vitro stimulated naïve T cells and CD4+ Treg cells of this strain performed poorly in terms of activation, proliferation and cytokine secretion (especially IL-2) in response to TCR triggering, this defect could be compensated by the addition of exogenous IL-2. Ex-vivo analysis of lymph nodes of immunized animals however showed only a minimal defect in all these parameters pointing to compensatory mechanisms in vivo. Even though Vav1 is also expressed by APCs, only B cells and macrophages but not dendritic cells showed impaired proliferation and T cell stimulatory capacity, respectively. Analysis of peripheral lymphoid tissue as well as CNS-infiltrating cells at later disease stages showed that inflammatory parameters remain high in the disease susceptible strain whereas they were promptly reduced in the disease protected strain.
Conclusions: Natural variations in *Vav1* can regulate a number of immune processes both during immune cell development as well as during immune responses not only in T cells but also in B cells and macrophages. These processes can jointly determine the outcome of an immune response.

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Interferon-beta therapy-induced elevation of serum IL-7 levels is blocked by anti-drug antibodies

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Objective: Interferon beta (IFN-β) is the most commonly used immunomodulatory drug for the treatment of multiple sclerosis (MS) patients. However, up to one third of IFN-β-treated patients develop neutralizing anti-drug antibodies (NAbs) against the drug. NAbs can alter the effectiveness of the drug by interfering with both the drug and its target receptor binding.

Studies have indicated that interleukin-7 (IL-7) could serve as a potential biomarker of IFN responsiveness in MS, and polymorphisms in the *IL7* and *IL7R* genes have been associated with increased susceptibility to a variety of autoimmune disorders. This study investigated the effect of IFN-β treatment on IL-7 biology in MS patients with and without NAbs present.
Methods: Swedish MS patients were screened for the presence of NAbs. Patient serum was analyzed for the presence of antibody neutralizing activity using the myxovirus resistance protein A (MxA) gene expression bioassay. Human serum was incubated with cell line A549 and the ability of patient's sera to neutralize the added IFN-β was measured. Quantification of serum IL-7 was carried out using commercially available human IL-7 ELISA kit (R&D Systems) in accordance with the manufacturer's instructions.
Results: Patients receiving IFN-β treatment had significantly increased serum IL-7 levels (Mean ± 95% CI: 15.9 ± 1.9 pg/ml) compared to natalizumab treated MS patients (5.6 ± 1.1 pg/ml) and healthy blood donors (7.6 ± 2.4 pg/ml). IL-7 levels peaked at 24 hours post IFN-β injection, but remained elevated throughout treatment. Furthermore, patients exerting high NAb titers against IFN-β had reduced IL-7 levels (14.3 ± 2 pg/ml) compared to NAb negative patients (19.3 ± 3.8 pg/ml), suggesting reduced IFN-β bioactivity ($p = 0.01$). On an individual MS patient level, a shift in treatment from IFN-β to natalizumab resulted in significantly reduced IL-7 level for most of the patients (21 of 23), suggesting the drug influences IL-7 levels and not vice-versa.
Conclusions: That the NAb status has an impact on the IFN-β abilities to modulate the IL-7 levels, indicate the disease relevant impact of anti-drug antibodies. Since IL-7 is a non-redundant, immunostimulatory cytokine, this may be an important biomarker for improving treatment outcome.

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High secreted levels of EGF from immune cells of patients with RR-MS may be related to the impaired neuronal repair

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Background: Epidermal growth factor (EGF) induces differentiation of neuronal precursor cells (NPCs) into astrocytes. In MS, there is a

failure of remyelination and insufficient differentiation of NPCs into oligodendrocytes. As there is an inflammatory infiltration in MS lesions, we studied the secretion of EGF from immune cells of RRMS patients, and its effect on neuronal morphology.
Methods: PBMCs of 39 RRMS patients (14 untreated, 25 IFN-β-treated) and 20 healthy controls (HC) were cultured for 24 h with anti-CD3/CD28 mAb or with isotype controls (IC) or LPS or diverse cytokines or without stimulation. EGF levels in the supernatants were measured by ELISA. The effect of supernatant's EGF on PC-12 neuronal-like cells morphology was studied by addition of anti-EGF neutralizing mAb or IC to the cultures.
Results: EGF levels were lower in HC (98.7 ± 6.7 pg/ml) vs. untreated patients (140.9 ± 12.7 pg/ml, $P<0.01$) and vs. IFN-β treated patients (151. 6 ± 10.9 pg/ml, $P<0.001$). Stimulation with LPS increased the EGF levels in HC (129.3 ± 8.0 pg/ml vs. 98.7 ± 6.7 pg/ml, $p<0.01$) but not in untreated patients (136.5 ± 10.2 pg/ml vs. 140.9 ± 12.7 pg/ml) or in IFN-β treated patients (151.4 ± 9.9 pg/ml vs. 151. 6 ± 10.9 pg/ml). Stimulation with anti CD3/CD28 mAb increased the EGF levels of HC (126.5 ± 7.4 pg/ml vs. 102.1 ± 6.2 pg/ml, $p<0.05$) but not in untreated patients (141.3 ± 12.3 pg/ml vs. 140.1 ± 11.1 pg/ml) or IFN-β treated patients (151.9 ± 8.9 pg/ ml vs. 152.1 ± 9.9 pg/ml). Anti-inflammatory cytokines reduced the EGF levels. In HC: IL-4 = 71.1 ± 9. 5 pg/ml and IL-10 = 52.2 ± 6.7 pg/ml vs. no cytokine = 116.2 ± 5.0 pg/ ml, $p<0.005$ and $p<0.0001$ respectively. In RRMS patients: IL-4 = 112.2 ± 6.5 pg/ml, and IL-10 = 93.9 ± 6.2 pg/ml vs. no cytokine = 146.5 ± 5.6 pg/ml, $p<0.005$, $p<0.0001$, respectively). Supernatants from PBMCs of HC that were incubated with neuronal-like PC-12 cells slightly induced neuronal phenotype, while addition of the anti EGF neutralizing mAb dramatically increased the neuronal phenotype of these cells. Supernatant of RRMS patients induced impairment of neuronal phenotype and death of PC-12 cells. Both were reversed by anti EGF neutralizing antibody.
Conclusion: We described an unreported aspect of the immune response of patients with RR-MS. Our data suggest a defective immune mediated-neuroregeneration in RRMS patients via increased EGF secretion. Neutralization of the EGF may affect neuronal survival and maturation.

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Increased secreted levels of bone morphogenic proteins 2, 4 and 5 by immune cells of patients with relapsing remitting multiple sclerosis

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Background: Neural stem cells (NSCs) are abundantly present in MS lesions but they fail to mature into active neurons and oligodendrocytes. Bone morphogenic proteins (BMPs) signaling was found to be involved in neuronal and oligodendroglial differentiation of NSCs. In order to examine the potential of immune cells to affect neurogenesis/oligodendrogenesis processes, we studied the expression and secretion profile of BMP-2,4,5 and 7 by PBMCs of RR-MS patients.
Methods: BMP-2,4,5 and 7 basal mRNA levels were detected in PBMCs from RR-MS patients −19 untreated and 27 treated with IFN-β and 27 matched healthy controls (HC) by real-time PCR and standardized for the expression of GAPDH mRNA. BMPs secreted levels were detected in 24 h PBMCs supernatants by ELISA. Cellular analysis of BMPs expression was performed in purified $CD3^+$ cells, $CD14^+$ cells $CD3^-CD14^-$ cells using MACS. The effect of stimulation via CD3/CD28, and of different cytokines on BMPs mRNA expression was studied after 2 h of incubation.
Results: Basal mRNA levels of BMP-2,4,5 were higher in PBMCs of untreated patients (39.6 ± 15.4, 83.1 ± 31.7 and 17.6 ± 4.9, respectively) compared to HC (3.8 ± 1.0, 5.4 ± 1.7 and 1.7 ± 0.4, $p=0.03$, $p=0.03$ and $p=0.004$, respectively). Protein levels of BMP-2,4,5 were elevated in the supernatants of untreated patients (52.7 ± 10.6, 143.8 ± 32.2 and 327.5 ± 106.3 pg/ml, respectively) compared to HC (21.39 ± 3.5, 40.06 ± 12.9 and 71.1 ± 17.3 pg/ml, $p=0.01$, $p=0.01$ and $p=0.03$, respectively). No differences were found between mRNA and protein levels of untreated and IFN-β treated patients. No induction of BMP-7 mRNA and protein levels were observed in PBMCs of all groups. Increased levels of BMP-2,4,5 in PBMCs of RR-MS patients were primarily derived from upregulation of BMPs in T cells. CD3/CD28 stimulated T cells induced BMP-2,4,5 mRNA levels only in PBMCs of untreated patients and not in IFN-β treated patients or HC. Stimulation with one of TNF-α, IFN-γ or IL-17, significantly induced BMP-2 and 4 mRNA levels in untreated patients but not in IFN-β treated patients or HC.
Discussion: Elevated levels of BMP-2, 4 and 5 from immune cells may be related to the reported increased levels of BMPs in MS lesions, contributing to both anti-neurogenic and anti-oligodendrogenic environment. The unregulated BMPs expression in RR-MS may be resulted from the deviated immunity of MS patients, characterized by the stimulated T cells via CD3/CD28 pathway, and the effect of pro-inflammatory cytokines.

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p38 MAP kinase signaling in myeloid cells controls autoimmune disease of the CNS in a sex-specific manner

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Objective: Multiple sclerosis (MS) is an autoimmune inflammatory disease of the central nervous system (CNS). Inhibitors of p38 MAP kinase (p38), a key inflammatory kinase, have shown promise in treatment of select autoimmune diseases, but their potential in MS or its models remains relatively unexplored. We have recently shown that inhibition of p38 ameliorated experimental autoimmune encephalomyelitis (EAE), the main preclinical model for MS. Here we aim to delineate the cell types in which p38 signaling was required for disease pathogenesis, and to determine the downstream effects on the autoimmune response.
Methods: p38 activity was genetically modulated in B10.BR mice by T cell-specific expression of dominant negative or constitutively active transgenes. p38α was deleted in T cells and myeloid cells/macrophages in C57BL/6 (B6) mice by crossing floxed p38α mice with Lck-Cre or LysM-Cre mice, respectively. EAE was induced in B6 or B10.BR mice by MOG immunization and, in some experiments, mice were treated with daily injections of SB203580, a p38 inhibitor. Immune responses in lymph nodes and CNS were evaluated by flow cytometry, ELISA, or qRT-PCR.
Results: Unexpectedly, pharmacological inhibition of p38 only prevented EAE in female, but not male B6 mice. However, genetic manipulation of p38 activity in T cells in B10.BR mice affected disease in both males and females, suggesting either a strain-specific or a cell type-specific sexual dimorphism. To address these possibilities, we conditionally ablated p38 in T cells and myeloid cells in B6 mice. Surprisingly, T cell-specific deletion of p38 had no significant effect on EAE in either sex. In contrast, deletion of p38 in myeloid cells resulted in reduced disease in females, but not males, suggesting that the

sexually dimorphic response to p38 blockade in EAE originates to a large extent within the myeloid cell compartment. Analysis of the inflammatory infiltrates in the CNS revealed that female, but not male mice lacking p38 in myeloid cells exhibited reduced immune cell activation compared with controls.
Conclusions: Our findings suggest that p38 signaling in myeloid cells promotes CNS inflammation in a sex- and strain-specific fashion. Sexual dimorphisms in autoimmune diseases are poorly understood. Our studies reveal new molecular mechanisms underlying such phenomena, and also suggest that the p38 MAPK pathway may present targets for sex-specific therapies in MS.

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IL-22 confers resistance to EAE and is induced in T cells by constitutive phosphoinositide-3-kinase activity

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Objective: T helper cells are major players in the initiation of the autoimmune CNS disease model EAE. Signals during priming determine their pathogenicity by inducing differential expression of cytokines such as IL-17, GM-CSF, IFNγ and IL-22. We found that the strength of costimulation by CD28 and thereby Phosphatidylinositol 3-kinase (PI3K) signaling has major impacts on differentiation of Th1, Th17 and Tregs. We therefore aimed to analyze the effect of constitutive PI3K activity in T cells on EAE development.
Methods: We crossed $CD4^{cre}$-mice to conditional loxP-flanked Rosa26-Stop-PI3K-p110 mice, thereby creating mice expressing a constitutive active form of PI3K (p110) specifically in T cells of CD4/p110 mice. These mice were tested in vitro for T cell differentiation and in vivo for EAE development by immunization with MOG_{35-55} peptide. Further, we used T cells of CD4/p110 as donors in T cell transfer colitis experiments in RAG1 deficient animals.
Results: T cells from CD4/p110 mice were refractory to Treg- and Th17-differentiation in vitro but biased to Th1 differentiation. Surprisingly, CD4/p110 mice were fully resistant to EAE induction and furthermore naïve CD4 T cells of these mice did not induce colitis in RAG1 knockout animals in transfer experiments. Importantly, priming of T cells was not hampered since many MOG specific T cells were recovered in the periphery in immunized CD4/p110 mice. We found that T cells of CD4/p110 mice were not only biased to produce IFNγ but surprisingly also to produce high levels of IL-22. Finally, blocking of IFNγ partially reversed EAE susceptibility of these mice but most importantly the neutralization of IL-22 in vivo fully reconstituted susceptibility to EAE induction of CD4/p110 mice.
Conclusions: Contrary to what we expected, we found that constitutive PI3K activity, renders mice resistant in T cell-mediated autoimmune models such as EAE and transfer colitis. T cells from these mice were prone to produce IL-22 and IFNγ, which appear to be major mediators of resistance in the EAE model. The finding that IL-22 has as strong protective effects in EAE is new and this may be connected to risk alleles of the inhibitory secreted IL-22 receptor 2 found in MS patients.

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NAD+-dependent histone deacetylase SIRT6 regulates immune cell function and omeostasis

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Objective: to investigate the role of the NAD+-dependent deacetylase SIRT6 in immune cells function. SIRT6 is a class III NAD+-dependent deacetylase with a role in genome stability, glucose homeostasis, and longevity. A SIRT6 dependent mechanism was recently shown to enhance TNF, IFN-gamma and IL8 production and secretion.
Methods: WT and SIRT6KO mice were immuno-characterized focusing on immune cell subset representation in thymus and spleen. Spleen-derived CD4+ T cells were purified, cultured in presence of anti CD3 (2, 5 μg/ml) alone or in presence of anti CD28 (1 μg/ml) and TNF levels in supernatants assessed by ELISA. DCs were generated from freshly isolated bone marrow cells in presence of GM-CSF (20 ng/ml), % of CD11c+ cells asserted by flow cytometry. ImDcs were than activated in presence of CpG (1 μg/ml) And TNF, CXCL1 and CXCL2 expression monitored by QPCR at different time point.

EAE was induced in 6–8 week-old female C57B6 mice by immunization with MOG and complete Freund's adjuvant. Clinical score determination and histological evaluations were performed according to established protocols. For systemic NAD+ depletion, the nicotinamide phosphoribosyltransferase inhibitor FK866 was administered at 10 mg/kg body weight, twice a day, five days a week for two weeks. NAD+, NADH, NADP+, and NADPH levels in isolated splenocytes were detected with enzymatic cycling assays.
Results: 17 days-old SIRT6KO mice exhibit a significantly higher percentage of double positive thymocytes ($p<0.001$) and a lower representation of CD4+ or CD8+ single positive ($p<0.01$) thymocytes as compared to control animals. A 30% reduction in B220+ cells in the spleen is also typically detected thus suggesting a possible role for SIRT6 in B cell homeostasis. SIRT6KO CD4+ T cell produced reduced levels of TNF. Finally, SIRT6KO DCs differentiate normally.and shown impaired induction of Tnf, Cxcl1 and Cxcl2 mRNA in response to CpG.

FK866 administration effectively depletes NAD+, NADH and, to a lesser extent NADP+ and NADPH levels in vivo. FK866 determines a striking reduction in EAE severity and reduces CNS demyelination.
Conclusions: Our preliminary results suggest a possible role for SIRT6 in immune cell homeostasis as well as a contribution for this sirtuin to the regulation of immune cell function.

The NAD+-lowering agents FK866 reduces EAE severity, possibly by interfering with SIRT6 activity, and appears as a strong candidate for the treatment of neuroinflammatory conditions.

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Defining the minimal signals necessary to generate an encephalitogenic T cell

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Objective: Identifying the molecular signals that generate encephalitogenic T cells may provide novel therapeutic targets for multiple sclerosis (MS). We had previously observed that anti-CD3/CD28 activation of myelin-specific T cell receptor (TCR) transgenic T cells was not sufficient to generate encephalitogenic T cells, yet antigen presenting cells (APC) plus myelin peptide activation of these myelin-specific T cells generated highly encephalitogenic T cells, indicating that the APC were providing critical signals beyond T cell receptor and costimulation to the development of an encephalitogenic phenotype. This study was designed to determine which cytokines produced by APCs could recapitulate the encephalitogenic phenotype when used to activate myelin-specific T cells with anti-CD3/CD28.
Methods: Myelin-specific TCR transgenic CD4 T cells were activated with anti-CD3/CD28 in the presence of various cytokines (IL-1β, IL-6, IL-12 and IL-23) typically produced by APCs to determine if any cytokine or cytokine combination could recapitulate the encephalitogenic phenotype. After activation, the T cells were transferred into mice and monitored for EAE. In addition, the ability of these cytokines to induce expression of IL-23R, T-bet and lineage-specific cytokines was determined by flow cytometry and ELISA.
Results: No single cytokine was sufficient to generate encephalitogenic CD4 T cells. Contrastingly, T cells differentiated with IL-6/IL-23 or IL-12/IL-23 combinations were able to induce EAE. Flow cytometric analysis confirmed that IL-6 and IL-12 induced IL-23R expression on T cells.
Conclusions: We identified that IL-6/IL-23 or IL-12/IL-23 combinations provided critical signal to generate encephalitogenic T cells in the presence of TCR and costimulation molecule activation. IL-6 and IL-12 were able to induce the initial expression of IL-23R on naïve T cells during differentiation. Newly expressed receptor was sequentially engaged with IL-23, and its downstream signaling further enhanced the transcription of *IL-23R* itself. Enriched IL-23R signaling increased encephalitogenic capacity in T cells. We have previously shown that T-bet directly binds to the promoter region of *IL-23R* and regulate its transcription. It was likely that IL-12 induces the expression of IL-23R though a T-bet-dependent manner.

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RGS10 as a regulator of classical vs. alternative activation of microglia/macrophages

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Objective: Cellular machinery exists to halt classically activated microglia/macrophages and restore tissue homeostasis after injury by changing their activation state away from a pro-inflammatory reactive state to a gene expression profile that supports repair and tissue reconstruction. The cellular mechanisms that govern this switching process are currently unknown, and redundant mechanisms are likely to exist, thus ensuring that resolution and repair occurs. Previously, our group has shown that Regulator of G protein Signaling 10 (RGS10), a microglia-enriched GTPase accelerating protein (GAP) for G alpha subunits, is an important regulator of microglia activation and that RGS10-null mice displayed increased microglial burden in the CNS from birth and developed a Parkinson's disease-like phenotype in response to chronic systemic inflammation. These data suggested an important regulatory role in microglia activation responses and a neuroprotective role for RGS10 in the nigrostriatal pathway during chronic neuroinflammation. The objective of this study was to investigate the role of RGS10 as regulator of microglia/macrophage activation not only for classical but also for alternative activation.
Methods: Previously, we have shown that Regulator of G protein Signaling 10 (RGS10), a microglia-enriched GTPase accelerating protein (GAP) for G alpha subunits, is an important regulator of microglia activation and that RGS10-null mice displayed increased microglia numbers in the CNS from birth as measured by FACS analysis and immunohistochemistry. Moreover, repeated intraperitoneal injections of lipopolisaccaride (LPS) (7.5×10^5 EU/kg for 6 weeks) induced a Parkinson's disease-like phenotype (loss of nigral dopaminergic neurons) in response to chronic systemic inflammation. Here, we evacuate features of the M1 phenotype of RGS10-null macrophages (both peritoneal and bone marrow derived) upon LPS treatments by performing multiplex ELISA to measure proinflammatory cytokine release and target effector assays to measure cytotoxicity on MN9D dopaminergic neuroblastoma cells. We also studied features of the M2 phenotype of RGS10-null macrophages upon IL-4 and IL-13 treatment by performing flow cytometry, quantitative real time PCR to measure M2 marker gene expression including YM1 and Fizz1, as well as protein expression by western blot to monitor IL-4 signaling pathway, phospho-STAT6 and total STAT6. IL-4 receptor expression. Lastly, we evaluated phagocytosis responses by performing Fc-receptor mediated and *E. coli* particle-mediated phagocytosis assays and lastly we measured chemotaxis responses *in vitro*.
Results: Our results showed that RGS10-null macrophages produced higher levels of proinflammatory cytokines including TNF, IL-1 and IL-12p70 in response to LPS treatment and exerted higher cytotoxicity on MN9D cells compared to WT macrophages. RGS10-null macrophages also displayed attenuated/blunted M2 phenotype upon IL-4 and IL-13 priming. LPS treatment after IL-4 priming lead to attenuated responses of YM1 and Fizz1 mRNA expression although IL-4 receptor levels were not different between RGS10-null macrophages and WT macrophages. Importantly, phagocytic activities to both FcR-mediated and *E. coli* particle of RGS10-null macrophages were blunted in response to IL-4 priming and/or LPS treatments. However, chemotaxis assays did not reveal any differences between RGS10-null and WT macrophages.
Conclusions: Here, we characterized the M1 vs. M2 phenotype of primary RGS-null microglia, peritoneal macrophages and bone marrow-derived monocytes. Our data indicate that RGS10 limits pro-inflammatory factor production and promotes alternative activation in both microglia and macrophages. This study implicates RGS10 as an important determinant of microglia/macrophage activation status during inflammatory responses in the CNS as well as in the peripheral immune system.

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Evaluation disease activity and staging in MS and NMO by T cell subset to predict therapeutic response

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Objective: Multiple Sclerosis (MS) are a heterogeneous autoimmune disease in clinical course, typically present relapsing and remitting course (RR-MS) initially and later exhibit progression (SP-MS).

MRI is useful tools to evaluate disease activity of MS, however, it does not always detect relapses or exacerbation of MS and magnitude of lesions does not always correlate with therapeutic responses.

We seek an easy-to-use immunological marker in peripheral blood that can classify type and stage of MS and possesses correlation with disease activity and therapeutic response.

Methods: We collected PBMC from MS and NMO patients and checked the frequency of $CD4^+CD25^+$ T cells and fractions of $CD25^{high}$ and $CD25^{low}$ among them.

We chose steroid pulse therapy against relapses or exacerbation at first and extend the choice to plasmapheresis/exchange, mitoxantrone or cyclophosphamide. We checked several markers related to Treg or Th17 to clarify the characteristics of fractions of $CD25^{high}$ and $CD25^{low}$ and also IL6 in CSF from some patients.

Results: 1) At steady state, $CD4^+CD25^+$ T cells were higher in MS than in NMO or collagen diseases. 2) At relapse or exacerbation, $CD25^{high}$ fraction decreased in RR-MS and NMO but not changed in SP-MS, whereas $CD25^{low}$ fraction increased in SP-MS and NMO but not changed in RR-MS. 3) The ratio of $CD4^+CD25^{high}/CD4^+CD25^{low}$ was correlated with therapeutic response in both RR-MS, SP-MS and NMO and maintained high in the patients with good therapeutic response. 4) $CD4^+CD25^{high}$ was mostly (80–90%) $Foxp3^+CTLA4^+$ Treg with capacity to produce IL10 and LAP and also contained $Foxp3^+RORgt^+$ subset producing both IL10 and IL17. $CD4^+CD25^{low}$ contained some (40%) $Foxp3^-CTLA4^+$ Treg and $Foxp3^-$ Th17 expressed high IL6R producing only IL17. 5) IL6 in CSF is high in exacerbation or relapse of SP-MS and NMO.

Conclusions: Foxp3 expression is not always constant in human Treg according to activation and may coexpress RORgt. Despite heterogeneous population more than Treg, focusing just on the expression level of CD25, we found that the disease activity in RR-MS involved with $CD4^+CD25^{high}$, which contains $Foxp3^+$Treg, the disease activity in SP-MS involved with $CD4^+CD25^{low}$, which contains pathogenic Th17, and the disease activity in NMO involved with the balance and transition between both fractions. Moreover, increase of $CD4^+CD25^{low}$ in SP-MS and NMO seems to be related to IL6 in CSF, which suggested the responsibility to poor response to traditional therapies.

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Altered T cell migration rather than induction of apoptosis is essential for glucocorticoid therapy of experimental autoimmune encephalomyelitis

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Objective: High-dose glucocorticoid (GC) therapy is widely used to interfere with acute relapses of multiple sclerosis (MS) patients but the exact mechanism remains unclear.

Methods: We have used experimental autoimmune encephalomyelitis (EAE) as a model of multiple sclerosis in combination with different mouse strains genetically modified in the locus of the glucocorticoid receptor (GR) to obtain new insight into the modes of action used by GC to ameliorate neuroinflammation.

Results: We have recently found that peripheral T cells are the main target cells of GC therapy of EAE. Surprisingly, it now turned out that induction of T cell apoptosis by GC is fully dispensable for their beneficial effects. Moreover, even a dimerization-deficient GR that lacks both, the capacity to induce apoptosis and the ability to activate gene expression in general, was sufficient to reduce leukocyte infiltration of the CNS and to ameliorate EAE. In part, this can be explained by repression of IL-17 and IFNγ but not GM-CSF production, a mode of GC action that is retained in mice exclusively expressing a monomeric GR. Further, we found that GC also exert profound and sometimes opposing effects on T cell migration along various chemokine gradients. These GC effects are also mediated by the monomeric GR, and administration of pharmacological inhibitors *in vivo* indicates that the modulation of T cell migration is indeed central to the capacity of GC therapy to ameliorate EAE.

Conclusions: Our findings suggest that GC act at least in part by redirecting T cell migration rather than by inducing T cell apoptosis.

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Paracaspase MALT1 deficiency protects mice from autoimmune-mediated demyelination

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The paracaspase mucosa-associated lymphoid tissue 1 (MALT1) mediates signaling to the transcription factor NF-κB and is central to lymphocyte activation and proliferation. Besides its scaffold function in T and B cell receptor signaling, MALT1 also has proteolytic activity and cleaves specific proteins that are believed to facilitate lymphocyte activation. These functions suggest an important role of MALT1 in autoimmunity but so far its in vivo role in autoimmune disease development has not been documented. Therefore, we studied experimental autoimmune encephalomyelitis (EAE), an animal model of multiple sclerosis, in mice deficient in MALT1. In contrast to wild-type and MALT1 heterozygous mice, MALT1 knockout mice were completely protected against the development of EAE. Lymphocyte and macrophage infiltration into the spinal cord parenchyma was completely absent in MALT1 knockout mice, as was the extent of demyelination and proinflammatory gene expression. Experiments with bone marrow chimeric mice showed that MALT1 deficiency in hematopoietic cells is sufficient for EAE resistance. Adoptive transfer experiments showed that MALT1 deficiency in splenocytes is sufficient for EAE resistance. Moreover, autoreactive T cell activation was severely impaired in MALT1 deficient T cells, suggesting the inability of MALT1 deficient effector T cells to induce demyelinating inflammation in the CNS. Finally, the MALT1 substrate A20 was completely processed in wild-type T cells during EAE, but not in MALT1 deficient mice, suggesting a contribution of MALT1 proteolytic activity in T cell activation and EAE development. Together, our data identify MALT1 as an essential modulator of the autoimmune process during the early priming phase of EAE disease, suggesting that MALT1 may be an interesting therapeutic target in the treatment of multiple sclerosis.

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MOG-antibodies trigger pre-active lesion formation in multiple sclerosis

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Objective: Multiple sclerosis (MS) is a complex chronic disease of the central nervous system characterized by lesions of inflammation, axonal loss, astrogliosis and demyelination. The lesions in the brain continually form and regress during disease and disease activity. Well before any obvious myelin damage, leukocyte infiltration or blood–brain-barrier breakdown, clusters of activated microglia are seen in the normal appearing white matter. These so called pre-active lesions are found during all stages of disease. The microglia clusters are associated with stressed oligodendrocytes that express alpha B crystallin (CRYAB), a stress-induced heat shock protein. In vitro, CRYAB induces an immune regulatory response in human microglia indicting the same might be true in vivo.

Here we hypothesize that antibodies against the myelin oligodendrocyte glycoprotein (MOG) may lead to oligodendrocyte stress and CRYAB expression in pre-active lesions.
Methods: Experimental autoimmune encephalitis was induced with wild-type or MOG-deficient myelin and serum applied to rat oligodendrocytes. Cerebrospinal fluid from MS patients and controls was examined for the presence of antibodies to native MOG-in-myelin. The presence of antibodies is compared with the pathology to examine the relationship of MOG antibodies to the presence of pre-active lesions. Cultured oligodendrocytes will be treated with CSF from control and MS patients, and serum from mice to examine the role of MOG antibodies in induction of CRYAB.
Results: We show that serum from mice immunised with WT but not MOG deficient myelin induce rearrangement of MOG in oligodendrocytes, changes in intracellular signaling and cell morphology. Analysis of human CSF IgG reactivity against the full range of human myelin-associated proteins and stress induction in in-vitro studies are on-going.
Conclusion: Our data points to a strong role for MOG antibodies in inducing oligodendrocyte stress in vitro. While it is still unclear whether CSF from MS patients can induce CRYAB expression in vitro we present the findings from screening CSF samples from MS and controls to the full range of human myelin proteins present in MS and controls.

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Analysing the functional phenotypes of the multiple sclerosis-associated gene variants of DNAM1 (CD226)

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Recent genome-wide association studies identified a SNP haplotype in the gene of the DNAX accessory molecule 1 (DNAM-1 or CD226), which shows an association with a variety of autoimmune diseases, including multiple sclerosis (MS). CD226 is constitutively expressed on the surface of NK cells, $CD4^+$ and $CD8^+$ T cells and is involved in various cell functions. However, the role of CD226 in autoimmunity has not yet been defined. Here we investigate the phenotype of the MS predisposing and protective *CD226* SNP variants and their functional relevance for the development of MS and its murine model experimental autoimmune encephalomyelitis (EAE). Comparison of CD226 expression levels on immune cells from peripheral blood of healthy donors showed that carriers of the risk haplotype possess a significantly reduced CD226 expression on the surface of memory $CD4^+$ T cells compared to carriers of the protective haplotype. Moreover, this reduced expression level can also be observed in MS patients carrying the protective haplotype. To understand the relevance of the phenotypical difference for the development of autoimmunity we translated these findings into the EAE model. First, we detected elevated CD226 expression almost exclusively on activated as well as memory T cells in the spleen and lymph nodes. Similarly, CNS infiltrating $CD4^+$ T cells show high CD226 expression, suggesting an important role during the effector phase, the extravasation of T cells into the CNS or their subsequent control. Active immunization with the MOG35-55 peptide resulted in an aggravated EAE disease course in *Cd226*-deficient mice in comparison to wildtype littermate controls. Additionally, passive immunization using adoptive transfer of encephalitogenic MOG35-55-specific 2D2-transgenic $CD4^+$ T cells into recipient wild type mice showed an earlier disease onset when we used *Cd226*-deficient 2D2 cells in a gene-dose dependent manner than mice that received wild type 2D2-transgenic cells. These results suggest that reduced expression levels of CD226 may lead to a dysregulated immune response culminating in enhanced autoreactivity of T cells. Currently, we are investigating the exact mechanisms of how reduced CD226 expression levels on T cells exacerbate autoimmune responses.

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Neuroinflammation induces GABA reuptake transporter-2 in multiple sclerosis: Immune regulation by the neurosteroid ganaxolone

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Objective: Neurosteroids modulate the actions of γ-aminobutyric acid (GABA) including its anti-inflammatory effects. The GABA Reuptake Transporters (GATs) regulate GABA's intra- and extra-cellular concentrations, although the impact of neurosteroids on GATs' expression and function are unknown. We previously reported that the neurosteroid, allopregnanolone, was depleted in brains from persons with multiple sclerosis (MS) and its replacement was highly neuroprotective in MS models. Herein, we hypothesized that GATs might participate in MS pathogenesis, which could be modulated by the synthetic allopregnanalone analog, ganaxolone (GNX).
Methods: Protein and mRNA expression in autopsied cerebral white matter from MS with matched control patients and cultured primary human cells was assessed by deep sequencing and RT-PCR. GABA levels were measured by HPLC. The MOG/PTX experimental autoimmune encephalomyelitis (EAE) model in mice was also used to test the present hypothesis.
Results: GAT-2 expression was selectively increased in cerebral white matter from patients with MS, particularly in macrophages within demyelinating lesions. siRNA knockdown of GAT-2 in human lymphocytes increased extracellular GABA levels with a concordant reduction in immune gene transcript expression. Human macrophages stimulated with IFN-γ exhibited increased GAT-2 together with HLA-DR and STAT-1 expression, which was suppressed by treatment with GNX. In EAE, treatment with GNX protected axons, preserved myelin, reduced GAT-2, CD3, Class II, IFN-γ, STAT-1, IL12p35 expression and improved neurobehavioral outcomes. In lymphoid tissues from EAE animals treated with GNX, the Th1 ($CD3^+$/T-bet^+) lymphocyte population was reduced.
Conclusions: These findings emphasized the protective effect of the synthetic neurosteroid ganaxolone, coupled to a reduction in neuroinflammation by targeting the GABAergic system. Targeting GABAergic pathways is a promising strategy for treating neuroinflammatory diseases such as MS.

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Aiolos promotes TH17 cell differentiation by directly silencing Il2 expression

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Objective: To study the role of the transcription factor Aiolos, encoded by the *ikzf3* gene, in the differentiation of pro-inflammatory Th17 cells.
Methods: IL-17 producing $CD4^+$ T (T_H17) cells are instrumental in the immune response to pathogens. However, an overactive T_H17 response results in tissue inflammation and autoimmunity and therefore it is important to identify the molecular mechanisms that control the development of T_H17 cells. We found that the transcription factor Aiolos is upregulated during the differentiation of Th17 cells. In these studies, we used Aiolos-deficient mice in *in vitro* and *in vivo* experimental systems to investigate the role of Aiolos in Th17 cells.
Results: Interleukin 2 (IL-2) suppresses T_H17 cell development, but how IL-2 production is actively suppressed during T_H7 cell differentiation is not understood. We found that under T_H 17-polarizing conditions the transcription factors STAT3 and AhR upregulate the expression of the Ikaros family transcription factor Aiolos. Using Aiolos-deficient mice, we demonstrate that Aiolos controls the epigenetic status of the *Il2* locus, actively inhibiting IL-2 production and promoting the differentiation of T_H17 cells *in vitro* and *in vivo*.
Conclusions: Thus, we have identified a module in the transcriptional program of T_H17 cells that actively limits IL-2 production and promotes their differentiation.

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Effect of HLA-G gene polymorphisms in the production of sHLA-G molecules in relapsing-remitting multiple sclerosis

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Objective: Multiple sclerosis (MS) is a chronic inflammatory demyelinating and neurodegenerative disease of the central nervous system with an uncertain etiology that is commonly considered to be autoimmune in nature. A growing body of evidence has indicated a possible involvement of HLA (Human Leukocyte Antigen)-G antigens where this molecule seems to exhibit anti-inflammatory properties. HLA-G is a HLA-Ib molecule with a physiological tissue-restricted distribution, membrane-bound and soluble isoforms and immuno-regulatory functions mediated by the interaction with specific immune-inhibitory receptors (ILT-2, ILT-4, CD8, KIR2DL4). Cerebrospinal fluid (CSF) levels of soluble HLA-G (sHLA-G) were more elevated in MS than in controls, and in MS patients without magnetic resonance imaging (MRI) evidence of disease activity. The 3′ untranslated region of the *HLA-G* gene is characterized by two polymorphisms, DEL/INS14bp and +3142C>G, which control soluble HLA-G (sHLA-G) production: INS allele destabilizes mRNA and +3142G allele interact with microRNA. We investigated the influence of these two *HLA-G* variants on sHLA-G serum and CSF levels in MS patients.
Methods: We analyzed 69 unrelated Relapsing-Remitting MS patients (75 females, 41 males; mean age 36.5 ± 11 years) classified following McDonald. They were grouped in MRI inactive and active disease by Gadolinium (Gd)-DTPA-enhancement on MRI T1-weighted scans (GE Sigma Horizon). HLA-G polymorphisms were analysed by Real Time PCR with specific primers. sHLA-G molecules were quantified by ELISA and Western Blot systems with an HLA-G specific monoclonal antibody (MEM-G9, Exbio, Praha).
Results: Serum and CSF sHLA-G levels were more elevated in patients with DEL and C alleles, high sHLA-G producers and were different among the various combined *HLA-G* genotypes in both MRI inactive and active diseases. The highest and the lowest values were identified in MS patients with C/C,DEL/DEL and G/G,INS/INS genotypes, respectively.
Conclusions: These results report that serum and CSF sHLA-G levels in MS could be influenced by *HLA-G* polymorphisms irrespective of the inflammatory microenvironment. Taken together, these findings suggest that *HLA-G* polymorphisms should be taken into account when serum and CSF levels of sHLA-G are analyzed in MS, since, not only are they regulated by local inflammatory microenvironment, but they are also influenced by 14bpDEL/INS and +3142C>G individual polymorphisms.

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Identification of circulating nonclassic human leukocyte antigen G (HLA-G)-dimer molecules in cerebrospinal fluids from MS stable patients

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Objective: HLA-G is a non-classical HLA class I antigen expressed as membrane bound and soluble (sHLA-G) isoforms with a restricted tissue distribution and anti-inflammatory functions due to binding affinity for immune inhibitory receptors (ILT-2, ILT-4, KIR2DL4). sHLA-G levels in cerebrospinal fluid (CSF) were demonstrated to be more elevated in multiple sclerosis (MS) patients than in controls, and in MS patients without magnetic resonance imaging (MRI) evidence of disease activity. Recently, it was discovered that HLA-G can exist as a dimer with a substantially increase binding to inhibitory receptors.
Methods: The presence of HLA-G dimers and monomers was evaluated in CSF samples from 52 MS patients subdivided according to MRI in active (N = 18) and stable (N = 34) disease, 50 patients with other inflammatory (OIND) and 50 patients with non-inflammatory

(OIND) neurological diseases. The samples were analyzed by ELISA test and Western Blot assay in native and denaturating conditions by means of two HLA-G specific monoclonal antibodies (ELISA and immunoprecipitation: MEM-G9, G233; Exbio, Praha).
Results: The ELISA and Western blot results reported increased sHLA-G CSF levels in MS patients in comparison with OIND and NIND patients ($p<0.0001$) and higher CSF concentrations in stable than in active MS patients ($p<0.001$). The native Western blot analysis revealed both the HLA-G monomeric (39 kDa) and dimeric (78 kDa) conformations in CSF samples from MS patients with a stable disease, in NIND and OIND patients. NIND and MS stable patients presented a prevalence of the dimeric isoform. The Western blot under reducing conditions showed also a faint band at 53 kDa. On the contrary, CSF samples from MS patients with an active disease presented only the HLA-G monomeric conformation. The different HLA-G structures are recognized by both the anti-HLA-G antibodies G233 and MEM-G9 in ELISA and Western blot assays.
Conclusions: These data demonstrate that HLA-G dimeric molecules are mainly present in CSF samples from stable MS and NIND patients, while CSF from active MS patients present only monomeric HLA-G conformation. The presence of dimeric HLA-G structure in CSF samples from stable MS disease status support the implication of this structure in immune and inflammation control in central nervous system. In fact, this conformation is characterized by an increased binding avidity than HLA-G monomers, which translates into augmented signaling through ILT-2 receptor.

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Bowman-Birk protease inhibitor (BBI) suppresses Th17 differentiation through IL-27 signaling pathway

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Objective: To study the effect of BBI on Th-17 cell differentiation.
Background: BBI is a soybean-derived serine protease inhibitor. We have previously shown that oral administration of BBI can dramatically ameliorate experimental autoimmune encephalomyelitis through an IL-10 dependent mechanism. We and others also have shown that IL-27 plays an important role in suppressing Th17 cells in CNS autoimmune inflammation. We hypothesized that BBI suppresses Th17 cell differentiation through IL-27 signaling pathway.
Methods: Splenocytes from C57BL6 and IL-27R KO mice were collected and stimulated with Anti-CD3 and Anti-CD28 (1 μg/ml) in Th17 polarization condition with TGF-β (2 ng/ml) + IL-6 (20 ng/ml) in the presence or absence of BBI (1st stimulation) for 72 h. To investigate the effect of BBI on committed Th17 cells, after the 1st stimulation, cells were rested 2 days with IL-2 and then reactivated with anti-CD3 and anti-CD28 antibodies plus IL-23 (±BBI 0.1 and 0.5 mg/ml) (2nd stimulation) for another 3 days. In both 1st and 2nd stimulations, cells were stimulated with PMA/Ionomycin/Golgi-plug for 4 h to perform intracellular staining of IFN-g, IL-17 and IL-10.
Results: BBI significantly inhibited differentiation of Th17 cells in C57BL6 mice after 72 h (21.1% vs 4.63%). Furthermore, BBI slightly suppressed committed Th17 cells (19.5% vs 14.8%) in a dose dependent manner. Although BBI suppressed Th17 differentiation in C57BL6 mice, it did not affect differentiation of Th17 T cells in IL-27R KO mice (22.6% vs 20.1%).
Conclusions: BBI suppresses CD4 + T cell polarization towards Th17 cells. IL-27 signaling pathway appears important in this process. BBI has the potential as a safe and oral therapy in autoimmune diseases like multiple sclerosis.

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Potential role of CCR6+ T cells in inflammatory demyelinating diseases of central nervous system

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Background: Recent genome-wide association studies revealed that a polymorphism in *CCR6*, the gene encoding chemokine (C–C motif) receptor 6, was associated with susceptibility of some autoimmune disorders such as rheumatoid arthritis, Graves' disease, and Crohn's disease. It has been reported that CCR6 is expressed in IL-17-producing $CD4^+$ T helper cells (T_H17) and innate T cells (*i*T) such as γδ T cells, suggesting that $CCR6^+$ T cells are critically involved in IL-17-driven autoimmunity in mouse models including experimentally autoimmune encephalitis.
Objective: To assess a role of $CCR6^+$ T cells in autoimmune inflammatory demyelinating diseases of central nervous system including multiple sclerosis (MS) and neuromyeltis optica (NMO).
Methods: Peripheral blood was obtained from 22 patients with MS, 16 patients with NMO spectrum disorders, and 30 healthy subjects (HS). We analyzed the expression of several chemokine receptors on the cell surface of peripheral mononuclear cells (PBMC) by flow cytometry.
Results: Within the $CD4^+$ T cell population ($CD3^+CD4^+$), the frequency of $CCR6^+CD3^+CD4^+$ T cells did not differ between NMO, MS, and HS. However, within the $CD4^{neg}$ T cell population ($CD3^+CD4^{neg}$), $CCR6^+CD3^+CD4^{neg}$ T cells decreased in frequency in PBMC from NMO patients as compared to MS patients and HS. More strikingly, $CD161^{high}CCR6^+CD3^+CD4^{neg}$ T cells decreased in frequency in PBMC from NMO patients as compared to MS patients and HS. Within the $CD19^+$ B cell population, plasmablasts ($CD19^+CD27^+CD38^{high}CD20^{neg}$) increased in frequency in PBMC from NMO patients as compared to MS patients and HS. In this cohort, the frequency of plasmablasts was negatively correlated with the frequency of $CCR6^+CD3^+CD4^{neg}$ T cells in peripheral blood.
Conclusions: The CCR6 ligand CCL20 was constitutively expressed not only in epithelial cells of choroid plexus but also in astrocytes of inflamed brains, and thus CCR6 and CCL20 may represent an evolutionary conserved axis that regulates the CNS entry and dissemination of T cells during inflammation. The *i*T cells including γδ T cells, Vα24*i*T cells, and Vα7.2*i*T cells constitute a large percentage of $CCR6^+CD3^+CD4^{neg}$ T cells. Therefore this study suggests that the $CCR6^+CD3^+CD4^{neg}$ T cell population including *i*T cells might be a role contributing to alterations in B cell subset composition in NMO pathogenesis. Furthermore our results provide evidence that compartmentalized immune responses within CNS might not be sustained in NMO, in contrast with MS.

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Evidence supporting the existence of a novel histaminergic pathway in the regulation of experimental allergic encephalomyelitis susceptibility

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Objective: Histamine (HA) is a key regulator of experimental allergic encephalomyelitis (EAE), the autoimmune disease model of multiple sclerosis (MS). Histidine decarboxylase deficient mice (HDCKO), which are unable to synthesize HA, exhibit more severe EAE. HA exerts its effects through four different G protein coupled receptors (GPCR): H_1, H_2, H_3 and H_4 ($H_{1-4}R$). Each HA-receptor has been shown to influence EAE pathogenesis. In the mammalian brain, however, there is evidence for the existence of non-GPCR signaling by HA which is picrotoxin-sensitive and mediated by chloride conductance. In addition, γ-aminobutyric acid ($GABA_A$) receptor subunits can form HA-gated chloride channels *in vitro* suggesting that an ionotropic HA-receptor might contain known ligand-gated chloride channel subunits. Here our objective is to test the hypothesis that non-GPCR signaling by HA plays a role in autoimmune responses.
Methods: H_{1-4}RKO mice were generated. We assessed the susceptibility to EAE by immunizing H_{1-4}RKO, WT and HDCKO mice with MOG_{35-55} + CFA + PTX. Changes in the immune response associated with EAE in H_{1-4}RKO and WT mice were analyzed both in the draining lymph node (DLN) and the central nervous system (CNS) by ELISA and flow cytometric analysis. Differentiation and cytokine production of $CD4^+$ T cells were also studied under MOG_{35-55} specific and polyclonal stimulation. We also assessed the encephalitogenic potential of T cells by adoptively transferring T cells from WT or H_{1-4}RKO donors to WT recepients.
Results: H_{1-4}RKO mice, which lack all four GPC-HRs, are remarkably resistant to MOG_{35-55}-induced EAE and exhibit less severe neuropathology compared to WT and HDCKO mice. Splenocytes and the CNS infiltrating $CD4^+$ T cells from immunized H_{1-4}RKO mice produce significantly less IFN-γ compared to WT mice. Polyclonally activated $CD4^+$ T cells or *in vitro* differentiated Th1 effector cells from H_{1-4}RKO mice produce significantly less IFN-γ. Furthermore, under adoptive transfer conditions Th1 effector cells from H_{1-4}RKO mice are less encephalitogenic when compared to WT mice.
Conclusions: We have studied the function of endogenous HA on EAE susceptibility. H_{1-4}RKO mice are highly resistant to EAE. Our findings strongly support the idea that HA can be an endogenous ligand or an allosteric modulator to the $GABA_AR$ expressed on immune cells. Understanding the existence of this novel inhibitory signaling pathway by HA might help in designing new therapies for EAE and possibly MS.

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Inhibitory pathway TIM-3/Galectin-9 in patients with Multiple Sclerosis

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Objective: To define the possible role of inhibitory pathway T cell immunoglobulin domain and mucin domain-containing molecule-3 (Tim-3)-Galectin-9 in progression of Multiple sclerosis (MS).
Methods: We analysed by flow-cytometry Tim-3 expression on MBP-stimulated CD4+ and CD8+ T lymphocytes and CD14+ cells, as well as Annexin V expression and IFN-γ production in 50 MS patients with a diagnosis of relapsing-remitting (RR), primary progressive (PP), secondary progressive (SP), or benign MS (BMS). Forty age and sex matched healthy controls (HC) were also enrolled in study.
Results: in BMS patients MBP-stimulated: 1) Tim-3-expressing CD4+ and CD8+ T-cells; 2) CD4+AV+Tim-3+ and CD8+AV+Tim3+ (apoptotic cells); were significantly augmented compared to the values observed in the other groups of patients; results observed in HC were comparable to those seen in BMS patients.
Conclusions: These data suggest that the maintenance of physiologic Tim-3 expression is associated with a benign course of MS, possibly via the induction of apoptosis of MBP-specific lymphocytes.

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Evaluation of Th17-related parameters in multiple sclerosis patients under immunomodulatory IFN-β1 therapy

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Objective: Multiple sclerosis (MS), the inflammatory demyelinating disease of the central nervous system has a certain autoimmune background. Recently the pivotal role of Th17 association pathway has been shown in MS immunpathogenesis.
Methods: In this study, Th17-related cytokines and cytokine receptors were evaluated in MS patients under immunomodulatory IFN-β1 therapy. Twenty one relapsing remitting MS patients were enrolled in the study and peripheral blood samples were collected during 2 years follow-up. IL-23, IL-26, and IL-17 levels were determined by ELISA; IL-17R and IL-23R expression was analyzed by flow cytometry.
Results: Before immunomodulatory treatment initiation; IL-23R expression was rarely detected on $CD4^+$ T cells of both controls and MS patients. $CD13^+$ myeloid cells were highly positive for IL-17R. No significant difference was found between the expression of IL-17R and IL-23R and the IL-23 levels of MS patients compared to controls.

Of three cytokines, IL-26 was the highest in the MS patients without IFN-β1 treatment when compared to controls. Additionally; there was no significant difference in sIL17 levels between MS patients and controls.
Conclusions: After treatment and during follow-up, the level of $CD13^+IL\text{-}17R^+$ myeloid cells was significantly decreased after the 3rd month of IFN-β1 treatment. Interestingly, serum IL-23 level gradually increased during IFN-β1 therapy. Following a drastic drop after the first month of treatment, a significant increase of serum IL-26 levels was observed starting from the 6th month. On the other hand, IL-17 levels were steadily decreased during the follow-up, especially after the first year. The gradual increase in IL-23 and IL-26 during the treatment may indicate a compensatory response of immune system to the inhibition of Th17 responses. These results support the therapeutic effect of IFN-β1 on Th17-associated autoimmune background of MS. Alternatively, the increase in cytokines such as IL-23 and IL-26 that are involved in Th17-related immune responses may constitute a salvage pathway restricting the efficacy of immunomodulatory therapy.

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Interferon-beta up-regulates suppressor of cytokine signalling 1 in T cells of multiple sclerosis patients

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Objective: Suppressors of cytokine signalling (SOCS) are key regulators of cytokine responses. SOCS-1 plays a beneficial role in experimental autoimmune encephalitis (EAE). Although interferon-beta (IFN-β) is commonly prescribed for the treatment of multiple sclerosis (MS), its mode of action is not fully understood. We postulate that IFN-β has a positive impact through the induction of SOCS-1. Our goals are to assess SOCS-1 expression in T cells of MS patients and to evaluate its anti-inflammatory functions.
Methods: Using qPCR, we quantified SOCS-1 mRNA levels in CD4 and CD8 T cells from untreated or IFN-β-treated MS patients and healthy controls. We tested the impact of a SOCS-1 mimic, tyrosine kinase inhibitory peptide, which protects mice from EAE, on CD4 and CD8 T cell responses to cytokines with pro-inflammatory properties using flow cytometry-based assays.
Results: We found that SOCS-1 is significantly more expressed by both CD4 and CD8 T cells from IFN-β-treated MS patients compared to untreated patients and healthy controls. Mimicking the effect of SOCS-1 in-vitro significantly reduced proliferation and production of IFN-gamma (IFN-γ) and granzyme B in human CD4 and CD8 T cells in response to pro-inflammatory cytokines.
Conclusions: Our results indicate that the IFN-β therapy in MS patients leads to elevated SOCS-1 levels in circulating T cells. Our in-vitro assays show that SOCS-1 interferes with cytotoxic T cell functions and the production of the Th1 signature cytokine IFN-γ. Hence, IFN-β may indeed mediate its anti-inflammatory effects via an upregulation of SOCS-1. In addition, mimicking SOCS-1 in MS may overcome previous drawbacks of IFN-β therapy such as non-responsiveness and the formation of neutralizing antibodies.

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Regulation of immune responses by neurons in the central nervous system

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The central nervous system (CNS) is now known to actively communicate with the immune system. While studies on glial cells, especially microglia, have highlighted the importance of this cell type in innate immune responses of the CNS, the immune functions of other cell types, especially neurons are largely elusive. However, recent findings suggest that neurons also actively participate in immune responses by controlling glial cells and infiltrated T cells. The aim of this presentation is to address the immune function of neurons, and the roles they play in regulating inflammatory processes and maintaining homeostasis of the CNS. The ability of neurons to limit immune responses in the CNS depends on their own integrity. Healthy neurons normally express many molecules, for example intercellular adhesion molecule-5 (ICAM-5), to constitutively down-regulate T-cell or microglial activation. However, injured neurons may upregulate pro-inflammatory cytokines and neurotoxic proteins, such as high mobility group box 1 (HMGB1), which eventually exacerbate neuronal damages.

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Multiple sclerosis-associated CLEC16A has a key role in processing and surface expression of HLA class II via late endosomes

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Objective: In previous GWA studies, genetic variation in C-type lectin domain family 16, member A (CLEC16A) has been shown to associate with several autoimmune diseases, including multiple sclerosis (MS). The function of CLEC16A in humans is unknown, but studies in Drosophila implicate a role in autophagy and endosomal maturation. In this study, we assessed the expression patterns of CLEC16A in MS and its biological function in immune cells.
Methods: We obtained post-mortem brain white matter (WM) tissues of MS patients and both age- and gender-matched non-demented controls (NDC) from the Netherlands Brain Bank. Human melanoma cell line MelJuSo and primary DC were used to study the effect of CLEC16A silencing on HLA class II biology. MelJuSo cells were transfected with siRNA duplexes against CLEC16A. Monocytes from healthy donors were transduced with CLEC16A shRNA lentivirus prior to differentiation into dendritic cells (moDC).
Results: CLEC16A mRNA expression levels were strongly increased in MS patients versus controls. Relative expression was 3-fold higher in brain WM ($n = 16$ vs 13, $p = 0.028$) and 2-fold higher in PBMC from peripheral blood ($n = 72$ vs 47, $p = 0.006$). Protein expression was mainly observed in HLA class II^+ perivascular areas of MS WM. We also found coexpression of CLEC16A with HLA-DR, indicating presence in microglia. In PBMC from healthy controls, CLEC16A was predominantly expressed by APC, with high abundance in monocytes, macrophages and moDC. In moDC, CLEC16A colocalized with HLA-DR and CD63, a late endosomal marker, suggesting involvement of CLEC16A in late endosomal processing of HLA class II. To test this, MelJuSo cells were treated with different CLEC16A siRNA, which caused a 70–80% reduction of target mRNA expression. As a result, cell surface expression of HLA-DR was decreased for ~20% ($n = 7$ experiments), as determined by flow cytometry. CLEC16A siRNA-treated cells further showed intracellular accumulation and more dispersed localization of $HLA\text{-}DR^+$ and both $CD63^+$ and $Rab7^+$ late endosomal compartments. Similar effects on HLA-DR phenotype were seen for moDC treated with CLEC16A shRNA, showing ~50% reduction in HLA-DR surface expression, of which validation experiments are ongoing.
Conclusions: The increased expression of CLEC16A in MS and its role in late endosomal processing of HLA class II in APC may point to an autoimmune mechanism in which CLEC16A promotes the function of late endosomes resulting in excessive HLA class II antigen presentation.

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Interleukin-17A promotes accumulation of activated microglia/macrophages in different models of inflammatory CNS diseases

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Objective: Interleukin-17A (IL-17) is a key player in many autoimmune and infectious diseases and bridges the innate and adaptive immune response. Though the pivotal role of this cytokine has clearly been demonstrated for several peripheral organs, we and others demonstrated only minor direct impact of IL-17 on the induction of CNS tissue destruction or neurological symptoms. Nevertheless there is still a convincing body of evidence for a key role of IL-17 during neuroinflammatory responses.
Methods: To clarify its function in CNS immunity, we generated and characterized a transgenic mouse with an astrocyte targeted production of IL-17 (GF/IL17) and applied different neuroinflammatory disease models.
Results: The transgenic GF/IL17 mouse does not develop neurological deficits or cellular infiltrations. During systemic endotoxemia, induced by LPS injection, GF/IL17 mice exhibited a significantly pronounced accumulation of activated $CD45^{high}/CD11b^{+}$ microglia/macrophages and an upregulation of the proinflammatory cytokines TNF-α and IL1-β compared to controls. Histological characterization revealed a pronounced microglial activation by morphological criteria. Furthermore during Cuprizone-induced demyelination, CNS production of IL-17 lead to significantly increased numbers of Iba1 positive microglia/macrophages in the corpus callosum throughout disease course. Colocalization of Iba1 with the lysosomal marker CD68 revealed a significantly increased microglial activation in GF/IL17 mice during toxic demyelination, respectively.
Conclusions: These data point toward a modulating effect of IL-17A on activation and recruitment of microglia/macrophages during neuroinflammation and might indicate a main cellular target population of the cytokine IL-17 in the CNS.

Genetics

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Neuinflam9/Toxo1 regulates the innate immune response after nerve injury and susceptibility to autoimmune neuroinflammation in rat

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Objective: The activation of CNS resident cells is a common theme in different neurological disorders exhibiting innate immune responses through activating toll-like receptors signaling pathways.
Methods: To identify the genes predisposing CNS disorders through innate responses regulation, we performed a genome-wide linkage scan for expression of innate immune molecules after a standardized nerve injury in a rat F2 (BNxLEW.1N) intercross.
Results: We identified multiple expression quantitative trait loci (eQTLs) responsible for the regulation of several toll-like receptors (TLRs) and interferon regulatory factors (IRFs). Among these were the previously identified *Neuinflam9* QTL on rat chromosome 10, which overlaps with *Toxo1* regulating resistance to Toxoplasma gondii, here found to also regulate the expression of *Tlr2* and *Irf7*. The influence of *Neuinflam9*, on the expression of these innate immune molecules after nerve injury was verified in two different BN.LEWc10 congenic lines (lineages B and E). The congenic BN.LEWc10–E also demonstrated resistance to experimental autoimmune encephalomyelitis strongly suggesting that the same gene(s) that regulated expression of innate immune molecules also regulates predisposition to autoimmune disease in the CNS.
Conclusions: Identification of the gene(s) underlying these QTLs may reveal common pathways regulating innate immune activation in the CNS and identify new therapeutic targets for neuroinflammatory disorders.

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Association of HLA-DRB1*14, DRB1*16 and HLA-DQ5 with MuSK-myasthenia gravis in Turkish patients

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Objective: A strong association of a relatively rare group of myasthenia gravis (MG), muscle-specific kinase antibody-positive (MP) has been reported with HLA-DR14-DQ5 followed by another report suggesting a role for HLA-DRB1*16 and DQ5. In this study, the reported association between *HLA-DRB1*14,*16* and *HLA-DQ5* with MP MG and double negative (SN) MG patient groups was investigated in a Turkish population.
Methods: The study group included 224 generalized MG patients (77 men/147 women, mean age: 42 ± 16.8 years). Among the MG group, 116 patients had antibodies against anti-acetylcholine receptor (AP), 48 were MP and 60 were SN. Thymoma was present in 26% of the AP patients and 20% had a disease with late onset (>50). Among the MP patients, only 7 out of 48 had late-onset MG (14.5%). The data of 250 healthy donors (HC, 109 men/141 women, mean age: 29.8 ± 8.6) were evaluated as control. Polymerase chain reaction with sequence specific primers (PCR-SSP) was used for typing of *HLA-DRB1*03, DRB1*14, DRB1*16* and HLA-DQ5 (DQB1*05) allele groups.
Results: We found a highly significant association of both *DRB1*16* and *DRB1*14* with MP compared to HC (37.5 vs. 10.8%, $p = 1.9 \times 10^{-5}$, OR: 4.95 95% CI: 2.4–10.1 and 29.2 vs. 11.6%, $p = 0.0028$, OR: 3.1, 95% CI: 1.5–6.5, respectively). *DRB1*16* was also more frequent in the SN group (23.3%, $p = 0.012$, OR: 2.5 95% CI: 1.2–5.2). At the DQB1 locus, *DQB1*05* was also associated with MP (73 vs. 36% $p = 2.5 \times 10^{-6}$ OR: 4.8 95% CI: 2.4–9.5). When *DRB1* and *DQB1* results were evaluated together, *DRB1*16*-DQ5 and *DRB1*14*-DQ5 putative haplotypes were also associated with MP (27 vs. 11%, $p = 0.006$, OR: 2.9 95% CI: 1.4–6.2 and 33 vs. 10.8%, $p = 0.0002$ OR: 4.1 95% CI: 2–8.5). On the contrary, *HLA-DRB1*03* was found to be negatively associated with MP patients (2.1 vs. 17.6%, $p = 0.006$, OR: 0.09 95% CI: 0.01–0.7).
Conclusions: Due to the linkage disequilibrium of HLA-DQ5 alleles with both *DRB1*14* and *DRB1*16*, the presented data confirm the strong association of both *HLA-DRB1*16* and *HLA-DRB1*14* with DQ5

in MP MG and provides a replication of these results in a Turkish population with MP MG. However, the association of *DRB1*16* also with SN MG may implicate a specific effect of *DRB1*14* and its related haplotype in MP MG.

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Natural variation in the murine Y chromosome influences gene regulation and susceptibility to experimental allergic encephalomyelitis

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Objective: Sex-specific differences affect many aspects of immune system physiology and although these differences may arise from multiple mechanisms, including differential effects of sex hormones, there is increasing evidence that genetic differences in the Y chromosome can influence immune-mediated diseases. In *Drosophila*, natural polymorphic variation in the Y chromosome can influence the epigenetic regulation of autosomal and X chromosome gene expression, thereby epigenetically regulating phenotypic differences in males. Therefore, we hypothesized that Y chromosome-regulated differences in experimental allergic encephalomyelitis (EAE) susceptibility, the animal model of multiple sclerosis, is the result of an evolutionarily conserved role for the regulation of autosomal and X chromosome gene expression by the Y chromosome.
Methods: Test a panel of C57BL/6J Y chromosome (B6-ChrY) consomic strains, in which B6 mice inherit the Y chromosome from diverse substrains of mice, for susceptibility to EAE. Examine gene expression differences using Affymetrix Mouse Gene 1.0 ST arrays in naive CD4 T cells from wild-type B6 and the consomic strain exhibiting the most dramatic difference in disease susceptibility.
Results: Natural genetic variation in the mouse Y chromosome directly impacts susceptibility to EAE and B6 mice with an SJL Y chromosome (B6-ChrY^{SJL}) exhibited the greatest increase in resistance among the consomic strains tested. Our gene expression analysis using a binary filter of $P<0.05$ and 2× fold change revealed that natural variation in the Y chromosome results in 884 differentially expressed transcripts in B6 vs B6-ChrY^{SJL} CD4 T cells. Clustering of these transcripts by biological function identified an enrichment for immune system, nervous system, and mitochondria/metabolism-related genes. Interestingly, the collapsin response mediator protein 1 gene, which is expressed by male germ cells and contributes to axonal pathfinding during neural development, had the largest fold change increase of 16.9× in B6-ChrY^{SJL} T cells making it our top candidate gene contributing to EAE susceptibility.
Conclusions: Natural genetic variation in the Y chromosome influences autosomal and X chromosome gene expression, which may lead to the identification of genes and pathways that can be targeted for mechanistic studies and therapeutic intervention using *in vivo* animal models of multiple sclerosis.

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Histamine H1 receptor allele-specific interactions regulate susceptibility to CNS autoimmune disease

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Objective: Polymorphisms in the third intracellular loop of histamine H_1 receptor (*Hrh1*/H1R), a seven-transmembrane G protein-coupled receptor (GPCR), regulate susceptibility to experimental autoimmune encephalomyelitis, a T cell-mediated animal disease model of multiple sclerosis. However, the underlying mechanism for this difference is not clear, in part because the function of this domain of H_1R is not known.
Methods: Super high-resolution stochastic reconstructive microscopy (STORM), Ca^{2+} flux assays, and mass spectrometry are used to evaluate allele-specific signaling and proteomics.

We also combine genotypic and phenotypic analysis of *B. pertussis*- induced histamine sensitization (Bphs) in wild-derived mouse strains to determine genes that functionally modify *Hrh1*.
Results: In cells expressing H_1R alleles, STORM revealed a subtle difference in surface clustering in the H_1R variants. Since GPCR multimerization can influence signaling we examined allele-specific histamine-elicited intracellular Ca^{2+} flux. The susceptible (H_1R^S) allele promoted greater Ca^{2+} flux in response to histamine compared to the resistant (H_1R^R) allele. To assess potential differences in protein-protein interactions between H_1R^S and H_1R^R, we affinity purified H_1R (and its co-associated proteins) from cells overexpressing either the H_1R^S or H_1R^R allele, followed by identification of associated proteins by mass spectrometric/bioinformatics analysis. We have identified annexin A1, an endogenous negative regulator of inflammation, as a novel specific partner for the H_1R^R protein product, correlating with this allele's role in mediating resistance to autoimmune disease.

We have also undertaken a genetic approach to extend our understanding of how other factors may modify H_1R function. We have further catalogued the *Hrh1* genomic sequence across several *Mus* species and subspecies combined with histamine sensitization phenotyping. Most (75%) strains are *Hrh1*^{S}, but we found several wild-derived strains harboring the *Hrh1*^{R} allele, yet are sensitive to histamine. We have identified a linkage disequilibrium domain on *Chr6* surrounding *Hrh1* which we have termed Bphs-enhancer (*Bphse*). Introgression of this region onto histamine-insensitive *Hrh1*^{R} backgrounds has revealed that genes in this region may functionally enhance the activity of a resistant *Hrh1* allele.
Conclusions: Our data collectively suggest that H_1R may engage allele-specific pathways to control immune activation and further our understanding of H_1R function.

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Genetic variations in C-type lectin receptors are involved in the regulation of neuropathic pain-like behavior after peripheral nerve injury

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Objective: The immune system is known to both mediate and maintain neuropathic pain. In this study we investigated the effect of a small rat chromosome 4 gene fragment containing C-type lectin receptors (CLRs) for development of neuropathic pain after a peripheral nerve lesion.

Methods: Two congenic rat strains on DA background with a gene fragment from the PVG strain containing seven (APLEC) and three (R11R6), respectively, CLR genes, as well as DA male rats from the same colony were used in this study. The rats were behaviorally tested for development of mechanical hypersensitivity with a set of von Frey monofilament applied to the hindpaws at baseline and at different time points after spinal L5 nerve transection. One set were behaviorally tested, a second set were used for flow cytomety analysis and a third set of rats were treated with the monoclonal antibody R73 that deplete αβ T-cells or an IgG isotype matched control.
Results: There was a prominent strain difference in development of neuropathic pain-like behavior between the strains. Thus, DA rats started to recover after 14 days whereas APLEC and R11R6 rats remained sensitive during the entire time of testing (35 days). However, APLEC and R11R6 rats treated with the T cell depleting antibody started to recover already after 10 days, suggesting involvement of T cells in development of neuropathic pain. Spinal cord tissue dissected from DA and R11R6 rats at 14 days post injury were used for flow cytometry. The pain sensitive R11R6 strain displayed increased numbers of activated microglia, NK- and T-cells compared to DA rats.
Conclusion: The APLEC strain harbor a small genetic chromosome 4 fragment derived from the inbred PVG strain and contains a cluster of seven CLRs that are expressed on antigen presenting cells and lymphocytes. These are known to modulate and initiate an immune response. The finding that the pain and immune phenotype is retained in the smaller R11R6 congenic suggests that one or all of the three CLRs (MCL, Mincle and Dcar1) regulate recruitment of immune cells and inflammation in the spinal cord after nerve injury, in turn of relevance for neuropathic pain-like behaviour. Thus, the results of this study lend strong support for the notion that naturally occurring genetic variations in CLRs are involved in the regulation of pain after nerve injury and that this involves interaction with immune cells, in particular T cells.

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Mapping genome-wide expression in experimental autoimmune encephalomyelitis highlights disease correlated gene networks and positional candidate genes regulating autoimmunity

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Objective: Multiple sclerosis (MS) and its animal model, experimental autoimmune encephalomyelitis (EAE), are chronic inflammatory diseases characterized by autoimmune destruction of myelin sheaths and neurons. A number of genetic loci that predispose for MS have been identified. However, knowledge of their functional outcome is still limited. The expression quantitative trait loci (eQTL) approach has been successfully used to characterize genes and genetically driven networks important for regulation of complex diseases. We utilized eQTL approach to facilitate identification of genes that predispose for EAE, to infer their functions and to define genetically driven networks that correlate with EAE.
Methods: We combined classical EAE QTL mapping with genome-wide expression profiling (Gene 1.0ST arrays, Affymetrix) in spleen in an experimental backcross between EAE-susceptible DA and EAE-resistant PVG rat strains during the chronic stage of EAE. We first characterized transcripts which expression is regulated in *cis* and *trans*. Then, we utilized the Weighted Gene Co-Expression Network Analysis method to construct gene networks. Moreover, the Pearson correlation coefficient was used to determine gene networks that significantly correlated with clinical EAE phenotypes. We performed pathway analysis using Ingenuity Pathways Analysis to identify functional properties of candidate genes and gene networks that correlate with EAE.
Results: Among 2285 eQTLs we found 599 to be *cis*-regulated, of which 60 *cis*-eQTLs were positional candidates for known EAE QTLs. These positional candidates include autophagy-related genes, C-type lectins, NK cell receptors and ligands. With gene clustering analysis we defined several disease correlated gene networks. The most significant disease-correlated network was enriched for T cell-mediated immune mechanisms, also implicated in MS, and revealed both established and novel gene interactions.
Conclusions: We detected a number of *cis*-regulated transcripts, both in EAE QTLs and outside, which may play important roles in controlling autoimmune mechanisms. These can serve to generate novel hypotheses for dissecting pathogenic mechanisms that are dysregulated during chronic inflammation. In addition, we defined several disease correlated gene networks. Some networks were enriched for pathways involved in cell-mediated immune mechanisms of relevance for EAE and MS, and also included genes or family members of genes associated with human disease.

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CD59 mutation is associated with chronic inflammatory demyelinating polyradiculoneuropathy and chronic hemolysis in North-African-Jewish infants

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Objective: Chronic inflammatory demyelinating polyradiculoneuropathy (CIDP) is an acquired immune- mediated disorder of peripheral nerves. In childhood it typically manifests by lower limb weakness; relapses are common and are usually preceded by viral infections. The response to immunomodulation is positive but in some patients there are significant neurological deficits. We attempted to identify monogenic gene in four families with childhood CIDP.
Methods: Since all the patients shared a common ethnic origin and patients 5078 and 2888 were sibs, we assumed an autosomal-recessive mode of inheritance and searched for a founder mutation in the DNA samples of 5078 and 2888.

We identified homozygous regions using Affymetrix GeneChip followed by a whole exome sequencing. The DNA sample of 2888 was enriched with the SureSelect Human All Exon v.2 Kit which targeted 44 Mb (Agilent, Santa Clara, CA, USA). Sequencing was carried out on HiSeq2000 (Illumina, San Diego, CA, USA) as 100-bp paired-end runs. Image analysis and base calling were performed with the Genome Analyzer Pipeline version 1.5 with default parameters. We performed read alignment with DNAnexus software (Palo Alto, CA) using the default parameters with the human genome assembly hg19 (GRCh37) as reference. A total of 19,564 SNPs and indels were identified in the coding sequence.
Results: We identified homozygous missense mutation in CD59 in five children. This gene encodes a cell surface glycoprotein, Protectin, which inhibits the final step of the membrane attack complex (MAC) formation following activation of the complement system. The mutation segregated with the disease in the families and had a carrier rate of 1:66 among Jews of North-African origin.

Conclusions: CD59 mutation is associated with a failure of proper localization of CD59 protein in the cell surface. This is clinically manifested in infancy by relapsing peripheral demyelinating disease accompanied by chronic hemolysis. It is plausible that MAC is a major drive to myelin and axonal damage in demyelinating-remyelinating diseases.

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Molecular network analysis of ChIP-Seq-based vitamin D target genes

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Objective: Vitamin D (VD) is a liposoluble vitamin essential for calcium metabolism and immunomodulation, available as a dietary supplement and also produced endogenously when the sunlight UVB triggers its synthesis in the skin, followed by consecutive hydroxylation in the liver and kidney to generate the physiologically active 1alpha,25-dihydroxyvitamin D3 designated as calcitriol. It binds to the cognate nuclear receptor (VDR) expressed on many cell types, when heterodimerized with retinoid X receptor by interacting with VD response elements (VDREs) to activate gene expression involved in cell proliferation, differentiation, and apoptosis. Insufficient exposure to the sunlight and VD deficiency are associated with an increased risk for multiple sclerosis (MS). To study a protective role of VD in development of MS, it is important to globally characterize VDR target genes (VDRTGs) and their networks.
Methods: We identified VDRTGs from two different ChIP-seq datasets of VDR binding sites derived from calcitriol-treated human B cell and monocyte cell lines (SRP002673, SRP005910; Illumina GAIIx) by mapping them on hg19 with Bowtie, detecting peaks with MACS (fold enrichment > 20, FDR < 1%), and identifying genomic locations by GenomeJack (Mitsubishi Space Software), a genome viewer for next-generation sequencing (NGS). Molecular networks were studied by using KEGG, IPA, and KeyMolnet.
Results: After omitting noncoding genes, we identified 2246 and 186 VDRTGs in calcitriol-treated B cells and monocytes, and 456 and 108 VDRTGs in control cells. The corresponding microarray data (GSE22176, GSE27270) verified greater than a 2-fold increase in the levels of expression of 17 and 16 VDRTGs in calcitriol-treated B cells and monocytes. MACS peaks were located chiefly in the promoter and the intron, exhibiting variable VDRE sequences around the summits. We extracted totally 1541 VD-responsive genes by excluding those having an overlap between calcitriol-treated and control cells. The molecular network of those showed significant relationships with the networks of leukocyte transendothelial migration, FcgR-mediated phagocytosis, and transcriptional regulation by VDR.
Conclusions: The molecular network of the comprehensive set of VDRTGs is closely linked to a wide range of immune regulation. Therefore, a persistent perturbation in the network of VDRTGs due to vitamin D deficiency could induce a harmful immune response causative for MS. Supported by MEXT HRC S0801043.

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Deep replication and extensive fine mapping using the Immunochip significantly expands the horizons in the genetics of multiple sclerosis — For the IMSGC

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Objectives: 1) To fine map confirmed multiple sclerosis susceptibility loci by genotyping all available flanking variation. 2) To replicate potentially associated Single Nucleotide Polymorphisms (SNPs) identified through GenomeWide Association Studies (GWAS) and thereby expand the catalogue of associated variants.
Methods: More than a third of the established multiple sclerosis susceptibility loci are also associated with at least one other organ specific autoimmune disease. This remarkable overlap enabled the development of the Immunochip, a custom built Illumina genotyping array that includes a total of 196,524 SNPs designed to allow the fine mapping of nearly 200 loci established to be relevant in at least one autoimmune (38 known to be relevant in multiple sclerosis) and the deep replication of several thousand SNPs from each of twelve primary autoimmune diseases. We have typed the Immunochip in 17,097 cases and 20,055 controls from twelve countries.
Results: We identified genome wide significant evidence of association in 18 of the fine mapped regions not already established as relevant in multiple sclerosis, including Chr2q32 containing the STAT4 gene ($p = 6.08 \times 10^{-9}$). In the replication section of the Immunochip a further 28 independent loci outside of these fine-mapping regions also show evidence of association with genome-wide significance.
Conclusions: The genetic architecture underlying susceptibility to multiple sclerosis is polygenic with implicated genes being overwhelmingly immunological. Through the Immunochip we have substantially expanded the list of associated loci and fine mapped more than 40 of these associated regions.

miRNA

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Natalizumab therapy restores expression of miRNAs in multiple sclerosis patients

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Objective: The anti-α4 integrin monoclonal antibody natalizumab exerts its therapeutic efficacy in multiple sclerosis (MS) by interfering with immune cell infiltration into the CNS. Recent evidence implicates mechanisms beyond adhesion blockade in contributing to natalizumab's effects. The mediators that are responsible, however, have not been fully clarified. We examined the role of microRNAs that are regulated by natalizumab in relapsing-remitting (RR-) MS patients.
Methods: We performed a longitudinal study of 17 RR-MS patients investigating blood miRNA expression profiles at baseline and after 1 year of natalizumab therapy by microarray technique and quantitative PCR (qPCR) validation. We also compared the baseline expression profiles of these MS patients to those of 18 age- and sex-matched healthy controls (HCs). The regulation of the miRNAs that were found was then analyzed in autoimmune demyelination using an animal model of MS, experimental autoimmune encephalomyelitis (EAE).
Results: MiR-18a, miR-20b, miR-29a and miR-103 were upregulated, whereas miR-326 was downregulated upon natalizumab treatment in the longitudinal analysis. All four of the upregulated targets were found to be downregulated in RR-MS patients at baseline, compared to HCs. It appeared that expression of these miRNAs was reverted

by natalizumab therapy. All confirmed targets were regulated in peripheral blood leukocytes of mice suffering from EAE and showed disease-dependent expression levels.
Conclusions: The data indicate that natalizumab restores the expression of miRNAs dysregulated in MS. These miRNAs may serve as markers for disease response monitoring and may constitute new therapeutic targets in RR-MS.

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Next generation sequencing identifies micro RNAs that associate with pathogenic autoimmune activation leading to neuroinflammation in rats

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Objective: MicroRNAs (miRNAs) are known to regulate most biological processes and dysregulation of their expression has been associated with a variety of diseases, including multiple sclerosis (MS). Here we characterized miRNAs that associate with susceptibility to develop experimental autoimmune encephalomyelitis (EAE), a well-established animal model of MS.
Methods: We used next generation sequencing (NGS) to detect and quantify all small RNAs in the lymph nodes of EAE-susceptible DA and EAE-resistant PVG rats during immune activation, before signs of clinical disease. Using TaqMan qRT-PCR differential expression of selected miRNAs was validated and expression kinetics investigated. Target genes likely regulated by differentially expressed miRNAs were identified using computational predictions combined with the whole-genome expression data. As miRNAs predominantly exert a negative regulation of their targets, only genes with inverse differential expression were considered targets of the miRNAs.
Results: We detected 544 miRNAs in the lymph nodes of the two rat strains. Forty-three of the miRNAs were differentially expressed between DA and PVG, of which 35 miRNAs showed higher expression in the susceptible DA rats. Several of the miRNAs, including miR-146a, miR-21, miR-181a, miR-223 and members of the let-7 miRNA family, have previously been implicated in immune system regulation. Moreover, 74% (32/43) of miRNAs showed association with MS and other common autoimmune diseases. In addition, we also detected miRNAs previously not associated to autoimmunity, such as miR-199a-3p and miR-872. Differentially expressed miRNAs and their targets implicate functions and pathways important for MS and EAE such as migration and homing of immune cells through targeting CXCR3 and CCL3, regulation of cell proliferation through PRKCD and interferon signaling by regulating STAT transcription factors. We are currently performing functional investigation of selected miRNAs and their target genes.
Conclusions: Taken together, our findings demonstrate the potential to detect relevant and novel miRNAs that control autoimmunity. Our study highlights the ability to dissect pathogenic autoimmune inflammatory mechanisms trough exploration of dysregulated miRNAs and their targets in experimental neuroinflammation.

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Decreased miR-219 expression in MS patients: Possible role of miR-219 in the pathogenesis of MS

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Objective: Oligodendrocytes synthesize myelin membranes that ensheath axons and play a critical role for development and function of the central nervous system. Interestingly, although oligodendrocyte precursor cells (OPCs) are present in multiple sclerosis (MS) lesions, less mature oligodendrocytes are found. This could suggest that OPCs in MS lesions do not mature, explaining the (gradual) lack of recovery in patients with MS. Nowadays, it is becoming increasingly clear that post-transcriptional mechanisms, such as modulation of gene expression by miRNAs, play an essential role in neurodegenerative diseases and immune responses. For example, it has been shown recently that miR-219 is essential for normal oligodendrocyte differentiation. The goal of this study is to investigate whether the dysregulated outgrowth of OPCs, found in patients with MS, is determined by decreased expression of miR-219.
Methods: *Post mortem* brain tissue was obtained from patients with MS and controls. Using immunhistochemistry (IHC), five chronic active lesions from patients with mainly white matter lesions, five subpial grey matter lesions from patients with mainly grey matter lesions, and five white and five grey matter brain samples from controls were selected. We used micro-array analysis to determine differences in miRNA expression. In addition, we used *in situ hybridization* (ISH) and IHC to correlate the expression of miR-219 with proteolipid protein (PLP) expression in grey and white matter lesions found in patients.
Results: Micro-array analysis of miR-219 expression showed a 7.93-fold and 4.80-fold decrease in white and grey matter lesions, respectively, compared to control tissue. In addition, using ISH we observed that the decrease of miR-219 expression is correlated to the decreased expression of proteolipid protein (PLP) in grey and white matter lesions found in patients with MS.
Conclusions: Taken together, these results demonstrate a possible role of miR-219 in the pathogenesis of MS. Furthermore, these findings support the idea that deepening our understanding of miRNAs in relation to MS contributes to a better insight in the etiology, and might eventually have clinical implications for patients with this disease.

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MiR-126 and miR-126* play a role in flow-based leukocyte adhesion on cytokine-activated human brain microvascular endothelium in vitro

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Objective: MicroRNAs (miRs) are small non-coding regulatory RNAs that act through repression of protein translation and/or mRNA degradation at the post-transcriptional level. MiRs are critical players in the pathogenesis of many diseases, including neuroinflammatory disorders such as multiple sclerosis (MS). MS is characterized by leukocyte adhesion and infiltration subsequently leading to demyelination of nerve fibres. Leukocyte adhesion on brain endothelial cells (BECs), the main cellular constituent of the blood brain barrier (BBB), is a

complex multi-step process where expression of endothelial adhesion molecules such as VCAM1 and ICAM1 is increased. Previous results from our group shown that the pro-inflammatory cytokines TNFα and INFγ induced changes in the levels of at least 100 miRs in human CECs *in vitro*.

Here we studied the role of specific deregulated endothelial miRs in the adhesion of leukocytes on cultured human BECs.
Methods: Static and flow-based leukocyte adhesion to BECs was characterised *in vitro* using the monocytic (THP1), T-cell (Jurkat) and human brain endothelial (hCMEC/D3) cell lines.
Results: Increased adhesion of both leukocytic cell lines to BECs was observed following treatment with TNFα and INFγ compared to unstimulated cells. Increased expression of both ICAM1 and VCAM1 by hCMEC/D3 cells was also observed following cytokine treatment. Cytokine-induced maximal VCAM1 and ICAM1 expression coincided with the observed maximal leukocyte adhesion to BECs at 24 h.

Following an initial screening of five cytokine-deregulated BEC miRS using this *in vitro* BBB model, mir-126 appeared to have the most significant effect on leukocyte adhesion to hCMEC/D3 cells. Elevated miR-126 and miR-126*, the complement of miR-126, levels significantly prevented Jurkat and THP-1 cell adhesion and VCAM1 expression to hCMEC/D3 cells both in unstimulated and cytokine-treated conditions. However, decreased levels of miR-126*, but not miR-126, increased THP-1 adhesion on cytokine-treated hCMEC/D3 cells whereas THP-1 and Jurkat adhesion to unstimulated BECs was increased by both miRs.
Conclusions: These data suggest that both miR-126 and miR-126* modulate leukocyte adhesion on human brain microvascular endothelium. To our knowledge, this study is the first to report of a role for miR126* in human brain leukocyte adhesion and the first to confirm the regulation of VCAM1 by miR-126 in brain endothelium.

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The effect of IL-1β-regulated MicroRNA34a on the expression of microtubule-associated protein tau

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Objective: Tau is dysregulated in several neurodegenerative diseases, including Alzheimer's disease (AD). Additionally, recent studies have revealed altered expression of multiple microRNAs (miRs) in brain tissue of AD patients. It is well known that the expression of several cytokines is elevated in AD, and it has previously been shown that pro-inflammatory cytokines, such as IL-1β, have a direct effect on the expression of a variety of miRs. The ultimate purpose of our work is to test the hypothesis that pro-inflammatory cytokines regulate specific miRs that target the 3′-UTR of tau mRNA and thereby inhibit the expression of the tau protein.
Methods: Candidate miRs predicted to target the human tau 3′-UTR were identified using several computational algorithms. Fragments of the human tau 3′-UTR predicted to contain the miR binding sites were cloned into a luciferase reporter vector. Human embryonic kidney (HEK) 293 cells were co-transfected with the luciferase reporter constructs and the candidate pre-miRs. Luciferase assays were performed 48 h after transfection.

To test the effect of a candidate miR on endogenous levels of the tau protein, SH-SY5Y human neuroblastoma cells were transfected with pre-miRs. Western blot analysis was performed on cell lysates 48 h after transfection. Levels of tau mRNA was quantified with qPCR and protein levels will be further quantified using ELISA.

To determine the effects of specific cytokines on miRs thought to affect tau expression, SH-SY5Y cells will be treated with IL-1β. Levels of miRs, tau mRNA, and tau protein will then be measured.
Results: HEK293 cells treated with miR-34a showed decreased luciferase activity from the luciferase reporter construct with the fragment that contains the predicted miR-34a seed region. A similar decrease was seen at the mRNA level using qPCR. The effect of miR-34a was not seen when mutations were made in the predicted seed region. Initial western blot analysis suggests a reduction in endogenous tau protein levels in SH-SY5Y cells transfected with miR-34a compared to negative control pre-miR. The western blot findings need to be replicated to substantiate the conclusion.
Conclusions:Our results suggest that miR-34a can specifically bind to the tau 3′-UTR and repress tau expression. Since other groups have shown that miR-34a is dysregulated by altered IL-1β levels in AD, our results prompt further investigations of the effect of cytokines on tau expression, which are underway.

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Differential expression of microRNAs in activated human myeloid cells and ex vivo monocytes in multiple sclerosis patients

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Objective: MicroRNAs (miRNAs) are short ribonucleic molecules and post-transcriptional regulators of gene expression, including genes influencing both innate and adaptive immunity. The innate immune response in the inflamed central nervous system (CNS) of patients with multiple sclerosis (MS) reflects contributions from both peripheral-blood derived cells (macrophages) and endogenous long-lived microglia. Human-derived macrophages and microglia show distinct phenotypic and functional responses following either classical (M1, pro-inflammatory) or alternative (M2, anti-inflammatory) activation. Our objectives were to define miRNA profiles of human microglia and macrophages under M1 and M2 conditions, and compare these profiles to monocytes derived from MS patients.
Methods: Polarization of human macrophages (derived from CD14+ monocytes) and microglia (derived from human CNS tissue), was performed using standard protocols: GM-CSF, IFNg and LPS for M1; M-CSF, IL-4 and IL-13 for M2. Relative expression levels of miRNAs were compared between unpolarized, M1 and M2 conditions using qPCR. miRNA profiles were also determined in ex-vivo CD14+ monocytes obtained from healthy volunteers, untreated relapsing-remitting MS (RRMS) patients, and fingolimod-treated RRMS patients.
Results: Human macrophages and human adult microglia expressed distinct miRNA signatures under different activating conditions; M1-polarizing conditions resulted in significantly increased mir-155 and mir-146a expression in both macrophages and microglia compared to unpolarized and M2 cells ($p<0.01$). Mir-124a levels were lower in M1 macrophages compared to M2; mir-124a was not detected in microglia. CD14+ monocytes derived from RRMS patients expressed significantly higher levels of mir-155 and mir-146a compared to monocytes of healthy controls ($p<0.05$), while fingolimod treatment resulted in normalization of monocyte miRNA expression levels ($p<0.01$).
Conclusions: In both human macrophages and microglia, miRNAs were differentially expressed following different modes of activation. We have extended these findings using CD14+ cells from both untreated and fingolimod-treated RRMS patients; miRNA levels were differentially expressed in untreated MS patients, however no differences were observed between fingolimod-treated patients vs. controls. Identification of individual miRNAs associated with myeloid

cell activation may provide insights into molecular mechanisms responsible for immune regulation.

Neuroinflammation

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The prokineticin system in experimental autoimmune encephalomyelitis: Possible novel targets for immune intervention

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Objective: Prokineticins (PK)s are newly discovered small secreted proteins (8–11 kDa), widely expressed in different tissues and involved in a wide spectrum of biological and pathological functions. They comprise two proteins, PK-1 and PK-2, signalling through two cognate G-protein coupled receptors (PK-R1 and PK-R2). It has been recently shown that prokineticins and their receptors can also modulate the immune response. In particular PK-2, highly expressed in inflamed tissues associated to infiltrating cells, promotes macrophage activation and migration, and can promote Th1 polarization by increasing the production of pro-inflammatory cytokines such as IL-1 and IL-12, while inhibiting that of anti-inflammatory cytokines such as IL-10 and IL-4. However, the role of PKs and their receptors has never been investigated in multiple sclerosis (MS) nor in its animal model, experimental autoimmune encephalomyelitis (EAE).
Methods: Chronic EAE was induced in C57Bl/6 mice by immunization with 100ug MOG_{35-55} in CFA and *B. Pertussis* toxin. Relapsing-remitting EAE was induced in SJL/J mice by immunization with 100ug of $PLP_{139-151}$ in CFA. Gene expression analysis was performed by real time PCR. Proliferative response of lymph node cells (LNC)s was assessed by [H^3] thymidine incorporation. Cytokine production was tested in parallel cultures by Elisa.
Results: During chronic MOG_{35-55} induced EAE, we observed an increased mRNA expression of PK-2 in LNCs and purified CD4+ T cells during disease. We also observed a significant increase of PK-2 at a protein level in serum of EAE mice. The increase of PK-2 was accompanied by a dramatic reduction of PK-R2 mRNA expression, while PK-R1 mRNA was not significantly modulated. To explore the possible role of PKs and PK-receptors in EAE, we treated mice with the non-peptide antagonist for PK-Rs PC7. Daily treatment with PC-7 was effective in reducing the severity of both chronic and relapsing-remitting EAE, when PC7 was used either as a preventive or as a therapeutic treatment. These *in vivo* observations were accompanied by *in vitro* results showing a dramatic reduction of Th1 cytokines and of IL-17, and an increase of the suppressor cytokine IL-10 in antigen-stimulated T cells of PC-7 treated mice compared to vehicle treated mice.
Conclusions: These results suggest that PK-2 and its receptors can play an important role in the development of EAE, and might represent a novel target of therapy in autoimmune demyelination of the CNS.

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CCR4 is required for experimental autoimmune encephalomyelitis by regulating GM-CSF and IL-23 production in dendritic cells

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Dendritic cells (DCs) are pivotal for the development of experimental autoimmune encephalomyelitis (EAE). However, the mechanisms by which they control disease remain to be determined. In this study we investigated the role of the CC chemokine receptor 4 (CCR4) in the pathogenesis of EAE. We demonstrate that expression of CCR4 by DCs is required for EAE induction. $CCR4^{-/-}$ mice presented enhanced resistance to EAE associated with a reduction in IL-23 and GM-CSF expression in the CNS. Restoring CCR4 on myeloid cells in bone-marrow chimeras or intracerebral microinjection of CCR4 competent DCs, but not macrophages, restored EAE in $CCR4^{-/-}$ mice indicating that $CCR4^{+}$ DCs are cellular mediators of EAE development. Mechanistically, $CCR4^{-/-}$ DCs were less efficient in GM-CSF and IL-23 production and also T_H-17 maintenance. Intraspinal IL-23 reconstitution restored EAE in $CCR4^{-/-}$ mice, whereas intracerebral inoculation using $IL\text{-}23^{-/-}$ DCs or $GM\text{-}CSF^{-/-}$ DCs failed to induce disease. These data demonstrate that CCR4 dependent GM-CSF production in DCs is required for IL-23 release in these cells and is a major component in the development of EAE. Our study identified a novel role for CCR4 in regulating DC function in EAE harbouring therapeutic potential for the treatment of CNS autoimmunity by targeting CCR4 on this specific cell type.

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Role of complement in an alpha-synuclein overexpression model of Parkinson disease

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Objective: Parkinson disease (PD) is a neurodegenerative disorder characterized by a loss of dopaminergic neurons in the substantia nigra pars compacta (SNpc) and intraneuronal aggregates of alpha-synuclein (a-syn), resulting in tremor, rigidity, bradykinesia and postural instability. In human PD and animal models of the disease, there is evidence of chronic neuroinflammation, including microglial activation, cytokine expression, and leukocyte infiltration. Inflammation appears to be an important contributor to dopaminergic neurotoxicity; however, the mechanism by which a-syn induced neuroinflammatory changes lead to neurotoxic effects is unclear. This study investigates a possible role for the complement system in a-syn based models of PD.

Methods: We used a mouse model of PD in which targeted a-syn overexpression in the substantia nigra is produced using an adeno-associated viral vector (AAV-SYN). We assessed complement component production via qPCR, ELISA, Western blot, and immunohistochemistry at 2 and 4 weeks post-injection in wildtype mice. We also assessed IgG deposition and microglial activation 4 weeks post-injection of AAV-SYN in wildtype and C5-deficient mice (C5 −/−).
Results: In wildtype mice, at 2 and 4 weeks post-injection, we observed induction of C3 mRNA which was around 3 fold over baseline. C3 protein was present in both AAV-GFP and AAV-SYN tissue 4 weeks and 6 months post-injection as observed by immunohistochemistry. At four weeks the distribution of the C3 staining in the AAV-SYN animals was similar to the AAV-GFP controls, while at 6 months there appeared to be increasing concentration of C3 in association with tyrosine-hydroxylase positive dopaminergic neurons. We observed little evidence for the presence of C5 in our mouse model: C5 mRNA was undetectable at 2 or 4 weeks post injection of AAV-SYN, C5 protein was not detected by western blot or immunohistochemistry, and C5a ELISA did not detect C5 cleavage in AAV-SYN injected animals. In C5 −/− mice injected with AAV-SYN, the inflammatory response appeared unchanged.
Conclusions: Our observations suggest that there is enhanced expression of C3 in this a-syn based mouse model of PD, with increased C3 mRNA as well as presence of the protein in association with neurons. In contrast, we found no evidence for participation of the C5 component in this PD model. It will be important to determine whether the enhanced expression of C3 contributes to dopaminergic cell loss in this model system.

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Beta-amyloid induces apoptosis of phagocytic microglia and macrophages

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Objective: In Alzheimer's disease, microglia are thought to protect neurons by clearing beta-amyloid (Aβ). Whether Aβ phagocytosis regulates microglial cell responsiveness, and whether macrophages participate, remains unclear. Here we investigated how *in vivo* uptake of Aβ affects the turnover of microglia and macrophages, by changing rates of proliferation and/or apoptosis.
Methods: Cellular responses were assessed in the neocortex of $APP_{swe}/PS1_{\Delta E9}$ Tg and wildtype control mice, as well as in post-mortem tissue from patients with Alzheimer's disease or normal controls. Aβ uptake by microglia/macrophages was assessed by confocal microscopy and flow cytometry. Proliferation and apoptosis of microglial and macrophage populations were evaluated using a combination of flow cytometry and immunofluorescence. *In vitro* experiments were carried out using microglia or macrophages prepared from adult mice.
Results: Plaque-associated microglia and $CD11b^{+}CD45^{high}$ macrophages were found to bind and ingest endogenously-produced Aβ in $APP_{swe}/PS1_{\Delta E9}$ Tg mice. Plaque-associated microglia and $CD45^{+}$ leukocyte-like cells were also observed in brains from patients with Alzheimer's disease. Notably, we found high proportions of Annexin V^{+} microglia and macrophages undergoing apoptosis in aged $APP_{swe}/PS1_{\Delta E9}$ Tg mice. Microglia and macrophages had greater caspase activity and increased p53 expression after *in vivo* uptake of Aβ, than microglia and macrophages that did not bind/ingest Aβ. Furthermore, microglia and macrophages had a lower rate of proliferation after taking up Aβ. *In vitro* analyses showed that caspase activation was dependent on Aβ uptake in microglia, whereas blocking Aβ uptake triggered apoptosis-induced cell death in macrophages.
Conclusions: Our data demonstrate that Aβ impairs microglial/macrophage viability and responsiveness. Amyloid clearance may fail as phagocytic microglia and macrophages succumb to Aβ-induced apoptosis.

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Exposure of microglia to GMCSF/IL4 impairs Toll-like receptor-induced transcription programmes without affecting binding of NF-κB to its consensus sequence

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Objective: Microglia are immune competent resident cells of the central nervous system (CNS). A recent study has demonstrated that microglia share ontological precursors with myeloid cells such as monocytes, macrophages and dendritic cells (DCs) but originate from a distinct progenitor. Microglia display remarkable plasticity and change their physiology in response to environmental cues. These changes affect amongst others their antigen presentation capacities and their responses to activating stimuli. Both GMCSF and IL4 can be produced endogenously within the CNS, and there is evidence that IL4/STAT6-mediated signaling plays a role in the pathogenesis of multiple sclerosis and brain gliomas. We hypothesized that GMCSF/IL4 (G4)-exposed microglia are different than myeloid-derived antigen presenting cells (APC) and suppress rather than support inflammatory responses.
Methods: We exposed primary microglia from adult rhesus macaques to G4 and analysed cell surface protein expression, APC capacities and responses to Toll-like receptor (TLR) ligands.
Results: G4-microglia acquire a DC-like cell surface protein expression profile. However, when used as APC in mixed lymphocyte reactions they potently induced the proliferation of regulatory Tcells. Intriguingly, TLR-induced pro-inflammatory cytokine production was severely impaired in G4-microglia, irrespective of the TLR ligand used. TLR-induced NF-κB activation, translocalization and binding to its DNA consensus site were intact, yet initiation of transcription was inhibited. We will present data on heterodimerization of NF-κB/p50 with other transcription factors that might explain this phenomenon.
Conclusions: We demonstrate that microglia that are exposed to G4 are characterized by severely impaired innate immune responses and are potent inducers of proliferation of Tcells with a regulatory phenotype. This suggests that such microglia primarily mediate tolerogenic responses and delineate distinctive properties of microglia compared with other myeloid cells.

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Epigenetic control of cytokine-induced cell death

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Objective: Inflammatory cytokines play a critical role in the pathogenesis of multiple sclerosis. In this study we examined the role of epigenetic control, particularly, DNA methylation, in the mechanisms of cytokine-induced cell death.
Methods: For the purpose of our study we employed a DNA methylation assay, which demonstrated that cytokine-induced cell death is associated with differential genome-wide methylation in gene promoters.
Results: Our results demonstrated that inflammatory cytokines induce cell death in human oligodendroglioma cells, and 5-azacytidine, a hypomethylation agent, can provide cell protection. Thus, they suggested that cytokines may cause cell injury and death by modulating the methylation status of genomic DNA. In corroboration, we also observed expression and subcellular localization changes of DNA methyltransferases (DNMTs) in response to cytokine treatment.
Conclusions: In conclusion, better understanding of the molecular mechanisms of DNA methylation associated with cytokine-induced cell death might have therapeutic implications in designing oligodendrocyte-protective agents in multiple sclerosis.

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Cannabidiol reduces host immune response and prevents cognitive impairments in Wistar rats submitted to pneumococcal meningitis

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Objective: The aim of the present study was to evaluate the effects of acute and extended administration of cannabidiol on the release of TNF-α, IL-1β, IL-6, CINC-1 brain-derived neurotrophic factor and behavioral parameters in adult Wistar rats submitted to pneumococcal meningitis.
Methods: Male Wistar rats underwent a cisterna magna tap and received either 10 μL of sterile saline as a placebo or an equivalent volume of *S. pneumoniae* suspension. Rats subjected to meningitis were treated by intraperitoneal injection with cannabidiol (2.5, 5, or 10 mg/kg once or daily for 9 days after meningitis induction) or a placebo. Six hours after meningitis induction, the rats that received one dose were killed and the hippocampus, frontal cortex and CSF were obtained to assess cytokines/chemokine and BDNF levels. On the 10th day, the rats were submitted to the inhibitory avoidance task. After the task, the animals were killed and samples from the hippocampus and frontal cortex were obtained.
Results: At 6 h after pneumococcal meningitis induction there was an increase in the levels of TNF-α and IL-1β in cerebrospinal fluid; TNF-α and CINC-1 in hippocampus and TNF-α, IL-1β and CINC-1 in frontal cortex. The acute administration of cannabidiol at different doses reduced the TNF-α and IL-1β levels in cerebrospinal fluid; TNF-α and CINC-1 levels in hippocampus and TNF-α, IL-1β and CINC-1 levels in frontal cortex. In the inhibitory avoidance test, there was no difference between training session between groups. In the training and test sessions there were not statistical differences between meningitis, meningitis + cannabidiol 2.5 mg/kg and meningitis + cannabidiol 5 mg/kg, showing memory impairment in these groups. Nevertheless, the extended treatment with cannabidiol + 10 mg/kg prevented memory impairment when compared to meningitis group in the training session.
Conclusions: Although descriptive, our results demonstrate that cannabidiol plays an anti-inflammatory role in pneumococcal meningitis. Furthermore, it prevents cognitive damage, possibly representing a new pharmacological approach towards pneumococcal meningitis. Future studies must be realized to understand the underlying mechanisms.

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A two-signal model for the activation of the blood–CSF barrier for immune cells trafficking

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The healthy brain is an immune privileged site, shielded by barriers from circulating immune cells. Nevertheless, numerous studies have suggested that a continuing dialogue between the brain and circulating immune cells is needed to maintain life-long brain plasticity, including neurogenesis, cognitive ability, and resilience to stress. Since the blood-cerebrospinal fluid barrier (BCSFB), at the brain's borders, is constantly exposed to circulating immune cells, we hypothesized that at this junction, the choroid plexus (CP) epithelium can sense signals coming from the central nervous system (CNS) parenchyma via the cerebrospinal fluid (CSF) and through dialogue with circulating immune cells, translate them into a reparative or protective mechanism. Here we found that different T cell populations, with distinct cytokine polarities, accumulate at the CP of healthy animals and affect the immunomodulatory properties of the CP epithelium and its ability to facilitate trafficking of leukocytes. We further show that activation of the CP for expression of trafficking molecules is tightly regulated by two signals; with the first signal coming from the CNS parenchyma, and the second from outside the CNS. Taken together, our findings demonstrate that the CP epithelium is endowed with immunological plasticity allowing it to serve not only as a filter for CSF nutrients, but also as an active interface for selection of circulating immune cells, regulating immune cells trafficking into the CNS.

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Monocyte-derived macrophages orchestrate repair under sterile and immune-mediated neurodegenerative conditions

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Objective: Recent studies have revealed the functional heterogeneity of monocyte-derived macrophages and its significance to wound healing in peripheral tissues. In the central nervous system (CNS), we have shown that monocyte-derived macrophages promote functional recovery after spinal cord injury by locally exerting an immunoregulatory phenotype. These results encouraged us to test whether these cells are involved in different types of injurious conditions, irrespective of their etiology. Using the retina as a relevant and convenient model to study diverse neurodegenerative conditions, we tested whether monocyte-derived macrophages are involved in resolution from of both biochemically-induced and immune-mediated insults.
Methods: C57BL/6J mice were subjected to retinal insult in models of glutamate intoxication or experimental autoimmune uveitis (EAU). CX_3CR1-GFP bone marrow chimeric mice were used to monitor the infiltration of monocyte-derived macrophages to the eye following the insults, and to characterize these cells. Monocyte depletion and augmentation experiments were performed to study the effects of monocyte-derived macrophages in the retina after insult.
Results: Glutamate intoxication and EAU induction both resulted in infiltration of monocyte-derived macrophages from the blood into affected retinas. Enhancing the monocytic population following glutamate insult increased the survival of retinal ganglion cells (RGCs) and the numbers of proliferating retinal progenitor cells (RPCs), whereas monocyte depletion resulted in diminished RGC survival and RPC renewal. The macrophages contributed to an anti-inflammatory and neuroprotective milieu in the injured retina. In EAU, the infiltrating macrophages could be divided into two subsets based on their expression levels of CX_3CR1. The CX_3CR1^{high} subset appeared later along disease and expressed higher levels of resolution-phase markers as compared to its CX_3CR1^{int} counterpart. Inhibiting monocyte infiltration at the induction phase prevented EAU onset, whereas monocyte depletion at the resolution phase of EAU resulted in a decrease in $FoxP3^+$ regulatory T cells.
Conclusions: Monocyte-derived macrophages display phenotypic and functional heterogeneity upon CNS insult. Although these cells are involved in the induction of an inflammatory autoimmune disease in the eye, certain subsets of these macrophages appear to be essential for the resolution of inflammation, regardless of whether it emerged as an outcome of sterile or immune-mediated insult.

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Variations in autophagy related gene Atg7 expression correlate to disease phenotypes in multiple sclerosis and experimental autoimmune encephalomyelitis

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Objective: The autophagocytic process has been linked to both innate and adaptive immunity and is implicated in neurodegeneration and autoimmunity, which are properties of multiple sclerosis (MS) and its animal model experimental autoimmune encephalomyelitis (EAE). Genome-wide expression analysis in a backcross (BC) population of rats induced with EAE revealed a genetic regulation of autophagy related gene 7 (*Atg7*) expression. Therefore, we aimed to investigate the involvement of *Atg7* in EAE and MS.
Methods: Genome-wide expression in 150 BC rats induced with EAE was used to define candidate *cis*-regulated EAE genes and to infer their functions. Cellular source and kinetics of *Atg7* expression was studied with quantitative PCR and Western blot in sorted cells from parental strains. The impact of *Atg7* on the immune system and EAE development was investigated in mice with the conditional *Atg7* deletion in T cells and APCs. The expression of *ATG7* was investigated in samples from MS patients and controls and the genetic association of autophagy genes was investigated in a Swedish cohort.
Results: We identified a genetic *cis*-regulation of *Atg7* that overlaps with a previously demonstrated EAE locus on rat chromosome 4, and further showed that higher *Atg7* expression correlates with less severe EAE. *Atg7* expression was higher in CD4+ and CD8+ lymph node T-cells from the EAE resistant rat strain. Furthermore, the mice with a conditional deletion of *Atg7* in T cells displayed decreased CD8+ T cell frequencies in lymph nodes of immunized mice. *ATG7* expression was increased in peripheral blood mononuclear cells (PBMCs) of MS patients compared to controls. Interestingly, we observed tendency for higher *ATG7* expression in PBMCs during clinical remission. Additionally, high *ATG7* expression in cerebrospinal fluid cells correlated with lower disability in MS patients. We observed evidence for association of *ATG7* and *ATG16L1*, the later a risk gene for Crohn´s disease, with MS in a Swedish population.
Conclusions: In conclusion, we demonstrate a potential involvement of *Atg7* in MS and EAE. Thus, the molecule and the autophagy pathway require further functional studies to elucidate the molecular basis of autophagy in neuroinflammation and degeneration.

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Allergy-induced cognitive abnormalities and neuroinflammation in an OVA-induced food allergy model are prevented by a dietary intervention

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Objective: Allergy is a chronic inflammatory disease and a major public health problem. Although the pathophysiology of allergic reactions has been well characterized, little is known about its consequences on brain function and cognition. The aims of this study were therefore twofold: 1) to investigate whether peripheral inflammation associated with allergy affects brain function and; 2) to examine whether dietary supplementation with a diet containing *Bifidobacterium breve* M-16V in combination with prebiotic non-digestible oligosaccharides (scGOS/lcFOS) (synbiotics) alleviates the allergy-induced pathologies.
Methods: Balb/c mice were fed a control or synbiotic diet for 2 weeks prior to and during a 5 week sensitization protocol to ovalbumin (OVA) by gavage. Anxiety levels were measured 48 h following each OVA challenge. Spatial memory was assessed using a T-maze 1 week after the first and fifth OVA challenge.
Results: The validity of the allergy model was confirmed by observing increased OVA-specific IgE levels in serum. Allergen exposed mice demonstrated higher anxiety levels and impaired spatial memory. These deficits were in parallel with decreased expression of brain derived neurotrophic factor (BDNF) messenger RNA (mRNA) and p-glycoprotein in the hippocampi. Importantly, synbiotics normalized OVA-induced aberrant cognitive and molecular changes. In addition, FACS analysis of the homogenized hippocampal cells revealed elevation in OVA-induced $CD11c^+F4/80^+CD68^-$macrophages, which were

attenuated by the synbiotic treatment, whereas synbiotics elevated $CD11b^{+}CD68^{+}F4/80^{low}$ cells.
Conclusions: The increased macrophage levels observed in the brains of OVA-allergic mice are likely derived from the circulation, which, in combination with observed decrease in brain blood barrier, implicate periphery-driven monocytes as potential key players in inducing robust decrease in brain function observed in the allergic mice. The present data support the notion that allergy-dependent peripheral inflammation modifies the brain inflammatory status and dampens the cognitive abilities of the animals, suggesting that allergy may play a role in the development and/or progression of neurological disorders.

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Transgenic inhibition of astroglial NF-kappa B protects from optic nerve demyelination and retinal ganglion cell loss in experimental optic neuritis

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Objective: Optic neuritis is an acute, demyelinating neuropathy of the optic nerve often representing the first appreciable symptom of multiple sclerosis. Although the specific mechanisms responsible for triggering optic neuritis are unknown, it has been suggested that a key pathological factor is the activation of immune–inflammatory processes secondary to leukocyte infiltration. However, to date, there is no conclusive evidence to support such a causal role for infiltrating peripheral immune cells in the etiopathology of the disease. On this basis, the objective of the present study is to dissect the contribution of the peripheral immune-inflammatory response versus the CNS-specific inflammatory response in the development of optic neuritis, by analyzing optic nerve and retinal ganglion cells pathology in wild type and GFAP-IκBα-dn transgenic mice following induction of EAE.
Methods: We induced EAE with MOG_{35-55} peptide in wild type and GFAP-IκBα-dn transgenic mice, where NF-κB is selectively inactivated in astrocytes, and examined optic nerve and retinal ganglion cells histopathology and gene expression.
Results: We found that, in wild type mice, axonal demyelination in the optic nerve occurred as early as 8 days post induction of EAE, prior to the earliest signs of leukocyte infiltration (20 days post induction). On the contrary, GFAP-IκBα-dn mice were significantly protected and showed a nearly complete prevention of axonal demyelination, as well as a drastic attenuation in retinal ganglion cell death. This correlated with a decrease in the expression of pro-inflammatory cytokines, chemokines, adhesion molecules, as well as NAD(P)H oxidase subunits.
Conclusions: Our results provide evidence that astrocytes, not infiltrating immune cells, play a key role in the development of optic neuritis and that astrocyte-mediated neurotoxicity is dependent on activation of a transcriptional program regulated by NF-κB. Hence, interventions targeting the NF-κB transcription factor in astroglia may be of therapeutic value in the treatment of optic neuritis associated with multiple sclerosis.

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Deficiency of the complement anaphylatoxin receptor C3aR on leukocytes worsens the outcome from spinal cord injury by promoting neutrophilia

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Objective: Inflammation following spinal cord injury (SCI) causes the propagation of pathology into neural tissue that was originally spared, thereby worsening neurological outcomes. With no effective anti-inflammatory treatments currently available, a better understanding of the key cellular and molecular mediators of post-SCI inflammatory pathology is critical for the development of new therapies. The complement cascade is thought to be major component of the innate immune response to SCI, but the contribution of its individual constituents remains ambiguous. This study aimed to examine the role of the complement anaphylatoxin receptor C3aR as a putative therapeutic target.
Methods: C57BL6/J (WT) and $C3aR^{-/-}$ mice on a C57BL6/J background were used in this study. Experimental mice were subjected to severe (70 kDyne) contusive SCI using the Infinite Horizons impactor device. Open-field locomotor scoring was used to monitor functional recovery for 5 weeks post-SCI. Magnetic resonance imaging, histology and flow cytometry were used to compare pathology and injury responses between genotypes. Bone marrow (BM) chimeric mice were also generated to more selectively determine the effect of C3aR deficiency on cells of myeloid ($[C3aR^{-/-} \rightarrow WT]$ BM chimeras) or CNS ($[WT \rightarrow C3aR^{-/-}]$ BM chimeras) origin compared to controls ($[WT \rightarrow WT]$ BM chimeras).
Results: $C3aR^{-/-}$ mice showed significantly worsened recovery of hindlimb function, along with greater lesion volumes, reduced white matter content and an increased presence of $Ly6b.2^{+}$ neutrophils/inflammatory monocytes at the lesion site at 1, 10 and 35 days post-injury compared to WT mice. Numbers of circulating $Gr1^{+}$ granulocytes were found to be significantly elevated in the blood of $C3aR^{-/-}$ mice compared to WT mice at 2 hours post-SCI. The functional phenotype of $C3aR^{-/-}$ mice was rescued in $[WT \rightarrow C3aR^{-/-}]$ BM chimeras but recapitulated in $[C3aR^{-/-} \rightarrow WT]$ BM chimeras.
Conclusions: Expression of C3aR on circulating myeloid-derived cells, but not CNS glia, serves an important regulatory role in the inflammatory response to SCI. Neutrophilia observed in $C3aR^{-/-}$ mice suggests that this role involves controlling the mobilization of inflammatory precursors from the BM niche to the site of SCI in response to chemotactic signals. Based on these findings, we propose that C3aR agonism during the acute phase of SCI may be a novel avenue to control the severity of the innate immune response and reduce inflammatory pathology.

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Absence of IGF1R from oligodendrocytes ameliorates EAE

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Objective: Signaling of Insulin-like Growth Factor 1 (IGF-1) through the insulin-like growth factor receptor 1 (IGF1R) mediates anti-apoptotic mechanisms in several cell types and regulates differentiation and myelination of oligodendrocyte (ODC) precursor cells (OPCs) during development and following injury. Interestingly, IGF-1 expression is increased in the CNS parenchyma during cuprizone intoxication and surrounding sclerotic lesions in Multiple Sclerosis and in its inflammatory animal model, experimental autoimmune encephalomyelitis (EAE). However, the role of IGF1R signaling in OPCs and ODCs in the context

of neuroinflammation remains unclear as published studies show contradictory results.
Methods: We used oligodendrocyte-specific deletion of IGF1R to investigate the role of IGF-1 signalling for disease course and recovery in the oligodendrocyte-ablating paradigms EAE and cuprizone intoxication.
Results: The use of a late-acting myelin-specific Cre strain (MOGi-cre) resulted in efficient ablation of the IGF1R gene without any clinical and histological abnormalities developing in the respective animals. We observed, however, upon cuprizone-mediated oligodendrocyte death impaired remyelination. EAE was, surprisingly, ameliorated. We found that microglia were less activated as shown by CD44 and MHC class II surface expression analysis
Conclusions: Taken together we show that specific deletion of IGF1R from mature oligodendrocytes increases oligodendrocyte susceptibility to death. Yet in EAE absence of IGF1R from oligodendrocytes results in significant amelioration of disease.

No conflict of interest.

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Induced Oligodendrocyte Death Does Not Elicit Anti-Myelin Autoimmunity

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Objective: Multiple Sclerosis is widely held to be driven by an anti-myelin immune response, yet the trigger of this autoreactivity remains elusive. While genetic and environmental factors contribute to the pathology, primary myelin-degeneration and oligodendrocyte apoptosis is acutely debated for initiating MS.
Methods: To address whether such a primary degeneration triggers autoimmunity, we used a mouse model allowing the specific and systematic induction of oligodendrocyte death.
Results: Sterile inflammation with gliosis and microglia activation followed ODC death and myelin lipids were found in deep cervical lymph nodes, making CNS-antigens available to lymphocytes. However, even under conditions favoring autoimmunity, like bystander activation, removal of regulatory cells, presence of myelin-reactive transgenic T cells, and application of demyelinating antibodies, no anti-CNS immunity was observed. Astonishingly, this ignorance was not mediated by tolerance towards myelin antigens.
Conclusions: We thus conclude that a lack of immunogenicity following degenerative oligodendropathy dismisses the notion that such a process *per se* could trigger any sustained inflammation as proposed for MS pathogenesis.

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Role of trans-signaling in mediating the actions of interleukin-6 in the central nervous system

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Objective: IL-6 is a pleiotropic cytokine implicated in the pathogenesis of many immunoinflammatory disorders of the CNS. IL-6 communicates with cells via two major modes termed either classical signaling or transsignaling. In classical signaling IL-6 binds to a membrane bound receptor termed IL-6Rα while for transsignaling IL-6 binds to a soluble form of IL-6Rα. These respective ligand/receptor complexes then associate with gp130 to activate the JAK/STAT and SHP2/MAPK signal transduction pathways, respectively. A soluble form of gp130 (sgp130) is able to block transsignaling specifically by binding to IL-6/sIL-6R complexes. Currently, the involvement of transsignaling in facilitating IL-6 cellular communication in the CNS is ill-defined and was the focus of this study.
Methods: A molecular genetic approach was employed using GFAP-IL6 and GFAP-sgp130 transgenic mice. Bigenic mice were generated with CNS-restricted, astrocyte-targeted production of murine IL-6 and co-production of the specific inhibitor of IL-6 transsignaling, sgp130 (termed GFAP-IL6/sgp130 mice). The brain from bigenic and control mice was analyzed for various parameters including transgene expression, gp130 coupled signal transduction pathway activation and molecular and cellular pathology.
Results: Transgene-encoded IL-6 mRNA and protein were similar in the brain of GFAP-IL6 and GFAP-IL6/sgp130 mice. GFAP-IL6/sgp130 mice had decreased pY-STAT3 in the brain with a marked reduction in the number of pY-STAT3-positive cells and the absence of detectable pY-STAT3 in specific cell types such as Bergmann glia. These changes in pY-STAT3 in GFAP-IL6/sgp130 mice were associated with a disproportionate reduction in the expression of various inflammatory markers as well as reduced astrocytosis, microgliosis, vascular pathology and blood–brain barrier leakage. Hippocampal neurogenesis which was markedly reduced in GF-IL6 mice was significantly increased in GFAP-IL6/sgp130 mice. Degenerative changes in the cerebellum characteristic of GFAP-IL6 mice were almost absent in GFAP-IL6/sgp130 mice.
Conclusions: We conclude that in the CNS: (i) sgp130-Fc is an effective means to block IL-6 trans-signaling, (ii) IL-6 transsignaling modulates selective cellular targets and molecular responses and, (iii) IL-6 transsignaling mediates degenerative changes in the brain, the blocking of which, could be an effective therapeutic approach to alleviate the detrimental effects of IL-6.

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Glycine receptor antibodies: Clinical spectrum and pathogenic mechanisms of the antibodies

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Objective: To describe the clinical features of patients with glycine receptor antibodies (GlyR) and characterize the antibodies.
Methods: Clinical data were collected using questionnaires and clinical history. Sera were examined using cell-based assays and rat central nervous system immunohistochemistry.
Results: Serum samples from 42 patients sent for routine antibody diagnostic testing bound the alpha 1 subunit of the glycine receptor

(GlyRα1) with titres between 1:20 and 1:60,000. 60% were males and ages ranged from 2–77 years. Spasms, stiffness (90%), excessive startle and touch sensitive myoclonus (80%), brainstem disturbance (65%), pain and sensory involvement (>50%), cognitive deficits with encephalopathy/seizures (50%) and autonomic signs (50%) were identified; only four patients had tumors (2 thymoma and 2 lymphoma). Paraclinical findings were normal (brain and spinal cord MRI) or unspecific (EEG). Most patients responded well to symptomatic treatments, immunotherapy (steroids with plasma exchange or IvIg), but relapses have occurred in a few patients after immunotherapy withdrawal and 3 patients died unexpectedly.

On fresh frozen rat tissue these patients' serum IgG bound to cell bodies and to the neuropil of the brainstem and the spinal cord, including the pontine reticular nuclei, locus coeruleus, solitarius nuclei in the brainstem, and ventral and dorsal horns of the spinal cord, where they co-localized with monoclonal antibodies to GlyRα1. However, some sera also bound to other regions particularly the cerebellum, hippocampus, thalamus, hypothalamus and caudate, sometimes without co-localization with monoclonal antibodies to GlyRα1, suggesting the presence of antibodies to other antigens which need further characterization.

In GlyRα1 transfected HEK cells these predominantly IgG1 antibodies caused internalization of the receptors which were targeted to the late-endosomal pathway for degradation over a period of 2–16 h. They activated complement deposition when incubated with a fresh source of complement.

Conclusions: Patients with GlyR antibodies have a subacute or chronic disease course with a wide clinical presentation; in addition to the motor symptoms some patients also exhibit brainstem and encephalopathic disturbance. Most patients showed a good response to immunotherapies. The GlyR antibodies bind to multiple regions in the CNS, which could explain the multifocal clinical features present in these patients. A detailed clinico-anatomical correlation is ongoing. The antibodies show evidence of pathogenicity in vitro and future studies will address their effects in vivo.

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Antigen-specific therapies in myelin oligodendrocyte glycoprotein-induced experimental autoimmune encephalomyelitis: A model for paediatric multiple sclerosis and acute disseminated encephalomyelitis

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Objective: A significant proportion of paediatric patients with multiple sclerosis (MS) and acute disseminated encephalomyelitis (ADEM) develop potentially pathogenic autoantibody responses that recognise the native, extracellular domain of myelin oligodendrocyte glycoprotein (MOG). The aim of this study was to develop an animal model of paediatric MS/ADEM that reproduces the pathological and serological features as a platform for the development of antigen-specific immunotherapies.

Methods: DBA/1 mice were immunized with the extracellular domain of myelin oligodendrocyte glycoprotein (MOG_{1-125}) or MOG_{79-96} peptide in Freund's complete adjuvant and development of clinical signs of EAE followed up to 30 days post immunization. Serum titers of MOG-specific antibodies were monitored by flow cytometry using live MOG transfected LTK3$^-$ cells and ELISA, and complement-dependent demyelinating activity assayed using myelinating cultures derived from embryonic rat spinal cord. The effects of low dose soluble MOG on disease development in MOG_{1-125} induced EAE was studied by treating mice with 25 μg soluble MOG (i.p.) every second day from day two post immunization. Treatment effects on antigen-specific lymphocyte proliferation and cytokine secretion in draining lymph nodes, inflammation within the CNS and the serum antibody response were determined between 9 and 14 days post-immunization.

Results: Immunization with MOG_{1-125} in adjuvant induced a rapidly fatal inflammatory demyelinating variant of EAE in DBA/1 mice associated with a high titer demyelinating MOG-specific antibody response that recognizes the extracellular domain of the protein as demonstrated by flow cytometry. In contrast, disease induced by MOG_{79-96} was far less severe and was not associated with a demyelinating MOG-specific antibody response. This was not due to intrinsic lack of immunogenicity as MOG_{79-96} immunized mice developed high-titer responses to both the immunizing peptide and MOG_{1-125} by ELISA. However these antibodies were unable to bind to native MOG at the surface of live cells. MOG_{1-125} induced EAE in DBA/1 mice therefore provides a model that reproduces the serological as well as clinical and pathological characteristics of pediatric MS/ADEM. In this model treatment with soluble MOG reduced disease progression; an effect associated with a global reduction in recruitment of both effector and regulatory T cell subsets into the CNS, as well as monocytes and B cells. However, although the total number of B cells in the CNS was reduced, the absolute number of a potentially regulatory CD1d$^+$CD5$^+$ B cell subset was increased 4-fold. These observations are in agreement with previous studies demonstrating treatment with soluble autoantigen suppresses the development of T cell mediated inflammation in the CNS. However, in the case of MOG-induced disease these beneficial effects an abrogated by 2-fold treatment-dependent increase in the demyelinating MOG-specific antibody titer.

Conclusions: Immunization with MOG_{1-125} induces an inflammatory demyelinating disease in DBA/1 that reproduces a specific serological response that characterizes many cases of pediatric MS and ADEM; namely, the presence of potentially pathogenic autoantibodies recognizing conformation-dependent epitopes exposed at the surface of the extracellular domain of MOG. Treatment with soluble MOG reduces disease progression in this model; an effect associated with a selective recruitment of potentially immunoregulatory CD1d$^+$CD5$^+$ B cells into the CNS. However, any beneficial effect provided by this cellular response is negated by a treatment induced increase in the demyelinating antibody titer. These studies demonstrate effective antigen-specific strategies for pediatric MS and ADEM must simultaneously target both T and B cell dependent effector mechanisms.

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Distinct subsets of interleukin-1 receptor antagonist producing cells are neuroprotective after focal cerebral ischemia in mice

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Objective: The cytokine interleukin-1 (IL-1) and its naturally occurring receptor antagonist (IL-1Ra) play a key role in determining

neuronal cell death and survival in focal cerebral ischemia. The objective of this study was to determine the cellular production of IL-1/IL-1Ra, and to test the neuroprotective potential of post-surgically injected IL-1Ra overexpressing bone marrow (BM) cells in a mouse model of focal cerebral ischemia.
Methods: C57BL/6 mice were injected i.v. with BM cells isolated from sIL-1Ra overexpressing mice, 30 min after permanent middle cerebral artery occlusion (pMCAo). Physiological parameters and behaviour were recorded post-surgically, infarct sizes were estimated, and microglial-leukocyte expression of IL-1/IL-1Ra was analyzed by flow cytometry, in situ hybridization and immunohistochemistry.
Results: We identify microglia, and not recruited leukocytes as the major producers of IL-1Ra after pMCAo in mice, and we show by using IL-1Ra knock out mice that microglial-produced IL-1Ra is neuroprotective. We report that the sIL-1Ra produced by the post-surgically injected BM cells, potentiates the neuroprotective effect of microglial-derived IL-1Ra, at both 24 h and 5 days, which is consistent with behavioural improvement at 5 days, and detection of recruited BM cells in the ischemic area 1.5 h after pMCAo. Interestingly, we also find that the sIL-1Ra overexpressing BM cells stimulate microglial production of IL-1Ra 6 h after pMCAo, at which time both IL-1α and IL-1β is upregulated.
Conclusions: Our results provide proof of principle that increasing the production of IL-1Ra by recruited BM cells can counteract the effect of IL-1α/β, increase neuronal survival and improve motor function alone or through induction of microglial-produced IL-1Ra after pMCAo in mice.

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Prolactin is dispensable to the development of experimental autoimmune encephalomyelitis

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Objective: Predominance of multiple sclerosis (MS) in women, reductions of relapses during pregnancy and their increase in post-partum period have suggested a hormonal influence on MS activity. The hormone prolactin (PRL) has long been debated as a potential promoting factor in several autoimmune diseases, including MS and the animal model for this disease, experimental autoimmune encephalomyelitis (EAE). However there are no available data that allow specifically ascribing a pathogenic role to PRL in the development of central nervous system autoimmunity. In the present work we investigated this issue by studying EAE in mice carrying genetic deletions for PRL-receptor (PRL-R) or PRL.
Methods: PRL$^{+/-}$ (B6.129S2(Cg)-*Prl*^{tm1Hmn}/J) and C57BL/6 mice were purchased from Jackson Laboratory. PRL$^{+/-}$ and PRL-R$^{+/-}$ mice were bred in our animal facility to give homozygotes. EAE was induced by immunization with 100 μg MOG_{35-55} in CFA and *B. Pertussis* toxin. Proliferative response of lymph node cells (LNCs) was assessed by [H^3]thymidine incorporation. Cytokine production was tested in parallel cultures by ELISA. Gene expression analysis was performed by real time PCR.
Results: In absence of PRL-R or PRL, EAE developed with a slight, significant delayed onset but with full clinical severity compared to their wild-type littermates. In line with these clinical findings, LNCs from PRL-R$^{-/-}$ mice restimulated *in vitro* with MOG_{35-55} exhibited reduced proliferation and decreased production of pro-inflammatory cytokines such as IFN-γ, IL-17A and IL-6 compared to those from control mice during disease priming 7 days p.i., but not at later time points (i.e. day 10 and 34 p.i). Moreover, PRL-R$^{-/-}$ mice and their controls displayed similar serum titres of anti-MOG_{35-55} IgG antibodies. The same findings were obtained in PRL$^{-/-}$ mice. In wild type naïve C57BL/6 mice, LNCs and CD4$^+$ T cells expressed transcripts for PRL-R. Expression of PRL-R was reduced in naïve LNCs stimulated *in vitro* with anti-CD3, as well as in LNCs and CD4$^+$ T cells obtained from mice 7 days p.i., suggesting that this receptor is down-regulated in stimulated T cells. We did not detect PRL either at the mRNA level in LNCs and CD4$^+$ T cells in any of the above conditions, or at the protein level in supernatants of cultured LNCs.
Conclusions: These data suggest that PRL plays a redundant role in the development of an optimal autoreactive Th1 and Th17 T cell response against myelin and in the clinical expression of EAE.

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SLAM (CD150) functions as an inflammatory modulator in the brain

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Objective: Signaling lymphocyte activation molecule (SLAM/CD150) is a mediator of innate and adaptive immune responses. It is expressed in immuno-competent cells, including lymphocytes, thymocytes, dendritic cells and macrophages. SLAM functions as a co-stimulatory molecule, through both homotypic and heterotypic interactions, to regulate responses to inflammatory mediators such as pathogens and proinflammatory cytokines. Importantly, SLAM plays a role in regulating phagocytosis, and in the bias of T cells towards a Th2 phenotype. In the current study we have assessed expression of SLAM and SLAM-mediated signalling in the CNS, and investigated its potential role in modulating inflammatory responses.
Methods: We have used Western immunoblot to assess the expression of SLAM and associated signalling molecules in brain tissue from adult C57BL6/J mice, and ELISA to evaluate cytokine release from cultures of isolated microglia, astrocytes and neurons from neonatal C57BL6/J mice. Cells were treated with recombinant SLAM to induce SLAM-mediated signalling, and β-amyloid (Aβ) to assess the effects of SLAM on modulating inflammatory insult. Additionally we carried out *in vitro* electrophysiological recording from mouse hippocampal slices to determine the effects of SLAM on the Aβ-induced impairment of synaptic function.
Results:Our findings illustrate robust SLAM expression in hippocampus and cortex of C57BL6/J mice. Analysis of the cellular localization of SLAM revealed it to be highly expressed in isolated microglia, relative to astrocytes or neurons. The evidence indicates that ligation of SLAM in microglia activates a downstream signaling pathway involving phosphorylation of Dok, Akt, ERK and activation of the transcription factor NFκB, which impact the production of proinflammatory cytokines. In light of its role in regulating peripheral immune responses, we assessed the effects of SLAM activation on the inflammatory response induced by the neurotoxic peptide, Aβ, the principal component of Alzheimer's disease plaques. Our data indicate that SLAM modulates Aβ-induced responses *in vitro*, and alleviates the Aβ–mediated impairment of hippocampal long-term potentiation.
Conclusions: This study has identified the expression of the immuno-regulatory protein SLAM in the brain, most abundantly in microglia. We have elucidated a potential signaling mechanism downstream of SLAM activation. Finally we provide evidence that activation of SLAM can alleviate the adverse effects of Aβ on brain function.

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Type-1 Interferon signalling plays a deleterious role in the outcome after stroke

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Objective: Type 1 Interferons (IFNs) are a super-family of pleiotropic cytokines that induce pro-inflammatory gene transcription via the classical JAK/STAT pathway. Increasingly, there has been evidence in the literature to suggest that neuroinflammation plays a key role in the progression of neural injury seen in stroke.
Methods: To address this $IFNAR_1^{-/-}$ mice underwent mid cerebral artery occlusion (MCAO) surgery and primary neuronal and glial cultures were generated to assess the cell type specificity in responsed to oxygen glucose deprivation.
Results: IFNAR−/− mice demonstrated a decreased infarct size (24.9 ± 7.1 mm^3 $n = 8$) compared to wild-type controls (65.1 ± 4.8 mm^3 $n = 8$). Western blot and immunohistochemistry showed alterations in the Stat-1 and 3 phosphorylation profiles in the $IFNAR_1^{-/-}$. Neuroprotection conferred by the absence of IFN signaling was confirmed in IFNAR-deficient primary cultures that were protected from cell death when exposed to oxygen glucose deprivation (OGD). Co-culture experiments using $IFNAR_1^{-/-}$ glia and WT neurons and WT glia and $IFNAR_1^{-/-}$ neurons were carried out in the OGD model. $IFNAR_1^{-/-}$ neurons in the presence of WT glia no longer displayed a neuroprotective phenotype suggesting the glia are a major driver of the neuroinflammatory response. In an attempt to block IFNAR signalling *in vivo* a blocking monoclonal antibody targeting the $IFNAR_1$ receptor was injected into WT mice via the tail vein (0.5 mg) 30 min prior to MCAO. This resulted in a 60% decrease in infarct size when compared to the IgG control.
Conclusions: Collectively these results indicate signalling through the $IFNAR_1$ subunit is deleterious in stroke. Furthermore, our findings suggest that therapeutic agents targeting the $IFNAR_1$ subunit may be beneficial in reducing the severity of a neuro-inflammatory event following stroke and in doing so limit infarct size.

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Antagonists to toll receptor nine in neuroinflammation

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It has now been confirmed that plasmacytoid dendritic cells (pDCs) are present in the brain lesions of multiple sclerosis (MS) patients (Balakov et al. *Ann Neurol* 2010; 68: 899)) and are also increased in the cerebrospinal fluid (CSF). pDCs produce large amounts of type 1 interferon (IFN) which are elevated in untreated relapsing remitting MS patients. pDCs also express toll receptor TLR9 Antagonists to this receptor have been found to reduce the level of interferon α in the CSF of MS patients, thus providing a rational approach to treatment.

pDCs have not been demonstrated in experimental allergic encephalomyelitis (EAE). Using human sensory peripheral nerve as antigen, as an autoimmune model of tuberculoid leprosy, we have developed an animal model of granulomatous hypersensitivity. Rabbits were injected with homogenates of sural nerve or dorsal roots plus adjuvant. After varying time periods, the injected rabbits were skin tested with dilute concentrations of sensory nerve in saline, resulting in the production of epithelioid granulomas. pDCs are considered to be the precursors of epithelioid cells. Some of the cells in the nerve antigen induced granuloma have a 'plasma like' appearance and have infiltrated the dermal nerves with associated axonal degeneration. If these 'plasma like' cells were to be confirmed as pDCs, then it should be possible to develop an *in vitro* animal model, in which the action of TLR antagonists can be studied, especially their ability to decrease the levels of interferon α. The most active fraction in producing both these cells in the endoneurium and the axonal degeneration is a deoxycholate extracted membrane fraction from non-myelin peripheral nerve. This is active at concentration levels of 1 μg/ml. A similar antigen may be present in the brain, in which case an *in vitro* model could be developed to study the effect of TLR9 antagonists in preventing central nervous system axonal degeneration.

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Toll-like receptor 9 ligands modulate the inflammatory reaction and functional outcomes in spinal cord injury

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Objective: The goals of the investigations were to determine the modulation of the inflammatory response that follows traumatic spinal cord injury (SCI) by a synthetic toll-like receptor 9 (TLR 9) agonist and antagonist and to define the effects of TLR9 ligands on the functional outcomes of acute SCI.
Methods: Adult female C57Bl/6 mice received a severe contusion at the T8 level using the Infinite Horizon Impactor. Mice received intrathecal injections of the TLR9 agonist (CpG ODN 1826), TLR9 antagonist (CpG ODN 2088), or vehicle (sterile, endotoxin-free water) at 1 day post-injury (dpi) and on alternate days thereafter. Mice were euthanized at various times after injury. A 3-mm spinal cord section containing the injury epicenter was dissociated. Cells were labeled with fluorescently-labeled antibodies, or isotype controls, and analyzed by fluorescence activated cell sorting. In addition, cytokine expression at the injury epicenter was evaluated by quantitative reverse transcriptase polymerase chain reaction (qPCR). Interleukin 1β (IL-1β), tumor necrosis factor-α (TNF-α), IL-10, or transforming growth factor-β1 (TGF-β1) transcript levels were evaluated. Functional testing including motor, sensory, and bladder function was also assessed.
Results: CpG ODN 1826 enhanced and CpG ODN 2088 attenuated the injury-induced increase in CD11b, CD45, and CD3-positive cell number at the epicenter compared to vehicle-treated injured mice. Both CpG ODN 1826 and CpG ODN 2088 differentially modulated cytokine expression at the injury epicenter. CpG ODN 1826 promoted IL-1β, TNF-α, and IL-10 expression but decreased TGF-β1 transcript levels. In contrast, CpG ODN 2088 reduced TNF-α expression and had no effect on TGF-β1 transcript levels. In addition, CpG ODN 1826 exacerbated and CpG ODN 2088 ameliorated the injury-induced bladder dysfunction. CpG ODN 2088 also reduced injury associated pain hypersensitivity. The TLR9 ligands did not induce systemic side effects as assessed by serum cytokine levels and spleen histology.
Conclusions: Intrathecal administration of TLR9 ligands modulates the inflammatory response and functional outcomes of SCI. The studies highlight the therapeutic potential of CpG ODN 2088 in bladder control and pain sensitivity in SCI. As some TLR9 ligands are evaluated in

clinical trials of cancer and infections, encouraging findings, such as those reported here, support the notion that inhibitory CpG ODNs could be attractive candidates for human SCI trials.

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Acidosis in the mouse model of multiple sclerosis and in in vitro neuronal networks

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Objective: Multiple sclerosis (MS) is an inflammatory demyelinating disease of the central nervous system (CNS) featured by severe axonal and neuronal degenerative processes. Beside classical mechanisms leading demyelination and neurodegeneration, the acidification of the extracellular matrix and the consequent activation of Acid sensing ion channels (ASICs) has been proposed as an emerging cause of neuronal derangement. Acidification-mediated activation of Asics induced overload of Na^+ and Ca^{2+} in neurons that might induce axonal swelling and neurodegeneration. Accordingly, the inhibition of Asics channels in EAE mice ameliorates the clinical severity, counteracting neuronal damage processes. The scope of this work is the investigation of electrophysiological effects elicited by Asics activation in primary neuronal cultures coupled with micro electrode array (MEA) devices and the effects of Asic1 selective blockers on Experimental Autoimmune Encephalomyelitis (EAE) mouse model.
Methods. Primary neuronal cell cultures obtained from the mouse embryonic cerebral cortex were plated onto poly-L-lysine and laminin coated MEAs. Long term recording were done before, during and after acidosis and in the presence of specific Asics antagonists (Diminazene and Psalmotoxin-1). Furthermore we tested the effects of acidosis on cell survival by using HTS/HCS immunofluorescence based technologies. Finally we administered, 2 days after the onset of the pathology in EAE mice, diminazene or psalmotoxin-1 to block selectively Asic1 channels at the peak of inflammation.
Results: Low pH elicits a substantial impairment of neuronal functioning that includes network synchronization defects. Although, acidosis inhibits firing activities of neurons, this phenomenon is transient and largely dependent of pH levels. The administration of both Diminazene and Psalmotoxin, partially rescued electrophysiological alterations occurring during acidosis. Low pH also elicited apoptosis, suggesting that the acidosis mediated activation of Asics channels participate to histo-pathogenetic processes occurring in EAE and possibly in MS.
Conclusions: Using MEA technology and EAE mouse model we explored the contribution of Asics to the maintenance of neuronal homeostasis, creating new perspectives for a comprehensive study of the role of pH variations in MS.

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Increased responsiveness to LPS in CD200-deficient mice is associated with increased blood brain barrier permeability and infiltration of peripheral cells

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Objective: The interaction between CD200, which is expressed on numerous cells, and CD200 receptor, which is expressed mainly on cells of the myeloid lineage including microglia, contributes to the maintenance of microglia in a quiescent state. Therefore, predictably, lipopolysaccharide (LPS) has been shown to induce greater activation of microglia prepared from $CD200^{-/-}$, compared with wildtype, mice. Here we assessed the effect of intraperitoneal (ip) injection of LPS on activation of microglia in hippocampus of wildtype and $CD200^{-/-}$ mice and investigated, using magnetic resonance imaging (MRI), the possibility that the changes were a consequence of increased blood brain barrier (BBB) permeability and the associated infiltration of immune cells.
Methods: Wildtype and CD200-deficient mice were injected ip with LPS (50 μg/mouse) and, after 3 hours, anaesthetized with isofluorane and positioned in a stereotaxic frame in a small rodent Bruker Biospec 7 Tesla MRI. BBB permeability was evaluated by examining extravasation of gadopentate dimeglumine, and hippocampal tissue, prepared from these animals, was assessed for CD11b and CD40 mRNA. IFNγ concentration was assessed in hippocampal tissue by ELISA. In separate experiments, a single cell suspension was prepared from whole brain of perfused wildtype, and CD200-deficient, mice and assessed for evidence of infiltrating cells using flow cytometry.
Results: LPS increased expression of CD11b and CD40 mRNA to a greater extent in cells prepared from hippocampal tissue of $CD200^{-/-}$, compared with wildtype, mice. Since IFNγ, which is a potent activator of microglia, was significantly increased in tissue from $CD200^{-/-}$ mice and since resident cells produce little IFNγ, we examined infiltration of IFNγ-producing peripheral cells and report a significant increase in $CD4^+$ cells and $CD11b^+$ $CD45^{high}$ macrophages in brain tissue from $CD200^{-/-}$, compared with wildtype, mice. The data indicate that infiltration was associated with increased BBB permeability in $CD200^{-/-}$ mice.
Conclusions: The findings demonstrate that LPS induces more profound microglial activation in the hippocampus of $CD200^{-/-}$, compared with wildtype, mice. We propose that this is a consequence of activation by IFNγ which is released from infiltrating T cells and macrophages and that the increase in BBB permeability in $CD200^{-/-}$ mice is a significant factor in allowing entry of these circulating cells.

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Modulation of microglial phenotypes by CD200

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Objective: Activated microglia, like macrophages, adopt different phenotypes depending on the stimulus; IFNγ, the Th1 cell-derived cytokine, induces classical activation of microglia, whereas, Th2 cell-derived cytokines, like IL-4, induce an alternative activation state. Acquired deactivation, mediated by TGFβ and/or IL-10, is associated with the down-regulation of the innate immune response. In addition, a deactivation state has been described in which the interaction between ligand–receptor pairs, e.g. CD200–CD200R, play a key role in suppression of the immune response. Here we investigate the modulatory effect of CD200, expressed on neurons and astrocytes, on the response of microglia to IFNγ and IL-4.

Methods: Mixed glia and purified microglial were cultured from neonatal C57BL/6 wildtype and CD200$^{-/-}$ mice and incubated in the presence or absence of IFNγ (50 ng/ml; 24 h), CD200Fc, a synthetic soluble activator of the CD200 receptor (2.5 μg/ml; 30 min pre-treatment) or IL-4 (200 ng/ml). Concentrations of cytokines were assessed in supernatant samples, and cells were harvested to investigate IFNγ Receptor1 and pSTAT1 expression using Western immunoblotting, and markers of microglia which identify classically- and alternatively-activated states using PCR.
Results: IFNγ increased mRNA expression of markers of classical activation, TNFα and iNOS to a greater extent in cells from CD200$^{-/-}$, compared with wildtype, mice. This may be accounted for by increased expression of IFNγR1 and activation of STAT1. In contrast, expression of mannose receptor, a marker of alternative activation, was selectively down-regulated by IFNγ. CD200Fc suppressed IFNγ-induced TNFα and IL-6 mRNA, and the supernatant concentration of these cytokines, in cells prepared from wildtype mice.

IL-4 increased mRNA expression of arginase 1, mannose receptor and chitinase 3-like 3 in cells prepared from wildtype and CD200$^{-/-}$ mice, whereas iNOS and TNFα mRNA were down-regulated by IL-4.
Conclusions: The data demonstrate that microglia from wildtype and CD200$^{-/-}$ mice respond to insults by altering their phenotypic state ranging from the characteristic pro-inflammatory profile associated with classical activation, to an alternative activation state resulting in tissue repair and extracellular matrix remodelling. Cells obtained from CD200$^{-/-}$ mice displayed an enhanced response to IFNγ suggesting CD200-related deactivation of microglia may specifically modulate the classically-activated pathway.

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S1P receptor internalisation attenuates IL17/TNFα-induced production of IL6 in human astrocytes

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Objective: The family of sphingosine-1-phosphate receptors (S1PRs) is G-protein coupled and composed subtypes S1P1R-S1P5R. These receptors are drug targets of Gilenya® (Fingolimod, pFTY720), the first approved oral therapy for multiple sclerosis (MS). To further examine the function of S1P1Rs in the CNS, their role in cytokine release from human astrocytes was demonstrated. Here, focus was given to an IL17-induced increase in levels of IL6, both of which have been implicated as pro-inflammatory cytokines in MS.
Methods: Human astrocytes derived from fetal cerebral cortex or primary mouse astrocytes from day 3 postnatal C57BL/6J pups were treated with or without IL17 +/− TNFα, in the presence or absence of S1PR compounds. The levels of IL6 protein in the culture media and levels of IL6 mRNA in astrocyte cells were examined using ELISA and quantitative PCR, respectively. The S1P1R internalisation was examined by immunocytochemistry.
Results: The data showed that human astrocytes expressed IL17 receptors and in vitro treatment of human or mouse astrocytes with IL17 increased the levels of IL6, which was greatly enhanced in the presence TNFα. Importantly, the treatment of human astrocytes with S1P also increased the levels of IL6. In contrast, pFTY720 inhibited the IL17/TNFα-induced increase in levels of IL6, as did the S1P1R selective compound AUY954. Notably, both pFTY720 and AUY954, but not S1P, internalised S1P1Rs which likely explained the opposing effects of these S1PR ligands on regulating the levels of IL6. The effects of IL17 did not involve the sphingosine kinase pathway.
Conclusions: Taken together, the data suggested that pFTY720 attenuates the IL17/TNFα-induced increase of IL6 in human astrocytes via a mechanism that involves internalisation and functional antagonism of S1P1Rs. The pFTY720-mediated inhibition of IL17/TNFα-induced increase in IL6 signaling in astrocytes likely contributes to the ability of this drug to reduce pro-neuroinflammatory pathways that sustain MS.

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A comparative study of two PET radioligands for the imaging of neuroinflammation in an animal model of multiple sclerosis

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Objective: Multiple Sclerosis (MS) is the most common cause of progressive disability in the western world. The exact causes of the disease remain unknown, however, MS leads to demyelination, axon loss and blood–brain-barrier breakdown within the CNS. Current diagnostic criteria relies on MRI imaging which is insensitive to disease progression within the normal appearing white water within the CNS which is thought to be especially important in progressive disease. We aim to study two different PET radioligands (^{18}F-GE180 and ^{11}C-PK11195) in an animal model of MS. These two PET radioligands target the peripheral benzodiazepine receptor (PBR) which is a marker of activated microglia cells.
Methods: Two animal models were used to test the relative binding characteristics of the different ligands. In both models *in-vivo* PET imaging and *ex-vivo* autoradiography techniques were used to assess the binding. Lewis rats (n = 12, ^{18}F-GE180 and 12, ^{11}C PK11195) were injected with LPS (10 μg in 1 μL) or saline (1 μL) into the left striatum. The animals were imaged 12 hours following this injection. A focal delayed type hyposensitivity EAE model of MS was also used. Lewis rats were injected intrastriatally with heat killed BCG (1 μL). The lesions were then activated *via* an intradermal injection of BCG in CFA. Animals were imaged on day 0, day 14 and day 28 (n = 18) following lesion activation. Animals were also killed for autoradiography at each time point (n = 12).
Results: ROI analysis of the autoradiography showed that there was an increase in the binding ratio across the whole hemisphere for the LPS injected animals (1.75 ± 0.54 PK11195; 2.32 ± 0.24 GE180). For the MS-model there was an increase in binding at all-time points when the ROIs were placed in the striatum (day 0 — PK11195, 2.44 ± 0.22, GE180 — 2.97 ± 0.73; day 14 — PK11195, 3.01 ± 0.33 GE180 — 3.31; day 28 — PK11195, 2.04 ± 0.03 GE180 — 2.68 ± 0.31) and cortex (day 0 — PK11195, 1.14 ± 0.06, GE180 — 1.58 ± 0.52; day 14 — PK11195, 1.26 ± 0.06 GE180 — 1.67; day 28 — PK11195, 1.23 ± 0.08 GE180 — 1.38 ± 0.14). From the *in-vivo* images of the DTH lesion, there was also an increase in TSPO binding at all timepoints in a ROI drawn around the lesion compared to the corresponding ROI on the contralateral side. Day 0 — PK11195 = 1.99 ± 0.42, GE180 = 1.57 ±

0.29; Day 14 — PK11195 = 1.63 ± 0.07 GE180 = 1.56 ± 0.26; Day 28 — PK11195 1.45, GE180 = 1.75 ± 0.26.
Conclusions: GE-180 and PK11195 both reveal the presence of neuroinflammation in two different animal models. The area of microglia activation in a clinically relevant model of MS is greater than the area revealed by conventional imaging techniques such as MRI. From the autoradiography results, GE-180 reveals a larger area of microglia activation surrounding the DTH lesion. This could be due to a number of factors, critically GE-180 is a fluorinated tracer and therefore, has a longer half-life compared to the carbon-11 tracer of PK11195.

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Effects of polarized T cell and macrophage supernatants on human fetal OPCs

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Objective: Neural-immune cell interactions are important determinants of the central nervous system microenvironment, with the potential to either induce injury or promote repair. Pro-inflammatory cytokines associated with Th1 polarized T cells and M1 polarized myeloid cells, including IFN-gamma and TNF-alpha, have been implicated as mediators of rodent oligodendrocyte progenitor cell (OPC) injury. Activated human B-cell supernatants have been shown to induce TNF-independent injury to rodent oligodendrocytes. Previous studies have shown that both Th2 and M2 polarized cells produce neurotrophic factors that support rodent OPC survival and differentiation. The objective of the present study was to use human fetal OPCs to compare the capacity for Th1/Th2 T cell supernatants and M1/M2 macrophage supernatants to induce injury or promote survival and differentiation.
Methods: Human fetal A2B5+ cells were isolated from brain specimens (16–20 weeks gestation) obtained from the Human Tissue Repository at the Albert Einstein College of Medicine. T cells and macrophages were polarized to the Th1/Th2 or M1/M2 lineages, respectively, as we have previously described. OPCs were grown on astrocyte-matrix or ECM in DMEM/F12/N1 with growth factors PDGF-AA and bFGF. After 4 days in culture, OPCs were treated with either 10% of AIM-V (media only), Th1, Th2, M1, or M2 supernatants for 2 days. Cells either were fixed and stained or changed to fresh media and grown for another 4 days, then fixed and stained for Olig2, O4 and GC by immunohistochemistry.
Results: Application of Th1 supernatants to human fetal OPCs reduced the number of O4+ cells and Olig2+ cells after 2 days in culture; after washing, O4+ cells remained lower 4 days later. Application of Th2 supernatants reduced only the number of O4+ cells after 2 days, and this effect was reversed 4 days later after washing. Neither M1 nor M2 macrophage supernatants induced a significant change. Both Th1 T cells and M1 macrophages produced comparable levels of TNF-alpha. Addition of recombinant IFN-gamma, a Th1-associated cytokine, reduced the number of O4+ cells in culture.
Conclusions: This data implicates pro-inflammatory T cells rather than macrophages as the predominate cell type mediating OPC injury. Whether soluble factors produced by Th2 or M2 polarized cells can protect progenitors from such injury remains to be determined.

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AlphaB-crystallin influences the autoimmune role of neutrophils in experimental autoimmune encephalomyelitis

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Objective: Multiple sclerosis (MS) is a neurological disease in which self-reactive immune cells target and destroy oligodendrocytes. It is therefore imperative to control the inflammatory response in MS. Neutrophils are the most prevalent immune cell in human blood and contribute to inflammatory environments by eliciting recruitment and activation of other immune cells, cytokine production and effector mechanisms such as granule secretion. Due to their absence in MS CNS tissue, a role for these cells in the disease has not been considered. It was recently discovered that neutrophils are more active in MS patients especially during relapses[1]. Also, a robust infiltration of these cells is observed in the CNS of mice with experimental autoimmune encephalomyelitis (EAE), a model of MS[2]. We are interested in further investigating whether neutrophils play a role in the initiation, development and/or progression of EAE. AlphaB-crystallin (alphaBC) is the highest up-regulated gene in active MS lesions and possesses a number of protective functions including chaperoning, anti-neurotoxic and anti-inflammatory capabilities. Studies have shown that alphaBC promotes the survival of astrocytes and suppresses symptoms of EAE. We hypothesize that alphaBC plays a protective role in EAE by, in part, down-regulating the neutrophil inflammatory responses.
Methods and results: In MOG_{35-55}-immunized 129S6 wild type (WT) EAE mice, migration and extravasation of $Ly6G^+$ neutrophils from venules into the brains of these mice was observed at peak of disease using intravital imaging. To determine if alphaBC reduces the activation of neutrophils, these cells were isolated from 1) the spleens and blood of naïve WT mice and stimulated with lipopolysaccharide (LPS) and/or formyl-Met-Leu-Phe in the presence or absence of alphaBC peptide and, 2) the spleens of EAE mice treated with 10 micrograms of recombinant human (rh) alphaBC or PBS everyday from day of immunization and restimulated *in vitro* with LPS. The secretion of pro-inflammatory cytokines, IL-1beta, IL-12p40, and TNF, as measured by ELISA, was reduced in neutrophils exposed to alphaBC.
Conclusions: Reduction of neutrophil responses may contribute to the amelioration of EAE clinical symptoms following alphaBC treatment. Ongoing studies are assessing neutrophil effector functions and influence on T cell activation in EAE.

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The effect of CNS inflammation on the onset of infectious and genetic prion disease

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Objective: Prion diseases manifest as infections, genetic and sporadic disorders caused by a misfolded protein denominated PrP^{Sc}. We used 2 mouse models, one which carry a mutation at codon 199 of the prion protein and succumb to disease at the age of 5–6 months and the other model is wt C57Bl/6J mice infected with prions, in order to investigate the effect of CNS inflammation on the early stages of disease. In previous work we show that in the infectious mouse model, CNS inflammation in the form of experimental autoimmune encephalomyelitis (EAE) accelerate disease manifestation, concomitantly with the deposition of PrP^{Sc} aggregates in inflamed white matter areas. In the present work, we asked whether CNS inflammation accelerate disease also in the genetic mouse model and whether the mechanism of acceleration relates to infiltration of activated prion infected immune cells to the CNS.

Methods: C57Bl/6J mice were inoculated intraperitoneally with scrapie brain homogenates and later induced for EAE by the inoculation of MOG_{35-55} in Complete Freund's adjuvant (CFA) supplemented with pertussis toxin. Lymphoid cells from the co-induced animals were injected into naïve mice as viable cells or as cell homogenates. In parallel Tg mice carrying a mutation at codon 199 representing a model of E200K genetic CJD were also induced for EAE and followed up for EAE and prion disease clinical signs.
Results: We show here that mice infected with activated prion–EAE viable cells succumb to prion disease considerably faster than mice infected with equivalent cell extracts or other control groups, probably due to an accelerated form of neuroinvasion, an important factor in infectious prion disease. Contrarily, in the genetic mouse model the pathological process could not be manipulated by CNS inflammation and the disease incubation period remained the same as in the untreated Tg mice.
Conclusions: We speculate that in the genetic disease PrP^{Sc} is generated spontaneously in the CNS and the prions entering the brain throughout the immune system by an accelerated neuroinvasion cannot interfere with the progression of disease. Whether these conclusions have important clinical implications as related to the risk of genetic or infectious prion disease remain to be established.

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ADAMTSs and extracellular matrix alteration in multiple sclerosis

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Objective: To determine a role for ADAMTS-1, -4, -5 in the pathogenesis of MS.
Methods: ADAMTS-1, -4 and -5 mRNA expression was studied by qRT-PCR in human neuroblastoma SHSY-5Y cells, undifferentiated or differentiated to a more neuronal phenotype using retinoic acid. Modulation by proinflammatory cytokines, which are involved in the pathogenesis of MS, was also studied. Cryostat sections of normal and MS CNS tissue white matter, obtained from the UK Multiple Sclerosis Tissue Bank, were used to determine ADAMTS-1 expression by Western blotting. Expression of neoepitopes of versican, derived by ADAMTS cleavage, was examined by immunohistochemistry.
Results: SHSY-5Y cells expressed ADAMTS-1, -4 and -5 constitutively. ADAMTS-1 was significantly increased on cellular differentiation with retinoic acid. IL-1 and TNF had no effect on ADAMTS mRNA expression. ADAMTS-1 protein was observed in SHSY-5Y cells and CNS tissue sections by Western blotting of extracted proteins. ADAMTS-mediated versican breakdown, as determined by neoepitope expression, appears to be increased in MS brain tissue compared to normal brain tissue. Differential expression in normal and CNS tissue is being investigated.
Conclusions: ADAMTS-1, -4 and -5 were constitutively expressed in the SHSY-5Y neuronal cell model, however they were not modulated by the cytokines tested. Neuronal cells may therefore not be a major source of ADAMTSs involved in MS pathogenesis. However ADAMTSs from other sources may be involved in ECM breakdown in MS brain and therefore MS pathogenesis.

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Endothelial ICAM-1 and ICAM-2 are essential for mediating neutrophil crawling and facilitate paracellular transmigration across the inflamed blood–brain barrier in vitro

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Neutrophils are early cellular participants in acute cerebral inflammation contributing to exacerbation of tissue damage and facilitation of monocyte recruitment. The entry of circulating leukocytes into the central nervous system (CNS) is strictly controlled by the blood–brain barrier (BBB) but the precise molecular mechanisms mediating the multi-step neutrophil migration across the inflamed BBB remain to be investigated. Here we studied the involvement of the neutrophil β2-integrins and their endothelial ligands intercellular adhesion molecule-1 (ICAM-1), ICAM-2 and junctional adhesion molecule-A (JAM-A) in neutrophil firm adhesion and crawling and in diapedesis across the highly specialized BBB under physiological flow *in vitro*. Employing highly purified mouse bone marrow derived neutrophils and primary mouse brain microvascular endothelial cells (pMBMECs) from adhesion molecule deficient mice, we found endothelial ICAM-1 to be critically involved in mediating neutrophil arrest on the BBB under flow. Post-arrest neutrophil crawling depended on endothelial ICAM-1 and ICAM-2, as neutrophil crawling was only completely abolished in the absence of both adhesion receptors. Neutrophil LFA-1 was found to be the major ligand of ICAM-1 and ICAM-2 in mediating neutrophil crawling with additional conributions by other β2-integrins. In the absence of these ligands neutrophils displayed an atypical adhesive behavior with the BBB characterized by a fast displacement (>16 μm/min) on the BBB in the direction of flow designated as *speeding*. We found no involvement of JAM-A in neutrophil arrest, crawling or *speeding* on the inflamed BBB under flow. Finally, by using pMBMECs derived from mice expressing a C-terminal GFP fusion protein of vascular endothelial (VE)-cadherin, endothelial ICAM-1 and ICAM-2 were found to guide neutrophils to diapedesis across the BBB via the paracellular route.

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Interleukin 26: A Th17 cytokine which impacts on the blood brain barrier

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Objective: Multiple sclerosis (MS) is an autoimmune disease characterised by the disruption of blood brain barrier (BBB) integrity. Our laboratory has previously shown that Th17 lymphocytes are implicated in this pathology and that IL-17 has a particular ability to affect the permeability of the BBB. However, little is known about additional Th17-related mediators and their impact on BBB integrity. IL-26 is a pro-inflammatory cytokine, so far only known to regulate epithelial cells functions. In this study, we aim to investigate whether IL-26 is associated with the human Th17 phenotype and whether it could impact on BBB functions.
Methods: We first performed a thorough characterization of IL-26 expression in human Th1, Th2 and Th17 lymphocytes using q-PCR and flow cytometry analysis. We also evaluated IL-26 receptor (IL-26R) expression on the BBB, using primary cultures of human brain-derived endothelial cells (BBB-ECs) and human brain tissue sections. To test the effect of IL-26 on BBB function we used an *in vitro* Boyden chamber permeability assay.
Results: We found that IL-26 expression is linked to Th17 phenotype and that IL-26 expression correlated with levels of IL-17, IL-22, IL-23R and RORγ. In addition, we found that both IL-26R α (IL-20Rα) and β

(Il-10Rβ) chains are expressed at the human BBB *in situ* and by BBB-ECs *in vitro*, suggesting that BBB endothelial cells can respond to IL-26. *In vitro* treatment of human BBB-ECs resulted in an increase in permeability and a down regulation of the tight junction molecule occludin.
Conclusions: Therefore, the Th17-secreted cytokine IL-26 might play a role in impacting BBB integrity and has potential implications in MS pathology.

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Comprehensive monitoring of intrathecal immune cells in patients with multiple sclerosis by 12-color flow cytometric immunophenotyping

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Objective:To devise method for comprehensive monitoring of intrathecal immune cells in clinical trials and to investigate their role in the pathogenesis of neuroimmunological diseases.
Methods: For immunological research of CNS inflammatory diseases, CSF cells are valuable because of their proximity to affected tissue. We devised 12 color flow cytometric immunophenotyping (FCI) method to reliably quantify 20 different populations of immune cells, including subtypes of T cells, B cells, NK cells, monocytes, myeloid dendritic cells (DCs), plasmacytoid DCs, granulocytes, basophils and assess their activation status based on the surface expression of activation markers CD25, CD80 and HLA-DR in a limited sample of >5000 CSF cells. Fresh CSF and uncoagulated (EDTA) peripheral blood from patients with untreated neurological diseases (N = 101) were collected and processed simultaneously. Cohort includes clinically isolated syndrome (CIS, N = 3), relapsing-remitting MS (RR-MS, N = 27), primary progressive MS (PP-MS, N = 20), secondary progressive MS (SP-MS, N = 14), other inflammatory neurological diseases (OIND, N = 29) and non-inflammatory neurological diseases (NIND, N = 8). FCI was performed and analyzed in a blinded manner.
Results: CSF has predominance of T cells while monocytes, granulocyte and B cells are under-represented in comparison to blood. CD56bright NK cells are highly enriched in CSF (1.39%) compared to blood (0.21%). CSF CD4/CD8 and plasmacytoid DC/myeloid DC ratio is significantly elevated. Significant positive correlation between blood and CSF monocyte, basophil, plasmacytoid DCs and myeloid DCs was observed. CSF HLA-DR + activated CD8 + T cells significantly correlated with blood HLA-DR + activated CD8 + T cells and terminally differentiated double negative T cells ($p<0.0001$), suggesting that the activation of cytotoxic T cells in periphery and CNS is closely linked.

Percentage of activated monocytes (i.e. CD25 + CD80 +) is significantly elevated in OIND compared to MS, indicating that full activation of myeloid lineage is not typical feature of the immune response in MS. Unsupervised hierachical clustering analysis according to cellular subpopulation revealed that 5 distinct clusters exist in the CSF and a cluster enriched with RR-MS patients is clearly separate from the other clusters enriched in progressive MS or OIND.
Conclusions: 12-color FCI provides valuable information about the phenotype of intrathecal immune responses in neuroimmunological disorders.

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Alpha-synuclein induces MHCII dependent CD4 T cell proliferation and activation of microglia

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Objective: Parkinson disease (PD) is a progressive neurodegenerative disorder characterized by a loss of dopaminergic neurons in the substantia nigra and intracellular aggregates of the protein alpha-synuclein (α-syn). Reactive microgliosis, production of inflammatory cytokines and chemokines and T cell infiltration are key mediators in PD pathogenesis however it is unknown how α-syn leads to immune system activation. In these studies we examined the role of the major histocompatibility complex-II (MHCII) in α-syn-induced neuroinflammation and neurodegeneration.
Methods: Using an AAV2-α-syn overexpression model of PD, we assessed MHCII expression, reactive microgliosis, and IgG deposition 4 weeks post-transduction as well as dopaminergic neuron loss at 6 months post-transduction in wildtype and MHCII deficient animals. In an *in vitro* co-culture system using primary microglia and OTII transgenic T cells we assessed the effects of α-syn on cytokine and chemokine expression and T cell proliferation.
Results: *In vivo*, we found that α-syn causes induction of MHCII expression in microglia, increased microgliosis and IgG deposition, whereas genetic knockout of MHCII prevents α-syn-induced microglial activation at 4 weeks post-transduction. In our *in vitro* co-culture system, α-syn treatment of microglia increased presentation of antigen to CD4 + T cells, expression of cytokines and chemokines, and induced CD4 + T cell proliferation.
Conclusions: Results from these studies indicate a crucial role for MHCII in the inflammatory and neurodegenerative processes mediated by α-syn. These studies may provide the groundwork for the development of a completely novel class of neuroprotective therapies targeted to inhibit MHCII in PD-specific inflammatory processes.

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Investigating T cell–Oligodendrocyte precursor cell interactions

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Objective: Although the immune system is known to be involved in multiple sclerosis (MS) pathogenesis, immune responses are also implicated in the repair of myelin (remyelination). *In vivo* studies have shown T cells to be necessary for efficient remyelination; however the mechanisms by which this occurs are poorly understood. We hypothesise that interactions between CD4 + T cell subsets and oligodendrocyte precursor cells (OPCs) may drive OPC maturation, alter T cell phenotypes and influence remyelination. In order to investigate this, we sought to develop an *in vitro* model whereby the reciprocal effect of T cells on OPCs could be further studied.
Methods: OPCs were isolated from neonatal murine forebrains and digestion, separation and culture conditions were optimised. Using magnetic beads, OPCs were selected for NG2 expression. OPCs were cultured in conditions which supported proliferation, and then co-cultured with CD4 + T cell subsets which were polarised to distinct subsets. As OPCs cannot be grown in serum, a range of co-culture conditions were tested to identify optimal OPC and T cell culture conditions.
Results: Neural medium, supplemented with B27, was sufficient to support growth of polarised T cells in the presence of additional cytokines. Maturation of OPCs was quantified by immunofluorescence

using Myelin Basic Protein (MBP) as a marker of differentiated oligodendrocytes. T cell phenotype was characterised by flow cytometry. After 96 hrs in co-culture conditions, Th1 cells were found to be producing high levels of both IFNγ and IL-10, as measured by ELISA, and were expressing T-bet, the classical Th1 transcription factor.
Conclusions: We have developed an experimental co-culture model whereby OPCs and CD4+ T cell subsets can interact and the reciprocal effects of these interactions can be examined. This novel T cell–OPC co-culture model lends itself to investigation of OPC maturation. This is a key process in remyelination and has implications for the treatment of demyelinating diseases such as MS.

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Acute peripheral and CNS immune responses in hypercholesterolemic mice after mild brain ischemia

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Objective: Peripheral inflammation contributes to ischemic brain injury, albeit underlying mechanisms are not fully understood yet. The reason of the latter is most likely due to insufficient experimental models which neglect co-morbidities such as hypertension and hypercholesterolemia. Although hypercholesterolemia is a well-established risk factor for stroke, its role in peripheral inflammation and subsequent ischemic brain injury remains elusive.
Methods: Wild type and ApoE$^{-/-}$ mice fed with high or normal cholesterol diet were exposed to 20-min of middle cerebral artery occlusion (MCAO). Acute ischemia-induced systemic immunological changes were characterized by flow cytometry in the spleen and the blood 24 h after ischemia. Infarct volumes, edema formation, cellular apoptosis and vascular adhesion molecule expression in the brain were determined via immunohistochemistry. Differential immune cell infiltration into ischemic and non-ischemic brain hemispheres was quantified by flow cytometry.
Results: We observed increased cellular frequencies of splenic CD11b+ cells and activated T cells in hypercholesterolemic mice after mild MCAO. These increased peripheral immune responses were strongly associated with increased infarct volumes and number of apoptotic cells as well as intracellular adhesion molecule-1 expression. In line with the enhanced vascular adhesion molecule expression immune cell infiltration in ischemic brain hemispheres of hypercholesterolemic mice was increased.
Conclusions: The close correlation between peripheral immunological responses and increased neuronal damage combined with enhanced inflammatory reactions in the brain suggest that stroke induced acute immunological changes in hypercholesterolemia might contribute to ischemia induced tissue injury. In order to develop more specific therapies targeting these inflammatory responses a greater understanding of the contribution of specific immune cell populations including their molecular pathways involved in these processes is still required.

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IFNγ induced gene expression during the course of experimental autoimmune encephalomyelitis; focus on microglia

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Objective: Multiple sclerosis (MS) is a complex neuroinflammatory disorder, characterized by multifocal inflammation within the CNS, demyelination and axonal damage. Cumulative axonal damage clinically manifests as a broad range of physical and cognitive disabilities. A usually overlooked and debilitating set of symptoms, associated with poor prognosis in MS patients, are balance and coordination deficits. Experimental autoimmune encephalomyelitis (EAE) is the most commonly used animal model for MS. Wild type (wt) mice with EAE develop ascending paralysis, which has been linked to inflammatory lesions in the spinal cord. In mice lacking interferon-gamma (IFNγ) signaling, however, EAE induction leads to the development of deficits such as axial rotation, circling behavior, and balance defects that are atypical of this disease model. These atypical deficits had been linked to inflammatory foci within the cerebellum/ brainstem. Interestingly, we previously showed that the instance and severity of atypical defects in IFNγR-knockout (KO) mice with EAE are not dependent upon the extent, composition, or areas of inflammatory foci formation in the CNS. Instead, we found that the onset of deficits is strongly linked to the absence of IFNγ signaling in the CNS resident microglia. In this study, we therefore hypothesized that IFNγ signaling in EAE would promote an anti-inflammatory/neuroprotective transcriptional profile. We set out to examine differences in the transcriptional regulation of IFNγ (a) in the inflamed CNS and (b) specifically in T-cell activated-microglia.
Methods: We induced EAE in mice by immunization with myelin-oligodendrocyte glycoprotein (MOG) peptide. CNS tissues were harvested from adult IFNγRKO mice and wt controls, both at onset and chronic phase of EAE and assayed by RT-PCR. Targets were categorized as: inflammatory/anti-inflammatory, and neuroprotective/neurotoxic.

To test IFNγ signaling effects in T cell-activated microglia, we established an ex-vivo co-culture system. Mixed glia isolated from CNS tissues of adult IFNγRKO and wt mice were cultured in the presence of M-CSF. Microglia were then purified and co-cultured with T cells isolated from IFNgKO or wt mice with EAE, with or without MOG. At several time points, T cells were removed and assayed for cytokine production by flow cytometry. RNA isolated from microglia was subjected to RT-PCR analysis for gene expression of the categories mentioned above.
Results: Results from this study will be instrumental in elucidating the mechanism by which IFNγ signaling affects the clinical course of EAE and provide a better understanding of the disease process that contributes to balance deficits in MS.
Conclusions: IFNγ signaling in microglia might prove an important targeting pathway for therapeutic intervention in the inflamed CNS.

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Vitamin D supplementation protects juvenile/adolescent, but not adult or 'in utero' treated rats against multiple sclerosis-like neuroinflammation

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Objective: Lack of vitamin D has been associated with a higher prevalence, relapse rate and progression of multiple sclerosis (MS). The ontogenetic timing of this association has remained unclear, as well as any potential effects of vitamin D supplementation in established MS. We here study the role of vitamin D during different developmental stages in myelin oligodendrocyte glycoprotein (MOG)-induced experimental autoimmune encephalomyelitis (EAE), an animal model of MS.

Methods: Juvenile, adult and the mothers of *'in utero'* treated rats were split into three groups and exposed to different levels of vitamin D in their diet: 10, 0 or 2 international units (IU) prior to subsequently inducing EAE in juvenile, adult and in *'in utero'* treated rats (the last ones were subjected to the regular laboratory diet (2 IU vitamin D) right after weaning until the end of the experiment).
Results: Vitamin D supplementation led to a milder disease course in juvenile/adolescent, but not in adult rats. Protected rats displayed less severe central nervous system (CNS) inflammation and demyelination. Moreover, juvenile rats subjected to vitamin D rich diet displayed lower number of IFN-γ producing MOG specific T cells and their cytokine expression pattern reflected a shift towards an anti-inflammatory phenotype. In contrast, treatment of the mothers with different levels of vitamin D did not significantly influence the susceptibility to EAE in their offspring.
Conclusions: We conclude that vitamin D plays an important role during development by affecting the susceptibility, as well as the severity of MS-like neuroinflammation in rats. These positive effects on clinical disease are predominant in juvenile/adolescent period. Our data emphasize the importance of the right timing for vitamin D treatment which makes an important contribution to current intense interest in use of vitamin D as a prevention, or even as a therapy against MS.

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Multivariate analysis of inflammatory and neuronal injury markers in cerebrospinal fluid of multiple sclerosis: Higher levels are associated with younger age

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Objective: The extent of neuronal injury and axonal loss determine physical disability in MS patients and are both associated with degree of CNS inflammation. Inflammatory mediators have crucial role in leukocyte recruitment and subsequent CNS neuroinflammation. The aim of the present study was to use data-driven multivariate statistics to explore possible associations between a panel of selected CSF biomarkers and robust clinical and demographic parameters in a large cohort of MS patients.
Methods: Levels of some of the most validated inflammatory biomarkers; matrix metalloproteinases-9 (MMP9), the B-cell chemokine CXCL13, osteopontin (OPN), and a marker of nerve injury, neurofilament-light chain (NFL) were measured by ELISA in 550 subjects: relapsing-remitting MS (RRMS; n = 210), secondary progressive MS (SPMS; n = 22), primary progressive MS (PPMS; n = 12), clinically isolated syndrome (CIS; n = 87), other non-inflammatory neurological diseases (ONDs; n = 92), and inflammatory/infectious neurological disease ONDs with signs of inflammation or viral/bacterial infections (iOND; n = 125). The association study was performed using principal component analyses (PCA) and orthogonal partial least squares (OPLS/O2PLS).
Results: Our main finding was that the variable age consistently segregated in the opposite way to both inflammatory (CXCL13/MMP9) and acute axonal damage (NFL) markers in younger patients, but only up to an age of approximately 54 years. CXCL13 and MMP9 co-varied well in the younger age groups, but less so in older patients, possibly because the levels of both markers were lower in the latter group. CXCL13 and MMP9 also correlated well with both NFL and OPN in younger patients. These results were validated in several ways, both by excluding age from the PCA models and also by using the model to predict the scores of a test set of cases that were not included in the construction of the original model.
Conclusions: This study indicates a clear effect of age up to the intervals between 50 and 55, with a greater degree of inflammation and axonal damage being associated with younger age. This indicates a potential benefit from early anti-inflammatory interventions, especially in younger age groups.

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Microglia–neutrophil interactions: A new field of application for the phagocytosis Marker pHrodo

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Objective: Microglia are playing an important role as the resident scavenger cells of the central nervous system. During ischemic episodes, such as stroke, the blood–brain barrier is impaired and leukocytes are able to infiltrate the tissue. Among these are neutrophil granulocytes, the most common white blood cells, as well as T-cells and monocytes. This results in a sterile inflammation and evokes the activation of quiescent microglia. Recently, we could show that neutrophil infiltration leads to strong neurotoxic effects which are reduced by the engulfment of living motile neutrophils by microglial cells. The underlying mechanisms are not sufficiently known. Since quantification in live imaging is time consuming and inconsistent we developed an alternative phagocytosis assay based on a new flow cytometric approach.
Method: We have applied pHrodo succinimidyl ester dye as pH-dependent amin-reactive marker for phagocytosis. Under acidic environment particularly phagolysosomes we were able to detect emission in the red light diapason corresponding to 585 nm wave length. Until recently staining with pHrodo was implemented only on prokaryonts and thymocytes (Miksa et al., 2009; Maneu et al., 2011). To mimic the in vivo events in vitro, freshly prepared murine neutrophils were added to cultures of murine microglia at different set ups and time points. The phagocytosis of pHrodo labeled neutrophils was quantified by flow cytometry and optically verified by fluorescence microscopy.
Results: We are the first to show that an amin reactive dye can be used for neutrophils, which are very active scavenger cells with a high membrane turnover. Our new staining protocol is highly effective and quick, which is important, due to the fact that the half-life of neutrophils is estimated in about 6–12 h. It was shown that the engulfment takes place rapidly after co-incubation, even though the neutrophils are still non-apoptotic at that very moment. This confirms our previous findings of in-vivo 2-photon microscopy. Thus we can determine the rate of phagocytosis in microglial cells using different conditions.
Conclusions: The new protocol enables a simple quantification of the process of phagocytosis. This allows answering questions about phagocytosis enhancing conditions and underlying mechanisms of engulfment.

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Antibodies to ion channels in limbic encephalitis

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Objective: Limbic encephalitis (LE) is an infectious or autoimmune CNS damage characterized by inflammation of limbic system and other parts of the brain. The main symptoms of LE are a severe impairment of short-term memory deficits plus confusion, psychiatric signs, and seizures. These symptoms typically develop over a few weeks or months, but they may evolve over a few days. Early detection may improve outcome of LE. All types of this nosology fall into 2 main categories: Infectious encephalitis – caused by direct invasion of the brain by a virus. Autoimmune encephalitis – caused by immune system reacting against certain oncogens in a case of paraneoplastic LE, or against a voltage-gated potassium channels (VGKC). The aim of our study was to elucidate a concentration of antibodies (AB) to human herpes virus 6 (HHV-6), contactin-2 associated protein (Caspr2), and to Kv1 subunits of VGKC.
Methods: Under our observation were 31 patients with LE, 9 from them demonstrated symptoms of LE version – Morvan's syndrome, which included irregular contractions of long muscles, cramping, weakness, hyperhidrosis and insomnia. 76 healthy donors comprised control group. We used both ELISA and Western Blot for detection of all three types of AB.
Results: It was found that AB against HHV-6 were detected in 26 (84%) patients, and AB against Caspr2 and Kv1 only in 9 (29%) patients. Concentration of all three types of AB in control group didn't exceed 13%.
Conclusions: Our new data reveal a role of infectious AB more often than paraneoplastic or ion channels AB in majority of LE cases.

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Anti-TNF therapy improves behavioural outcomes after focal cerebral ischemia in mice

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Objective: Tumor necrosis factor (TNF) plays an important, but complex role, in the pathophysiology of stroke. TNF exists in two biologically active forms, soluble (solTNF) and transmembrane (tmTNF). Anti-TNF therapy using Etanercept, which blocks both forms, is neuroprotective in models of acute CNS injury and is clinically effective in the treatment of several autoimmune diseases. However, Etanercept also triggers several serious side effects, such as congestive heart failure, demyelinating disease, lupus syndrome and also infections, which represent a considerable risk for stroke victims. solTNF-specific inhibitors, sparing tmTNF, have the potential to inhibit deleterious inflammation without compromising the immune system's response to infections. In the present study, we tested the effect of XPro1595, a new class of dominant-negative TNF inhibitors that selectively inhibit solTNF and block several of TNF's well-known functions, such as nuclear NF-κB translocation and caspase-mediated apoptosis, in mice subjected to focal cerebral ischemia.
Methods: Infarct volumes were compared 6 h, 1 and 5 days after permanent middle cerebral artery occlusion (pMCAO) in C57BL/6 mice treated with an i.v. injection in the tail vein 30 min after surgery of 1) XPro1595 (10 mg/kg/dose), 2) Etanercept (10 mg/kg/dose), or 3) vehicle (saline). Mice were weighed, temperature monitored and tested regarding behavior/improved recovery using the horizontal rod, grip strength and rotarod test. Acute phase response (APR) proteins and microparticle analysis was also investigated in serum samples after surgery.
Results: Comparison of the mean infarct volume for each group revealed no significant difference. However, mice treated with anti-TNF therapy performed significantly better than saline-treated mice in the grip strength test on day 1 and 3 and the total number of footfaults on the horizontal beam was reduced in the anti-TNF treated group, with the Etanercept-treated animals performing slightly better than the XPro1595-treated animals. The APR was reduced by the Anti-TNF, but the magnitude of the response was far less pronouced than in other brain injury models. The number of microparticles in the circulation was also unchanged at day 1 but the number increased significantly in XPro1595-treated mice at day 5, and with a tendency to increased numbers in Etanercept-treated mice compared to saline-treated mice.
Conclusions: Anti-TNF therapy did not reduce infarct volume in this model, but the changes in behavioural outcomes were significant. While the CNS inflammatory response, and the APR is less pronounced in this model compared to others, it is still the case that some benefit from anti-TNF therapy may be accrued.

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The effect of extracellular superoxide dismutase 1 on microglia/macrophage phenotype in the context of amyotrophic lateral sclerosis

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Objective: The toxic properties of mutated superoxide dismutase 1 (SOD1) contribute to disease progression in familial amyotrophic lateral sclerosis (ALS). SOD1 protein can be secreted from cells and is detected in the cerebral spinal fluid of ALS and healthy patients. It has been shown that extracellular mutant SOD1 protein activates microglia/macrophages to become neurotoxic. Work in our laboratory has determined that Ly6C^{Hi} inflammatory monocytes exhibit pro-inflammatory microRNA (miRNA) and genetic profiles two months prior to disease onset in ALS mice. Spinal cord microglia, on the other hand, exhibit pro-inflammatory miRNA and gene profiles only one month prior to disease onset in ALS mice. The objective of this work is to determine whether extracellular SOD1 contributes to the activation and role of microglia/monocytes in disease progression of ALS mice.
Methods: Bone marrow derived macrophages (BMDM) and resident microglia were cultured from adult mice. Adult microglia were collected for culture by sorting with 4D4, a novel microglia-specific monoclonal antibody generated in our laboratory. Cells were stimulated with wild type or mutant SOD1 protein purified form yeast.

For control, cells were stimulated with lipopolysaccharide. The effect of extracellular SOD1 on miRNA and gene expression in microglia and BMDM was measured by Taqman qPCR analysis. Taqman analysis was performed on selected miRNAs and genes that were found in our laboratory to be dysregulated in microglia or Ly6C^{Hi} monocytes *in vivo*.
Results: Many pro-inflammatory miRNAs were affected in BMDM and microglia after stimulation with mutated SOD1 protein. The time and dose sensitivity to SOD1 protein was different in microglia and BMDM, suggesting that the cells may express different levels of receptors or signaling molecules involved in sensing extracellular SOD1 protein.
Conclusions: Extracellular SOD1 protein may activate microglia and peripheral monocytes *in vivo*. The earlier activation of Ly6C^{Hi} monocytes in ALS mice may be due to potential differences in concentration of extracellular SOD1 in the periphery versus spinal cord and/or differences in expression of SOD1-sensing machinery in Ly6C^{Hi} monocytes versus microglia.

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MCAM is a novel adhesion molecule expressed at the BBB that regulates inflammatory immune cell migration

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Objective: Expression of adhesion molecules by blood–brain barrier endothelial cells (BBB-ECs) and leukocytes regulates immune cell trafficking to the CNS. In inflammatory conditions such as multiple sclerosis (MS), CD4 T lymphocytes polarized into auto-aggressive encephalitogenic T_H17 and T_H1 are known to transmigrate across BBB and accumulate in central nervous system (CNS) lesions. While CD8 have been implicated in MS pathogenesis as well, the role of CD8-T_c17 or T_c1 lymphocytes is still unclear. Identification of adhesion molecules specifically involved in the recruitment of highly inflammatory immune cell populations through the BBB would provide new therapeutic targets to impact on neuroinflammatory lesions without impairing CNS immunosurveillance. We hypothesize that MCAM plays a role in CNS inflammation by promoting recruitment of IL-17-producing lymphocytes through the BBB into the CNS parenchyma.
Methods: In a proteomic screen and analysis, we identified expression of MCAM/CD146 on human BBB-ECs and on a subset of human effector CD4$^+$ and CD8$^+$ T lymphocytes. We characterized the phenotype and the function of MCAM-expressing cells using a combination of *ex vivo*, *in vitro* and *in situ* studies using human and mouse material from healthy controls, MS subjects and Experimental Autoimmune Encephalomyelitis (EAE) animals.
Results: We demonstrate that MCAM is co-expressed by human effector CD161$^+$ and CCR6$^+$ T lymphocytes, and that both CD4 and CD8 MCAM$^+$ lymphocytes express more IL-17, IL-22, GM-CSF and Granzyme B than MCAM^{neg} lymphocytes. Moreover, MCAM is strikingly up-regulated in human during MS relapses on CD4$^+$ and CD8$^+$ T lymphocytes, while treatment decreases MCAM expression. In addition, MCAM blockade reduces CD8 T_c17 and CD4 T_H17 lymphocyte transmigration across human BBB-ECs *in vitro*.

In vivo, depletion of MCAM$^+$ cells from MOG_{35-55}-reactivated CD4 T lymphocytes decreases clinical symptoms in adoptive transfer EAE experiments. Furthermore, expression of MCAM is strongly up-regulated on CD8 T lymphocytes in the TCR1640 transgenic mice, a unique model of spontaneous relapsing-remitting EAE.
Conclusions: Our data demonstrate that encephalitogenic IL-17-producing CD8 and CD4 T lymphocytes express MCAM, and that MCAM could serve as a biomarker for MS and a valuable target for the treatment neuroinflammatory conditions.

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Aberrant expression of interleukin-1 by neoplastic human astrocytes: Implications for glioma progression and therapy

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Objective: Interleukin 1 is a major proinflammatory cytokine produced by myeloid cells and is implicated in neurodegeneration. In human brain, IL-1 is expressed by microglia and contributes to neuroinflammation and neuronal toxicity. IL-1 is also the major activator of astrocyte innate immune responses through the MyD88 pathway. Although human astrocytes do not produce IL-1, reports indicate neoplastic astrocytes might. We systematically investigated whether human glioma cells produce IL-1 and the mechanisms that regulate IL-1 biosynthesis in glioma.
Methods: Human glioblastoma cell lines (U251, U87, and SNB19) were stimulated with cytokines (IL-1/IFNg) or TLR ligands (LPS or poly IC) and the expression of IL-1a and IL-1b was studied by real-time PCR, western blot analysis and ELISA. Functional assay was performed using glioma-derived culture supernatants and human fetal neuronal cultures as targets. The role of the innate transcription factor IRF3 was tested by adenovirus-mediated gene transfer, and the inflammasome modulator annexin A2, by siRNA. The glioma secretome has been investigated using quantitative proteomic approach.
Results: Cytokine activated glioma cultures produced IL-1 protein in amounts comparable to activated microglia. Similar to microglia, the bulk of IL-1b was intracellular, suggesting the involvement of inflammasome in IL-1 processing. Glioma supernatants induced neuronal killing which was reversed by IL-1 receptor antagonist. Annexin A2, a suppressor of macrophage inflammasome, *promoted* glioma IL-1 production and release. In contrast, IRF3 suppressed glioma IL-1 production, similar to macrophages. We are currently analyzing glioma secretome which includes cytokines, chemokines, proteases, protease inhibitors, tumor-associated proteins, growth factors, extracellular matrix components, and matrix remodeling proteins.
Conclusions: To our knowledge, this is the first study demonstrating the striking finding that cytokine-activated human glioma cells produce IL-1 protein (intracellular and secreted) in amounts comparable to activated microglia. As IL-1 is a major trigger for glioma angiogenesis, invasion, and proliferation, our data indicate the presence of glioma-microglia connection that promotes tumor progression.

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Comparative analysis of human and mouse astrocytes as components of the brain innate immune system

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Objective: Astrocyte cultures derived from neonatal rodent or human fetal brains are readily available systems that have aided our

understanding of astrocyte responses to various insults. Treatment of cultures with toll-like receptor (TLR) ligands or cytokines has become a popular approach to investigate astrocyte responses to innate immune factors. One of the highly debated points in glial biology is the extent to which astrocytes can participate in the innate immune response. We compared highly purified primary murine and human astrocyte cultures in their responses to LPS, poly IC and cytokines (IL-1 ± IFNg), as the three main immune triggers.
Methods: Primary astrocyte cultures (>99% GFAP+, Iba-1 negative) were obtained from mouse neonatal brains and from human fetal brains by serial passages of initial mixed cultures. Inflammatory gene expression was examined by real-time PCR and ELISA. Primary microglia (human and murine) as well as a microglial cell line BV2 were also analyzed in comparison to address the potential "contaminating microglia" problem.
Results: Human astrocytes were unresponsive to LPS, but were highly responsive to IL-1/IFNg. Mouse astrocytes were highly responsive to LPS and much less responsive to IL-1/IFNg, but the response also depended on specific target genes. For example, the levels of LPS-induced astrocyte IL-1 and TNFa production were comparable to those of LPS-stimulated microglia. However, IL-1/IFNg was a better inducer of iNOS than LPS. Poly IC induced high levels of TNFa and iNOS in murine astrocytes but not in human astrocytes.
Conclusions: Our side by side experiments using highly pure astrocyte cultures demonstrate that unlike human astrocytes, murine astrocytes are capable of responding to LPS, similar to microglia. We suggest that the most convenient marker of murine astrocyte LPS response is TNFa release (ELISA). Initial analyses show that mouse astrocyte LPS response is mitigated by TLR4 siRNA suggesting that the TLR4 signaling pathway is preserved in murine astrocytes. Poly IC induces a more proinflammatory, toxic gene profile in murine compared to human astrocytes. Our study has implications for the interpretations of experimental results derived from various culture systems and supports that astrocyte innate immune responses are species-dependent.

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TGF-beta1-induced astrocytic release of interleukin-6: A possible role in epielsptogenesis

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Objective: It is becoming increasingly evident that inflammatory mechanisms promote neuronal hyper-synchronization and epileptogenesis following brain insults. Our recent studies identified serum albumin as a key player in the inflammatory cascade leading to epilepsy, at least partially through the activation of TGF-β signaling pathway in astrocytes. In this study we set out to explore the mechanisms by which TGF-β1 induces glial activation and epileptogenesis.
Methods: Primary cultures of microglia and astrocytes were treated with TGF-β1 and gene expression analysis was performed by quantitative PCR. Electrophysiological response to IL-6 was obtained in the acute slice preparation (field potential recordings) and in-vivo (subdural corticoencephalography in C57/BL6 mice implanted with osmotic pumps, 1–5 μg/ml into the lateral ventricle for 7 days).
Results: In glia cultures TGF-β1 induced early and rapid up-regulation of IL-6 at both mRNA and protein levels whereas the upregulation of other pro-inflammatory cytokines such as IL-1β and TNF-α was milder and at a later time point. Notably, SMAD2/3-dependent TGF-β1 signaling induced the expression of IL-6 primarily in astrocytes and to a significantly lesser extent in microglia. IL-6 was sufficient to induce epileptiform activity in acute brain slices 2–3 h after exposure. Finally, a continuous intracerebroventricular injection of IL-6 (1 and 5 μg/ml) induced recurrent seizures within 3–4 days in more than 75% of treated animals.
Conclusions: Taking into account the robust induction of IL-6 by albumin, we suggest astrocytic release of IL-6 as a potential key player in epileptogenesis.

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TNF-α regulation of corticostriatal synaptic strength: A homeostatic response in movement disorders

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Objective: Neuro-inflammatory processes are considered key elements of the pathophysiology of movement disorders such as Huntington's and *Parkinson's disease*. In particular, increased levels of the proinflammatory cytokine, tumor necrosis factor (TNF)-α are found in the brains of patients. In addition to its immune function, TNF-α has been shown to have a neuro-regulatory function at synapses, increasing AMPA receptor (AMPAR) trafficking to the cell surface on hippocampal and cortical pyramidal neurons. Altered corticostriatal synaptic function is receiving increasing attention as an early correlate of neurodegenerative diseases associated with movement disorders. However, whether TNF-α contributes to these changes has not been investigated.
Methods: In the present study, we use patch-clamp recordings from striatal medium spiny neurons in acute brain slices to examine the contribution of TNF-α to the regulation of AMPAR trafficking.
Results: In acute slices, exogenous TNF-α treatment reduces the magnitude of corticostriatal AMPAR excitatory postsynaptic current (EPSC) relative to the NMDAR EPSC, a correlate of synaptic strength. We find that this occurs through a preferential removal of AMPAR lacking the GluA2 subunit. In addition, we show that TNF-α is involved in a homeostatic response to prolonged blockade of D2 dopamine receptors, and is required to reduce extrapyramidal motor symptoms.
Conclusions: These data suggest that TNF-α is a regulator of glutamatergic synaptic strength in the medium spiny neurons of the corticostriatal pathway in a manner distinct from its regulation of synapses on pyramidal cells, and that TNF-α may mediate an adaptive response during early stages of pathological conditions.

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Changes in beclin 1 associated with Alzheimer's disease negatively regulate retromer and impair phagocytosis of amyloid-beta

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Objective: Phagocytosis plays an important role in maintaining brain homeostasis and when impaired can result in the accumulation of unwanted cellular material, dysfunctional synaptic pruning,

inflammation and tissue damage. In Alzheimer's disease (AD), microglia (i.e., the phagocytes of the brain) surround β-amyloid (Aβ) plaques yet seem unable to eliminate these deposits, suggesting phagocytosis may be impaired in AD. Mechanisms that might contribute to reduced phagocytosis in AD are currently unclear.
Methods: Beclin 1 was reduced in vitro in BV2 microglia cells using lentiviral constructs. Phagocytic efficiency was determined by live cell imaging and flow cytometry. In vivo phagocytosis was determined histologically after intracerebral injection of fibrillized Aβ or pH-sensitive latex beads into beclin 1 deficient mice. Protein expression was determined by western blot. Retromer localization to the phagosome was visualized by live cell imaging using RFP-tagged constructs. CD36 receptor recycling was quantified using immunocytochemistry approaches and a well-established receptor recycling assay. Human microglia were isolated from postmortem AD brains using standard Percoll gradients.
Results: Here, we identify a novel function for the autophagy protein beclin 1 in regulating phagocytic efficiency and phagocytic receptor recycling. Specifically, we demonstrate that beclin 1, which is dysregulated in AD, is necessary for efficient phagocytosis of latex beads and Aβ in vitro (using BV2 microglial cells) and in vivo within mouse brains. Using live cell imaging we observe that reducing beclin 1 also results in diminished retromer protein expression and localization to the phagosome. We further show that beclin 1-mediated impairments in retromer are sufficient to reduce the recycling of the phagocytic receptor CD36, a receptor known to facilitate Aβ uptake, and diminish phagocytosis. Interestingly, microglia isolated from postmortem AD brains show significantly reduced beclin 1 and retromer protein levels.
Conclusions: Together these findings position beclin 1 as a link between autophagy, retromer trafficking, and receptor-mediated phagocytosis and provide insight into mechanisms by which phagocytosis may be impaired in AD. Therefore, strategies that enhance beclin 1 levels may provide a novel approach for the treatment of AD.

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Addition of cerium oxide nanoparticles reverses adverse effects of diesel exhaust on AP-1 transcription factor activity in the mouse brain

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Objective: Cerium oxide (CeO_2) nanoparticles are used in diesel fuel as a catalyst to improve fuel efficiency. Studies show that CeO_2 can provide neuroprotection and this may be due to its antioxidant capability. AP-1 is a dimeric transcription factor formed by Jun and Fos family of proteins. AP-1 is important in regulation of cellular processes and response to stressful stimuli. Neuroinflammatory events and oxidative stress are factors which can activate AP-1. We examined the effect of diesel exposure with or without CeO_2 nanoparticles on AP-1 and its upstream activated proteins.
Methods: Atherosclerosis-prone apolipoprotein E knockout (ApoE −/−) mice were exposed by inhalation to exhaust from an engine using standard diesel (DE) or diesel containing CeO_2 (DCeE). Changes in brain and cerebellum were assessed using gel shift mobility assay for transcription factor activity and western blots for protein levels.
Results: AP-1 activation was significantly decreased in the brain fraction of DE groups compared to control group but addition of CeO_2 nanoparticles to the diesel caused recovery back to control levels. Similar recovery was seen in c-Jun and phosphorylated-c-Jun levels in the nuclear fractions of the brain samples. In the cytoplasmic fraction, we showed *de novo* synthesis of c-Jun only in DCeE group.
Conclusions: The potential of CeO_2 to counteract the effect of DE exposure is not only by activating the signaling pathway responsible for phosphorylation and activation of c-Jun but also by increasing *de novo* production of the c-Jun protein. This may be one mechanism by which CeO_2 nanoparticles are able to provide neuroprotection during stressful conditions.

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Unravelling the novel role of neuronal cell adhesion molecular heparan sulfate proteoglycan N-Syndecan in inflammatory response

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Objective: The crosstalk between the CNS and the immune system is important for both systems to keep their own homeostatic functions, as well as to control inflammations under pathological conditions. It is therefore pivotal to understand how neurons regulate inflammation in the CNS. However, this has not been thoroughly addressed so far. The aim of our research is to characterize how neuronal cell surface receptor/adhesion molecules are involved in neuroimmune crosstalk. N-Syndecan (Syndecan-3) is a major transmembrane heparan sulfate proteoglycan abundantly expressed in the central and peripheral nervous systems. It has been shown to regulate neuronal cell adhesion, migration and axonal outgrowth. N-Syndecan deficiency has been associated with impaired feeding behavior, learning and memory formation in mice. Little is known about its association with the CNS inflammation so far. However within this protein family, both Syndecan-1 and -4 have been shown to participate in the peripheral inflammatory responses through regulation of leukocyte transendothelial migration. We therefore aim to address how N-Syndecan regulates inflammation in the CNS.
Methods: In vivo, using the LPS-induced peripheral inflammation model (1 mg/kg, i.p. injection) to study the expression of N-Syndecan in inflammatory responses by Western blot (WB), in situ hybridization and qPCR, and using N-Syndecan knockout (NSKO) mouse line to compare the CNS immune status under the steady state and the inflammatory conditions by flow cytometry and immunohistochemistry. In vitro, using hippocampal neurons to study the expression of N-syndecan in response to proinflammatory cytokine stimulation by WB etc.
Results: The expression of N-Syndecan in the mouse cerebral cortex is upregulated in response to LPS stimulation in vivo. N-Syndecan is also induced in rat hippocampal neurons by cytokines TNFα and IFNγ in vitro, and the total heparan sulfate content is simultaneously upregulated. Microglia of P8 NSKO mice are more proinflammatory, with larger cell size, and enhanced expression of CD11b, and MHCII, especially in noncortical segments.
Conclusions: N-Syndecan is upregulated in response to inflammation induced by LPS and proinflammatory cytokines. The absence of it in mouse brain results in increased microglia proliferation and enhanced microglial proinflammatory properties at the early postnatal stage. These data suggest that N-Syndecan is involved in regulation of the CNS immune responses.

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Thy1+ Sca1+ innate lymphoid cells infiltrate the CNS during autoimmune inflammation, but do not contribute to disease progression

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Objective: Innate lymphoid cells (ILCs) are a recently discovered family of lymphocytes being involved in early host defense, particularly at mucosal surfaces such as lung and gut. Several reports have suggested a critical role for RORγt$^+$ ILCs during intestinal autoimmunity, and type 2 ILCs have been implicated in mouse models of allergen-induced airway inflammation. However, it has not been tested yet whether ILCs also participate in other autoimmune reactions. In the current study we investigated the involvement of Thy1$^+$ Sca1$^+$ ILCs in autoimmune neuroinflammation, using the mouse model of multiple sclerosis, experimental autoimmune encephalomyelitis (EAE).
Methods: To investigate the role of Thy1$^+$ Sca1$^+$ ILCs during neuroinflammation we used a 14-parameter flow cytometric approach. Furthermore, the functional relevance of ILCs was tested using an adoptive transfer model and depleting antibodies.
Results: Surprisingly, we could detect a significant population of ILCs within the central nervous system (CNS) even under physiological conditions. Their number expanded within the inflamed CNS tissue, accompanied by production of IL-17 and IFN-gamma. However, functional studies using a depleting antibody specifically targeting Thy1$^+$ ILCs revealed that their presence or absence does not modify disease progression.
Conclusions: Our results suggest that Thy1$^+$ Sca1$^+$ ILCs can be found not only at epithelial surfaces, but also in other non-lymphoid tissues such as the CNS. During autoimmunity, ILCs did not contribute to disease progression, but it remains unclear whether ILCs have a role in the defense against invading pathogens.

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Elucidating mechanisms of neuroinflammation in a Parkinson's disease model

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Objective: Parkinson's disease (PD) is a common, age-related neurodegenerative disorder characterized primarily by the loss of dopaminergic (DA) neurons in the substantia nigra (SN) in the ventral midbrain. The mechanisms that cause DA neuron death are multi-faceted and remain incompletely understood; however, many pathogenic processes have been implicated in both genetic and toxin models of parkinsonism including mitochondrial dysfunction, oxidative stress, neuroinflammation, and protein aggregation. Neuroinflammation has been observed in postmortem brain tissue from PD patients and in several models of parkinsonism, including the rotenone model. In this study, we sought to test the overall hypothesis that the CD200/CD200 receptor (CD200R) system plays a key role in regulating the local neuroinflammatory status of the substantia nigra and the sensitivity of dopaminergic neurons to rotenone-induced neurotoxicity. The CD200/CD200R system is an important regulator of inflammation and impaired signaling of this system has recently been shown to enhance DA neurodegeneration. Binding of CD200, which is localized to the neuronal membrane surface, with its cognate receptor CD200R, which is expressed on microglia, the putative inflammatory cell type in the central nervous system, maintains microglia in a non-activated state, resulting in attenuated neuroinflammation.
Methods: Primary neuron/microglia mixed cultures from rat embryonic ventral mesencephalon (VM) were used to determine the effects of rotenone, a mitochondrial complex I inhibitor and DA neurotoxin, on the CD200/CD200R system and microglial activation *in vitro*. Immunocytochemistry was used to detect CD200 ligand expression and microglial activation in primary neuron/microglia mixed cultures and cytokine antibody arrays were used to measure and quantify cytokine levels in these cultures.
Results: Our data indicate that treatment of primary neuron/microglia mixed cultures from embryonic VM with rotenone decreases CD200 expression on DA neurons and causes concomitant microglial activation. Cytokine assays indicate that rotenone-treated primary neuron/microglia mixed cultures exhibit increased levels of inflammatory cytokines and decreased levels of pro-survival cytokines.
Conclusions: Collectively, our observations support our hypothesis that the CD200/CD200R system contributes to regulation of the neuroinflammatory environment of the SN and the sensitivity of DA neurons to rotenone-induced neurotoxicity. Ongoing studies aim to determine the specific mechanism(s) by which rotenone perturbs CD200 expression and signaling.

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Th17 bias renders mice susceptible to a viral model for multiple sclerosis

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Objective: Theiler's murine encephalomyelitis virus-induced demyelinating disease (TMEV-IDD) is a viral model for multiple sclerosis (MS). T helper (Th) 17 cells are a new subset of Th cells that express the transcription factor RORγt, and secrete proinflammatory cytokines, such as interleukin (IL)-17. Th17 cells have been suggested to play an effector role in immune-mediated diseases, including MS, while the role of Th17 cells in viral infections is unclear. With our collaborators, we have established transgenic (Tg) mice overexpressing RORγt on the C57BL/6 background, which is normally resistant to TMEV-IDD. The RORγt Tg mice have significantly higher amounts of IL-17 in the serum and higher ratios of Th17 conversion in vitro. In TMEV-IDD, in theory, Th17 cells can function as effector cells and antagonize anti-viral Th1 immune response. We hypothesized that RORγt Tg mice on the normally resistant C57BL/6 background would become susceptible to TMEV-IDD.
Methods: RORγt Tg and wild-type mice were infected with TMEV and killed 2 months post infection. We harvested the sera and spleen cells to characterize the anti-viral immune responses. Cytokines and anti-TMEV antibodies were measured by enzyme-linked immunosorbent assay (ELISA). Luxol fast blue staining and anti-TMEV immunohistochemistry were used to assess demyelination and viral persistence, respectively.
Results: We detected demyelinating lesions with virus persistence in the spinal cords of TMEV-infected RORγt Tg mice, but not in wild-type mice. Splenocytes from TMEV-infected RORγt Tg mice stimulated with TMEV had higher IL-17 and lower interferon-γ (Th1 cytokine) production, compared with wild-type mice. Additionally, the RORγt Tg mice had less anti-TMEV antibody in the serum than wild-type mice. This was mainly due to significantly lower amounts of anti-TMEV IgG2c (a Th1-induced IgG isotype) and lower amounts of anti-TMEV IgG1 (a Th2-induced IgG isotype) in RORγt Tg mice compared with wild-type mice, while similar amounts of anti-TMEV IgG2b were found in RORγt Tg mice compared with wild-type mice. The RORγt Tg mice did not have an enhancement of anti-viral lymphoproliferation responses compared with wild-type mice in a [^{3}H]-thymidine incorporation assay.

Conclusions: RORγt Tg mice became susceptible to TMEV-IDD. Since Th17 cells can inhibit anti-viral Th1 immune responses, the susceptibility may be due to enhanced Th17 proinflammatory immune responses and reduced Th1 anti-viral immune responses.

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In vivo induction of Tr1 cells, attenuates animal model of secondary progressive MS, via regulation of the CNS innate system

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Multiple sclerosis is an inflammatory disease of the central nervous system (CNS) that begins as a relapsing-remitting disease and is followed by a progressive phase (SPMS). The progressive phase causes the greatest disability and has no effective therapy. It had been suggested that the CNS innate system (microglia and astrocytes) drive SPMS progression, yet the mechanisms underlying SPMS are mostly unknown. The mucosal immune system is unique in its ability to induce regulatory T cells, and tolerance, following antigen activation; suggesting that it might also have an immunotherapy potential in SPMS.

Here we demonstrate that in an autoimmune encephalomyelitis model of SPMS, nasal treatment of mice with an anti-CD3 antibody, resulted in an *in-vivo* induction of Tr1 cells, which attenuated the disease course and severity. Moreover, we showed that at least one the mechanisms in which the Tr1 cells act, is by regulating the activation state of the astrocytes and microglial cells in the CNS, affecting different functions of the CNS innate cells, such as the production of chemokines and cytokines. In line with these findings, we have further demonstrated that the beneficial effects of the Tr1 cells can be reverted by blocking of IL-10 signaling, the main cytokine produced by the Tr1 cells. These findings identify a new immunologic approach to treat SPMS, demonstrating that (1) nasal anti-CD3 treatment induce Tr1 cells *in-vivo* that suppress the progressions of an encephalomyelitis model of SPMS, (2) identify new functions of Tr1 cells in the CNS, and (3) contributes to our growing understanding of the mechanism that underlay the progression of SPMS.

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Increased expression of the hypoxia related genes in peripheral blood cells of subjects with acute ischaemic stroke

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Objective: After stroke there is modulation of the peripheral immune system, with early immune suppression followed by increased levels of activated T cells. After an acute ischaemic stroke, some of the blood that flows through the brain could be subject to hypoxia. Hypoxia can modulate leukocyte function, so this study was performed to determine whether the peripheral blood leukocytes in subjects with acute ischaemic stroke show changes due to hypoxia at the level of gene expression.
Methods: RNA expression profiles were examined in peripheral blood cells of subjects at day 1, week 1 and week 3 after acute ischemic stroke, as well as healthy controls and subjects with motor neurone disease. A human qPCR array kit containing 84 different hypoxia pathway related genes was used in the primary screening of biological triplicates from each time point after stroke. Twelve selected genes were then tested in 37 stroke patients, 19 healthy controls and 28 subjects with amyotrophic lateral sclerosis.
Results: There was upregulation of hypoxia related genes after stroke. The most upregulated gene was *ADM*, the gene encoding adrenomedullin which showed the significant fold change in the first day of stroke onset compare to controls. Other genes that were significantly upregulated were *HIF3A*, *VEGFA*, *NPY*, *BIRC5* and *ECE1*.
Conclusions: There is evidence that there is upregulation of hypoxia related genes in peripheral blood cells after acute ischaemic stroke. This could influence the function of the cells.

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Neuroinflammation in systemic parasitic diseases: Brain cytokine profile in canine visceral leishmaniasis

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Objective: To evaluate the cytokine profile in the brain of dogs naturally infected with *Leishmania chagasi*.
Methods: Nineteen dogs with visceral leishmaniasis (VL) and six uninfected dogs were included in this study. The brain was collected in buffered-formalin to obtain Hematoxylin–Eosin stained sections and also in RNA*later* to proceed with RNA isolation. The RNA was extracted, reverse transcript to cDNA and it was submitted to qPCR to quantify the gene expression of the pro-inflammatory cytokines IL-1β, IL-6, IL-12, IFN-γ, TNF-α, and the anti-inflammatory IL-10 and TGF-β, using specific primers and Taqman probes. G3PDH was used as a housekeeping gene.
Results: The gene expression of the pro-inflammatory cytokines was detected up-regulated in the infected dogs (IL-1β: 8.0-fold more; IL-6: 3.5-fold more; IFN-γ: 5.9-fold more; and TNF-α: 6.3-fold more), except IL-12, which gene expression was down-regulated (6.8-fold less) in comparison with the uninfected dogs On the other hand, there was noticed a down-regulation of the anti-inflammatory cytokines gene expression in the infected dogs when compared to the uninfected animals, with IL-10: 6.1-fold less; and TGF- β: 5.3-fold less. Regarding the histopathological analyses of the brain, there was noticed high amount of mononuclear cells infiltrating the leptomeninges, the periventricular area and the perivascular space of parenchymal blood vessels.
Conclusions: IL-1β, IL-6 and TNF-α are the main cytokines related to opening of the blood–brain barrier (BBB), especially by up-regulating the expression of adhesion molecules, needed for leukocyte migration, while IFN-γ is associated with glial activation. The reduction of IL-10 and TGF-β are also critic for brain inflammation since IL-10 is related to modulate the cytokine production by microglial cells and TGF-β maintains the integrity of the BBB tight junctions as well as controls glial activation. The down-regulation of IL-12 may be due to a TNF-α-mediated inhibition. Altogether these results give support to the hypothesis that there are in course inflammatory changes in the nervous milieu during the occurrence of VL, indicating that there is an active immune-communication between the periphery and the central nervous system.

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Spontaneous demyelinating autoimmune neuropathy in ICAM-1 deficient NOD mice

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Objective: In chronic inflammatory demyelinating polyneuropathy (CIDP) an autoimmune mediated damage to the peripheral nervous system (PNS) causes chronic progressive pareses and sensory impairments. Further studies are impeded by the lack of adequate animal models. The autoimmune prone 'non-obese diabetic' (NOD) mouse strain spontaneously develops autoimmune diabetes. NOD mice deficient in 'intercellular adhesion molecule 1' (ICAM-1) – mediating costimulation and endothelial cell adhesion – are protected from diabetes, but instead develop progressive pareses.
Methods: We examined ICAM-1$^{-/-}$NOD mice by phenotype analysis, PNS electrophysiology and histology and flow cytometry. We quantified cytokine production and performed different adoptive transfer studies as well as thymic transplantation experiments.
Results: We surprisingly observed that ICAM-1$^{-/-}$NOD mice – instead of diabetes – spontaneously developed a chronic inflammatory demyelinating peripheral polyneuritis. T cells, B cells and macrophages were the predominant PNS infiltrating cells. All other organs including brain and spinal cord did not exhibit any abnormalities. Adoptive transfer only of CD4 + T cells, but not of other lymphocyte populations, induced an inflammatory neuropathy in immunodeficient recipients. Peripheral myelin proteins were antigenic targets. T cells in neuropathic ICAM-1$^{-/-}$NOD mice exhibited a Th17 and Th1 bias. Adoptive transfer of both Th1 and Th17 skewed CD4 + T cells from ICAM-1$^{-/-}$NOD mice increased the severity of recipients' neuritis, but again did not induce diabetes. Antibody mediated ICAM-1 inhibition generated neuritis only in juvenile NOD mice. Transplantation of thymocyte depleted ICAM-1$^{-/-}$NOD thymi induced clinical signs of neuritis in nude mice recipients.
Conclusions: We conclude that in mice generally prone to autoimmunity, deficiency in ICAM-1 shifts autoimmunity specifically from endocrine pancreas to peripheral nerves possibly due to an altered selection of neuritogenic instead of diabetogenic T cells in the thymus. This introduces a potential novel animal model of human CIDP.

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Iron oxide magnetic nanoparticles highlight early involvement of the choroid plexus in central nervous system inflammation

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Objective: Neuroinflammation during multiple sclerosis (MS) involves infiltration of immune cells and disruption of the blood–brain barrier (BBB). Both processes can be visualized by magnetic resonance imaging (MRI), in MS patients and in the animal model experimental autoimmune encephalomyelitis (EAE). Leakage of contrast agents into the CNS parenchyma reveals focal BBB breakdown and permits visualization of acute inflammatory lesions. However, discrepancies between MRI lesions and clinical symptomatology emphasize the need for novel MRI methods to better monitor CNS inflammation, especially early during disease progression. We previously showed that very small superparamagnetic iron oxide particles (VSOP) enhanced MRI can reveal CNS lesions in EAE which are not detectable by routinely applied contrast agents, e.g. gadopentate dimeglumine. Thus, we hypothesize that VSOP may help detect early or subtle inflammatory events that would otherwise remain imperceptible.
Methods: To investigate the capacity of VSOP to reveal early events in CNS inflammation, we induced EAE in SJL mice by adoptive transfer of encephalitogenic T cells, and administered VSOP prior to onset of clinical symptoms. In parallel, we administered VSOP to mice at peak EAE severity, and to unmanipulated controls. We then examined the dynamics and distribution of VSOP in the CNS by MRI and histology.
Results: In EAE, VSOP accumulated in the choroid plexus prior to disease onset and in the absence of overt inflammation. VSOP was also present in spinal cord meninges, but was not detectable in healthy control CNS. At peak disease VSOP was broadly distributed; we observed VSOP in lesions with a perivascular accumulation of immune cells but a preserved glia limitans. VSOP was prominently present in the choroid plexus. Moreover, at peak disease VSOP was seen as discrete puncta in elongated endothelial structures, co-localized with phagocytes, and also diffusely disseminated in the parenchyma, suggesting various entry mechanisms of VSOP into the CNS.
Conclusions: Using VSOP, we demonstrate *in vivo* that alterations at the choroid plexus represent a very early feature during CNS inflammation preceding clinical disease onset. The observation of VSOP in perivascular lesions with preserved glia limitans illustrates the potential for VSOP to reveal early stages in the pathological process.

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Magnetic resonance elastography reveals altered brain viscoelasticity in experimental autoimmune encephalomyelitis

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Objective: Cerebral magnetic resonance elastography (MRE) measures the viscoelastic properties of brain tissue in vivo. Since recently, it has been known that brain viscoelasticity is reduced in patients with multiple sclerosis (MS), highlighting the potential of cerebral MRE to detect tissue pathology during neuroinflammation.
Methods: To further investigate the relationship between inflammation and brain viscoelasticity, we applied MRE in the mouse model of MS, experimental autoimmune encephalomyelitis (EAE). EAE was induced and monitored by MRE in a 7-Tesla animal MRI scanner over four weeks.
Results: At peak of disease (day 14 after immunization), we detected a significant decrease in both the storage modulus (G') and the loss modulus (G''), indicating that both the elasticity and the viscosity of the brain is reduced during acute inflammation. Interestingly, these parameters normalized at a later timepoint (day 28) corresponding to the clinical recovery phase. Consistent with this, we observed a clear correlation between viscoelastic tissue alteration and the magnitude of perivascular T cell infiltration at both the day 14 and day 28. Hence, acute neuroinflammation is associated with reduced mechanical cohesion of brain tissue, while the structural complexity of the tissue remains unaffected. Moreover, the reduction of brain viscoelasticity appears to be a reversible process, which is restored when inflammation resolved.
Conclusions: For the first time, our study has demonstrated the applicability of cerebral MRE in EAE, and showed that this novel imaging technology is highly sensitive to early tissue alterations

resulting from the inflammatory processes. Thus, MRE may serve to monitor early stages of perivascular immune infiltration during neuroinflammation.

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Blood brain barrier permeability is enhanced in APP/PS1 mice and associated with neuroinflammation

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Objective: Alzheimer's disease (AD) is a progressive neurodegenerative disorder that is characterised not only by amyloid-β (Aβ) pathology but evidence of neuroinflammation. It has been proposed that inflammatory changes, consequent to persistent glial activation, may contribute to the early pathogenic changes. We set out to assess whether there were differential age-related changes in glia from a transgenic mouse model of AD compared with their wildtype counterparts and to investigate possible mechanisms which might explain such differences.
Methods: Mice overexpressing amyloid precursor protein (APP) with the Swedish mutation and exon-9-deleted presenilin (PS1) at 14 and 24 months of age were used in this study. Blood–brain barrier permeability was assessed by magnetic resonance imaging (MRI) and assessment of soluble and insoluble Aβ concentration was determined using 96-well multi-spot 4 G8 Aβ triple ultra-sensitive assay kits (MesoScale Discovery, USA). Hippocampal IL-1β and TNFα concentrations were measured by electrochemiluminescence assay (MesoScale Discovery, USA) and phagocytosis assay was performed in microglia isolated from brains of wildtype and APP/PS1. Real-time PCR was used to determine mRNA expression of *CD11b*, *GFAP* and *MIP-1α*.
Results: APP/PS1 mice displayed greater BBB permeability and increased Aβ accumulation in the hippocampus than their wildtype counterparts at both 14 and 24 months of age and this was associated with infiltrating cells. These changes were accompanied by enhanced hippocampal expression of IL-1β, TNFα, and MIP-1α. Interestingly, CD11b and GFAP mRNA expression correlated with changes in BBB permeability while expression of NGF and BDNF were decreased in APP/PS1, compared with wildtype, mice.
Conclusions: APP/PS1 display enhanced BBB permeability compared to wildtype mice — an effect that is exacerbated with age and accompanied by greater accumulation of Aβ. We propose that this increase in BBB permeability and accumulation of Aβ combine to induce a shift towards a proinflammatory phenotype.

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Protective role of astrocyte specific leptin signaling during experimental autoimmune encephalomyelitis

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Leptin, an adipokine, produced in periphery and transported across CNS, plays a vital role in regulating food intake and energy homeostasis. It is also a molecule that links metabolism to immunity. Leptin is associated with the inflammatory response as the leptin receptor (ObR) is expressed by various leukocytes. It is thought to play a detrimental role in exacerbating experimental autoimmune encephalomyelitis (EAE), an animal model for multiple sclerosis. However, it is not clear how leptin signaling specific to astrocytes affects the outcome of EAE. Here, we show that during EAE reactive astrocytes in the hippocampus and to a lesser extent in the hypothalamus had increased ObR immunoreactivity as shown by colocalization with GFAP (+) intermediate filaments. This astrocyte-specific increase of immunofluorescence coincided with a higher level of ObR protein expression in tissue homogenates shown by western blotting. To determine the functional role of the astrocytic ObR, we induced EAE in the astrocyte specific ObR "knockout" (ALKO) mice being bred in our laboratory. In ALKO mice, astrocytes express a truncated membrane-bound ObR without intracellular signaling capacity. The ALKO mice had higher EAE behavioral scores, increased infiltration of inflammatory cells, and more demyelination in comparison with wildtype littermate controls. The worsening of EAE in the ALKO mice indicates a protective role of astrocytic ObR signaling against EAE. We conclude that cell-type specific leptin signaling in the CNS can play an essential role in this autoimmune disorder.

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NLRP3 inflammasome targeting nanoparticles: A potential therapeutic for the treatment of ischemia

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Objective: Ischemic events firstly induce neuronal cell death in the ischemic core followed by increased activation of microglia and neuroinflammation in the region surrounding the core, the penumbra. Activation of microglia is thought to lead to an inflammatory cascade which may perpetuate cell loss post-ischemia in the penumbra thus the need for an effective post-ischemic therapeutic is of great importance. The NLRP3 inflammasome is a collection of proteins which includes the apoptosis-associated speck-like protein (ASC), the protease caspase 1 and the NOD receptor, NLRP3, recruitment of which leads to the proteolytic cleavage of caspase 1 to its active form and thereby the processing of pro-IL-1β to active IL-1β. Recent studies have suggested that activation of this inflammasome by ATP, released from dying neurons, plays a role in ischemic-induced injury. Here we propose the use of NLRP3 inflammasome targeting nanoparticles as a possible post-ischemic therapeutic.
Methods: Nanoparticles were synthesized by the aqueous route. $Cd(ClO_4)_2 \cdot 6H_2O$ and 1.3 molar equivalents of thio-glycolic acid (TGA) stabilizer were added to degassed water. H_2Te gas was generated from Al_2Te_3 *via* addition of a 0.5 M H_2SO_4 solution and was bubbled through the cadmium/thiol solution under a slow argon flow. The resultant solution was heated to reflux, following which fractions were precipitated *via* the addition of isopropanol and further purified on a Sephadex G25 column. Caspase 1 inhibitors or an anti-ASC antibody were subsequently conjugated to these nanoparticles.

Mixed glia, microglia and astrocytes from neonatal Wistar rats were cultured for 14 days at 37 °C and 5% CO_2. Cultures were treated with ATP and inflammasome-targeting nanoparticles, control nanoparticles or inhibitors and antibodies alone, and IL-1β and caspase 1 activity and cellular fluorescence were assessed.
Results: Caspase 1 inhibitor- and anti-ASC conjugated nanoparticles were preferentially taken up by microglial cells compared with astrocytes or neurons. The caspase 1 inhibitors- and anti-ASC-conjugated particles inhibited ATP-induced release of IL-1β to a greater extent than the unconjugated compounds. NLRP3-targeting nanoparticles

were also shown to be significantly more effective at inhibiting IL-1β release for up to 6 h after ATP treatment compared with the unconjugated inhibitors and antibodies.
Conclusions: NLRP3-targeting inflammasome particles are a potentially novel, cell specific, therapeutic for the treatment of ischemia induced chronic inflammation. Nanoparticles that were conjugated with compounds that target the NLRP3 inflammasome more effective at reducing the ATP induced release of IL-1β from glial cells than the compounds alone. Their ability to also reduce IL-1β, subsequent to ATP treatment, provides the potential for these nanoparticles to be an effective post-ischemic therapeutic strategy.

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FTY720 has the effects of anti-inflammation and neuroprotection on microglia

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Objective: The immunomodulator, Fingolimod (FTY720) is phosphorylated in vivo by sphingosine kinase, and functions as sphingoshine-1-phospate (S1P) receptor agonist. It has affinity with $S1P_{1,3,4,5}$ receptors, but not with $S1P_2$. FTY720 suppresses lymphocytes egress from lymphoid tissues by down-regulating S1P receptors, and suppresses invasion of lymphocytes into the central nervous system (CNS). FTY720 is an only oral medicine to treat multiple sclerosis (MS). Although it passes through blood–brain-barrier, the effects of FTY720 on CNS cells remain to be elucidated. We, thus, examined the effects of FTY720 on microglia, resident immune cells in the CNS.
Methods: Microglia were isolated from primary mixed glial cell cultures prepared from newborn C57BL/6 mice at 14 days in vitro using the "shaking off" method. mRNA expression of S1P receptors, proinflammatory cytokines and neurotrophin factors was assessed by RT-PCR, and protein expression was assessed by Flow Cytometry, ELISA and Western blotting.
Results: Microglia expressed $S1P_{1,2,3,4}$ and $_5$ receptor. Stimulation with LPS down-regulated microglial expression of $S1P_{2,4}$ and $_5$. FTY720 down-regulated production of proinflammatory cytokines such as TNF-α, IL-1β and IL-6, and up-regulated neurotrophin factors such as BDNF and GDNF.
Conclusions: In addition to the immunoregulatory functions in the periphery, FTY720 exerts anti-inflammatory and neuroprotective functions via microglia in the CNS. These functions of FTY720 in the CNS may also contribute to the therapeutic effects against MS.

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Toll-like receptor 2 (TLR2) stimulation regulates the balance between Th17 and Treg function in multiple sclerosis: A key role for IL-6/STAT3 signalling

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Objectives: Reduced immunoregulatory activity of regulatory T cells (Tregs) has been reported to increase susceptibility to autoimmune diseases, including multiple sclerosis (MS), an inflammatory demyelinating disease of the central nervous system. We reported that TLR2 is highly expressed by naïve and effector Tregs and that stimulation with Pam3Cys, an agonist for the TLR1/2 heterodimer reduced the suppressive functions of Tregs and skewed them into a Th17-like phenotype (Nyirenda et al., 2011). The objective of this study was to compare the effect of TLR2 stimulation on the suppressive functions of Tregs from MS patients and healthy controls (HCs). Basing on the observation of increased TLR2 expression by Tregs from MS patients than HCs, we hypothesised that Tregs from MS patients may be more susceptible to TLR2-induced loss of their suppressive functions and differentiation towards a Th17 lineage.
Methods: The study included 26 MS patients with clinically definite MS and 25 HCs with no history of autoimmune diseases or recent infection episodes. $CD4^+$ T cells were enriched from PBMCs then FACS-sorted as $CD4^+CD25^{hi}CD127^{neg}$ Tregs or subpopulations of $CD4^+$ T cells ($CD4^+CD45RA^+CD25^{++}$, naive Tregs; $CD4^+CD45RA^-CD25^{+++}$, effector Tregs) and $CD4^+CD45RA^+CD25^-$ cells, responder T cells, Tresp). For proliferation assays, Tresp were co-cultured with $CD4^+CD25^{hi}CD127^{neg}$ Tregs, naïve Tregs or effector Tregs at 1:16, 1:8 and 1:4 Treg/Tresp ratios in the presence or absence of Pam3Cys on plate-bound anti-CD3/CD28. Cells were stained for the expression of IL-17, RORC, CCR6 after 96 h culture in the presence or absence of Pam3Cys, neutralising anti-TLR2 mAb or a Th17 cocktail of antibodies and cytokines. In separate experiments, $CD4^+$ T cells were also stained for the expression of pSTAT3, IL-6R and gp130.
Results: The suppressive functions of all Treg populations studied obtained from MS patients were more susceptible to TLR2-induced reduction of suppressive function and expression of Th17 cytokines than those obtained from HCs. Stimulation of $CD4^+$ T cells with Pam3Cys enhanced the expression of IL-6, IL6R, gp130 and pSTAT3.
Conclusions: These data suggest that IL-6/gp130/STAT3 signalling is involved in TLR2-mediated reduction of Treg function and Th17 skewing of Tregs, and also in the differentiation of Th17 cells. In MS, infections could thus modulate the Treg/Th17 balance with implications for disease activity and progression. Therefore, TLR2 blockade could be a potential target for controlling unwanted Th17-mediated responses in autoimmune diseases such as MS.

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Interleukin-1 (IL-1) may contribute to develop classical and alternative activation of microglia/macrophages after spinal cord injury

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Objective: Microglia and macrophages (MG/MΦ) have a diverse range of functions depending on unique cytokine stimuli, and contribute to neural cell death, repair, and remodeling during central nervous system disorders. While interleukin-1 (IL-1) is shown to enhance acute inflammatory response, it is also reported to stimulate neuroregeneration. At last meeting, we reported that IL-1 contributed to inflammatory response to enhance cell death because IL-1 knock-out (KO) mice after spinal cord injury (SCI) improved the locomotor activity and lesion size associated with a decrease of TNFα level. However, the contribution of IL-1 on other MG/MΦ activation is still unclear. We determined, in the present study, that IL-1 participated in the classical and alternative activation of MG/MΦ in SCI model and in an adult MG primary culture system.

Methods: SCI was induced by transection of the spinal cord between the T9 and T10 vertebra in wild-type and IL-1 KO mice. Locomotor activity was monitored and lesion size was determined for 14 days. An alternative activating MG/MΦ marker, Ym1 levels were monitored with immunoblotting. Primary cultures of MG were prepared from adult mice brain, and were exposed to IFNγ or IL-4 with and without IL-1β. The media and cells were measured classical and alternative MG/MΦ activating markers. Moreover, cultures were exposed to IL-4 and/or IL-13 in the presence and absence of IL-1β with similar system.
Results: As shown previously, IL-1 KO mice improved SCI compared with wild-type mice. However, Ym1, an alternative activating MG/MΦ marker, was less in IL-1 KO mice. The Ym1 immunoreaction which was observed peri-injury area and was merged with IGF-1 was also less expressed in KO mice. We treated primary MG cultures with IFNγ or IL-4 in the presence and absence of IL-1β. Increased nitric oxide and TNFα was present in the culture media and increased inducible NO synthase was detected in cell suspensions following co-treatment with IFNγ and IL-1β. Expression of the alternative activation markers Ym1 and arginase-1 was increased after exposure to IL-4 and further increased after co-treatment with IL-4 and IL-1β. The phenotype was not observed after exposure of cells to IL-13.
Conclusions: We demonstrate *in vivo* experiments that IL-1 suppresses SCI in a process mediated by the reduction of inflammatory responses. Moreover, it is suggested that IL-1 participates in both the classical and alternative activation of MG in both *in vivo* and *in vitro* systems.

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Neuroprotective potential of estrogens and estrogenic compounds against amyloid-beta and oxidative stress

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Objective: Amyloid-beta (Abeta) peptide, which accumulates in the brains of Alzheimer's disease patients, can induce oxidative stress by itself or by activating microglia to oxidant release. Estrogenic compounds may protect neurons against Abeta and oxidative stress induced cell death. We compared the neuroprotective potential of 17beta-estradiol (E2), estrone (E1), tamoxifen (Tam), 4-OH-tamoxifen (4-OH-Tam), diethylstilbestrol (Des) and genistein (Gen) in cell culture models of oxidative stress. These compounds have differences in estrogen receptor alpha and beta (ER-alpha, -beta) binding affinities and their number of –OH-groups varies.
Methods: Cell damage was induced by exposing either rat primary hippocampal neurons, which express both ERs, or differentiated human SH-SY5Y neuroblastomas, which express mainly ER-beta, to H_2O_2 or $Abeta_{1-42}$. Lactate dehydrogenase release into culture medium was used as a damage indicator. Protein levels of Bcl-xL, Bcl-2, Bag-1, Abeta precursor protein and presenilin-1 were detected with Western blots.
Results: Treatments with 5 nM E2, Gen or 4-OH-Tam for 24 h before and after the H_2O_2-insult were neuroprotective in both hippocampal and SH-SY5Y cultures. E2 and Gen were also neuroprotective against $Abeta_{1-42}$ mediated toxicity when used 24 h prior and during the 48 h $Abeta_{1-42}$ exposure. Protein levels of anti-apoptotic Bcl-xL, Bcl-2 and Bag-1 as well as amyloid precursor protein and presenilin-1 were also examined in H_2O_2 exposed and estrogenic compound treated neuron-like SH-SY5Y cells.
Conclusions: The deleterious effects of $Abeta_{1-42}$ are, in part, likely mediated via mechanisms other than an increase of oxidative stress. ER-beta specific Gen was shown to be almost as good a neuroprotector as E2 with protective effects via the endogenous antioxidant defence systems.

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High body mass index before age 20 is associated with increased risk for MS in both men and women

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Objective: The relationship between obesity during adolescence and MS risk has previously been investigated using two large cohorts of American women[1] in which obese female adolescents displayed an increased risk of developing MS. The aim of this study was to investigate whether the finding could be replicated and if it applies to men as well as to women.
Methods: The report is based on a population-based, case–control study, with 1571 incident cases and 3371 controls matched by age, gender and residential area. By means of logistic regression, the occurrence of MS in subjects belonging to different BMI groups was compared with that of normal weight subjects with BMI between 18.5-21 kg/m^2.
Results: Subjects whose BMI exceeded 27 kg/m^2 at age 20 had an increased risk of developing MS compared with normal weight subjects with BMI ranging between 18.5 and 21 kg/m^2 (OR 2.2 (95% CI 1.6–3.0) among those who had BMI between 27 and 29, and OR 2.2 (95% CI 1.5–3.0) among those who were obese). MS risk in subjects with BMI between 25 and 27 kg/m^2 was modestly increased (OR 1.4, 95%, CI 1.1–1.8). The pattern of association was similar among men and women and the observed trend of a higher BMI resulting in a higher risk of developing MS was significant for both groups. There was no difference in current BMI between cases and controls.
Conclusions: Speculatively, the obesity epidemic may explain part of the increasing MS incidence as recorded in some countries. Measures taken against adolescent obesity may thus be a preventive strategy against MS.

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Reversed causality might explain the association between reproductive history and MS

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Objective: Possible associations between childbearing patterns and MS risk have been studied for a long time, with conflicting results. The aim of this study was to investigate the influence of reproductive history among women and men on the risk of developing MS.
Methods: A population-based case–control study involving incident cases of MS (1301 cases, 2826 controls) was performed in Sweden. We calculated odds ratios (OR) for MS comparing parents with

childless subjects together with 95 % confidence intervals (CI) by means of unconditional logistic regression models.
Results: Overall, there was an association between having children and reduced MS risk among both men (OR 0.6, 95% CI 0.5–0.8) and women (OR 0.7, 95% CI 0.6–0.9). In both sexes, subjects who had become parents within 5 years prior to index had a substantially reduced risk of developing MS (OR 0.4, 95% CI 0.3–0.6 for men, and OR 0.6, 95% CI 0.5–0.8 for women). No association between having children and MS risk was observed when more than 10 years had passed since the birth of the last child). We found no association between increasing offspring number and MS risk (p value for trend was 0.1 among both sexes).
Conclusions: The observed association between reproductive history and MS risk is thus restricted to a limited time period preceding index, with similar findings in both sexes, which contradicts biologic impact of pregnancy on MS risk and argues in favor of reversed causality.

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IL-23 activated γδ T cells may restrain regulatory T cell responses during autoimmune responses by limiting IL-2 in the extracellular environment

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Objective: IL-23 receptor (IL-23R) deficient mice are resistant to the induction of several T cell mediated autoimmune diseases including experimental autoimmune encephalomyelitis (EAE). We have recently shown that a subset of γδ T cells express the IL-23R constitutively. During inflammation γδ T cells respond fast and vigorously to IL-23, accumulate early in the central nervous system (CNS) and boost, in an IL-23 dependent manner, immune responses. More precisely, they propagate the inflammation directly by secreting large amounts of cytokines as IL-17 and interferon-γ, and indirectly by compromising both, induction and function of regulatory T cells (T-reg). In the present study we further investigated the mechanism how γδ T cells might restrain T-reg responses.
Methods: We analyzed the transcriptome and secretome of γδ T cells that were stimulated polyclonally in the presence or absence of IL-23.
Results: Both, microarray and mass spectrometry approaches revealed increased levels of IL-2Rα in IL-23 activated γδ T cells. Furthermore, using IL-23R reporter mice we demonstrated that all IL-23R-positive γδ T cells express IL-2Rα chain on their surface, which allows them to shed the soluble form of the IL-2R in the environment. Therefore, we propose that a γδ T cells might modulate the availability and bioactivity of IL-2 at sites of inflammation and thus restrain the development and function of Tregs.
Conclusions: In conclusion, IL-23-activated γδ T cells accumulate in the target organ during autoimmune tissue inflammation and secrete the soluble IL-2Rα. It remains to be determined whether this is the potential mechanism how IL-23R+ γδ T cells decrease the availability of IL-2 and restrain Treg responses. We propose that the crucial role of IL-23 during chronic inflammatory processes might be partially explained by its impact on the availability of IL-2 at sites of inflammation where effector and regulatory T cells differ in their sensitivity to IL-2 deprivation.

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A potent and comparable degree of neuroinflammation in ALS and progressive MS

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Objective: To verify possible similarities between the immunopathogenesis of Amyotrophic Lateral Sclerosis (ALS) and Primary Progressive Multiple Sclerosis (PP-MS).
Methods: Immunophenotypic and functional analysis were performed in peripheral monocytes and T lymphocytes of 24 ALS and 25 PP-MS patients, 25 age- and sex-matched healthy controls (HC) were enrolled as well.
Results: Compared to HC: 1) IL-17- ($p<0.001$), IFNγ-($p<0.01$), and T-bet-expressing (TH1)($p<0.05$) CD4+ T lymphocytes, as well as IL-6-expressing CD14+ cells, were increased in ALS and PP-MS; 2) IL-21-expressing CD4+ T cells were augmented in ALS and PP-MS, whereas IL-22-expressing T cells were increased in PP-MS alone ($p<0.05$); and 3) Treg cells ($CD4^+/CD25^{high}/Foxp3^+$) were decreased in both patients ($p<0.05$). An attempt to counteract this neuroinflammatory scenario was suggested by the observations that GATA3- (TH2) as well as BDNF-expressing T lymphocytes were increased in ALS and MS patients compared to HC ($p<0.05$). These results, showing that a similar degree of neuroinflammation is shared between ALS and PP-MS, patients were confirmed by analysis of cytokine production.
Conclusion: A profound, TH1-, TH17-, and IL-6-driven inflammation characterizes both ALS and PP-MS; this process is unsuccessfully hampered by TH2 activation and BDNF secretion. These results shed light on the pathogenic similarities shared by ALS and PP-MS and could help the design of novel diagnostic and therapeutic approaches to ALS.

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Investigating the role of prostaglandin E2 mediated neuroinflammation in models of Parkinsonism

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Objective: Recent studies have identified non cell-autonomous mechanisms such as inflammation as major contributors to Parkinson's disease (PD) pathology. Neuroinflammation in PD is primarily mediated by significant increases in reactive microglia, which secrete pro-inflammatory cytokines and chemokines, and reactive oxygen and nitrogen species (ROS/RNS). The prostaglandin E_2 (PGE_2) receptors EP2 and EP4 can respectively amplify or dampen microglial inflammation in a disease setting. The objective of this study is to investigate the role of microglial EP2 and EP4 in the pathogenesis of PD.
Methods: Conditional microglial knockouts of EP2 and EP4 were generated by crossing Cd11b-Cre transgenics with floxed EP2 and EP4 mice. To induce dopaminergic (DA) neuron loss and microglial activation we used the acute 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) neurotoxin model of Parkinsonism. Briefly, mice were injected with MPTP 4 times at 2 h intervals on Day 1, and tissues were harvested 3 or 7 days following MPTP treatment and analyzed using immunohistochemistry, HPLC, and qRT-PCR. To activate the EP4 receptor mice were injected with an EP4 agonist once daily. *In vitro* experiments including ELISAs, Greiss assay, and qRT-PCR were carried out using the BV2 murine microglial cell line or primary peritoneal macrophages isolated from wt mice.
Results: MPTP induced loss of DA neurons and striatal dopamine levels is correlated with a significant increase in activated microglia

in the substantia nigra pars compacta and striatum. Modulating EP2 and EP4 signaling can regulate microglial activation in a diametrically opposing fashion in the MPTP model. Furthermore, our data shows that microglial EP2 and EP4 signaling regulates DA neuron survival, loss of striatal DA neuron projections, and levels of striatal dopamine. Lastly, we show that PGE_2 signaling regulates microglial oxidative stress and cytokine production *in vitro*.
Conclusions: Our data indicates that EP2 and EP4 receptors may influence mechanisms of secondary neurotoxicity in a Parkinsonian model by regulating multiple inflammatory pathways in microglia. Microglial EP2 and EP4 may play a critical role in the progression of PD associated neurodegeneration. Targeting PGE_2 signaling may be a potential therapeutic avenue for PD and other neurodegenerative disorders with a neuroinflammatory component.

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Immune adaptation in the central nervous system in response to systemic infections

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Objective: Systemic bacterial infections are a common cause for morbidity and mortality in the elderly, and in particular those with a neurodegenerative disease, but the mechanism underlying these observations remain unclear. Experimental models of neurodegeneration have shown that LPS-induced systemic inflammation increases neuronal damage, a process that is believed to be mediated by activation of primed microglia. The effects of a systemic bacterial infection on the innate immune cells of healthy and diseased brain are less well described, and therefore, in this study we investigate the acute and long term effects on microglia and brain vascular endothelial cells in response to an experimentally induced bacterial infection.
Methods: Mice with or without a pre-existing neurodegenerative disease, were given a single systemic injection of live *Salmonella typhimurium*. Inflammatory cytokines were measured in serum, spleen and brain, and microglia and endothelial cell phenotypes studied by immunohistochemistry.
Results: Serum cytokine levels (i.e. IFN-γ, IL-1β, IL-6) peaked at day 7 after infection with *S. typhimurium* and were significantly reduced three weeks post- infection. In contrast, brain cytokine levels (IL-1β, IL-12) in *S. typhimurium* infected mice increased over three weeks, following high circulating IFN-γ levels. Intracerebral injection of LPS resulted in an exaggerated inflammatory response when compared to non-infected mice, suggesting priming of innate immune cells in the CNS in response to a systemic bacterial infection. Infection of mice with a pre-existing neurodegenerative disorder showed increased phenotype changes of microglial cells and a significant increase of infiltrating CD3 + T cells into the brain parenchyma.
Conclusions: These studies reveal that the innate immune cells in the brain are activated by systemic infections, which may lead to prolonged and damaging cytokine production. This exacerbation of the inflammation and the priming of the brain innate immune cells may have a profound effect on the progression of pre-existing neurodegenerative disease.

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Taking neutrophils out of play in developing secondary neuronal damage after stroke by anti-VLA-4 treatment

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Objective: Ischemic stroke is accompanied by a marked early infiltration of neutrophil granulocytes into the injured parenchyma. We assume this event as neurotoxic and their contribution to the secondary neuronal damage after ischemia.
Methods: We investigated the association of neutrophil granulocytes and inflamed vessels within the penumbra 24 h after permanent occlusion of the middle cerebral artery by using Lys-M-eGFP-mice and intracranial two-photon microscopy.
Results: We found within the penumbra in close proximity to the ischemic core especially strongly elevated slow rolling of neutrophil granulocytes on the endothelium. After setting a vessel lesion (irritation, thrombus, bleeding) we found a shift to firm adhesion and later an infiltration into the brain parenchyma. In the penumbra, more close to the healthy parenchyma just a few neutrophil granulocytes had been detected on the endothelium, but there was a dramatic recruitment to the vessel wall after setting distinct pathological events (see above) in contrast to healthy conditions where recruitment had not been observed. This indicates the highly alertness of the interplay between inflamed vessels and neutrophil granulocytes and in turn potentially contribution to secondary neuronal damage. Using an approach of intravenously application of VLA-4-antibody 24 h after ischemia during two-photon microscopy imaging, we showed for the first time the effectiveness of shifting adhesion and slow rolling of neutrophil granulocytes to fast rolling or detachment from the endothelium by this treatment, and thus potentially avoiding infiltration.
Conclusions: This approach would be suitable for a potential clinical application as add on therapy after stroke to reduce the development of secondary neuronal damage by taking neutrophil granulocytes out of play.

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Interleukin-17 inhibits oligodendrocyte progenitor cell proliferation and maturation

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Inflammatory cytokines such as interferon-gamma (IFN-g) and interleukin-17 (IL-17) are produced by T cells within and around lesions in the central nervous system (CNS) during multiple sclerosis (MS). Oligodendrocyte progenitor cells (OPCs) migrate to lesions in order to differentiate into mature oligodendrocytes (OLs) and remyelinate denuded axons. This complicated endogenous process of remyelination is rarely complete, as OPCs are often found surrounding lesions, while there are low levels of remyelination within the lesion. Due to evidence that remyelination in a lesion often occurs simultaneously with T cell mediated inflammation, it is important to understand how OPCs respond to inflammatory signals. IFNg is known to inhibit OPC proliferation at high concentrations but protect mature OLs at low concentrations. It is unknown how IL-17 affects OPCs, and whether or not it enhances or inhibits remyelination. OPCs and mature OLs express the IL-17 receptor, IL17RA, and this expression increases during peak experimental autoimmune encephalomyelitis (EAE), a mouse model of MS. To determine how vulnerable OPCs are affected by IL-17 signaling, primary murine OPCs were isolated and cultured in the presence or absence of IL-17

during proliferation and maturation states. Proliferation, quantified by Ki67 expression, decreased in a dose dependant manner over 48 h. This decrease in proliferation was not due to apoptosis or maturation. Maturation, quantified by myelin basic protein (MBP expression), was delayed in maturing OPCs in response to IL-17. IL-17 is inhibitory to OPC proliferation and maturation, suggesting that it has a detrimental effect on remyelination.

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IL-23/GM-CSF axis in IFN-β-treated relapsing-remitting multiple sclerosis patients

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Objective: In animal model of multiple sclerosis (MS), experimental autoimmune encephalomyelitis, IFN-γ and IL-17A double knockout T helper cells (Th) were proven to be encephalitogenic due to GM-CSF production. GM-CSF expression in Th depends on IL-23 signaling and RoRγt. Although IFN-β, first line therapy in relapsing-remitting (RR) MS, changes the expression of over a hundred genes, there is no data on how it acts on GM-CSF mRNA levels yet. Thus we assessed IL-23/GM-CSF axis in IFN-β-treated RRMS patients.
Methods: We followed the group of 45 RRMS patients starting IFN-β1b therapy, naïve for previous immunomodulatory treatment for 2 years. Patients without relapses or progression on Expanded Disability Status Scale during 2 years were considered responders (R, n = 20) and the rest as non-responders (NR, n = 25). Gene expression relative to GAPDH was determined by TaqMan qPCR for MX1 (marker of IFN-β biological response), IL-23 subunits p40 and p19, IL-23R, RoRγt and GM-CSF in peripheral blood mononuclear cells before start and at 6, 12 and 24 months of IFN-β therapy.
Results: R and NR had similar MX1 mRNA levels. In both groups IL-23 subunits mRNA levels were decreased at 6 months. After a year of IFN-β treatment, p19 expression remained significantly decreased and reached baseline levels at 24 months while p40 expression reverted to baseline values at 12 months. By contrast, IL-23R mRNA levels significantly increased during the first year of follow-up in R and NR, but significantly decreased after this peak only in R. Also, after statistically significant increase at 6 and 12 months RoRγt mRNA levels returned to start values at 24 months in both clinical groups. Conversely, GM-CSF expression significantly decreased in R and NR at 6 months, yet reverting to baseline at 12 months in R. Similar to IL-23R, GM-CSF expression had a tendency of higher values in NR than in R at 24 months.
Conclusions: Different gene expression in R and NR was not due to difference in IFN-β biological response, at least according to MX1 levels. Similar pattern of gene expression was observed in R and NR for all examined genes except IL-23R, which retained up-regulated in NR, but notably decreased in R at 24 months. For the first time we examined the effect of IFN-β therapy on GM-CSF expression, but the relevance of a trend towards higher GM-CSF and IL-23R mRNA levels in NR has to be further evaluated.

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TNF-alpha induced upregulation of T-type calcium channels via zinc increase through GluR2 lacking AMPA receptors

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Objective: Neuroinflammation plays an important role in a number of neurological disorders such as epilepsy, Alzheimer's disease and multiple sclerosis. In all these disorders, profound changes in neuronal excitability occur, and are proposed to contribute strongly to the clinical symptomatology of these disorders. Neuroinflammation and the release of inflammatory mediators such as TNF-alpha by microglia are thought to constitute a key mechanism that contributes to altered neuronal excitability. Low voltage activated T-type Ca^{2+} channels play a crucial role in the modulation of the excitability of neuronal membranes. Here, we have studied a potential role of TNF-alpha to induce upregulation of T-type Ca^{2+} channels underlying substantially altered excitability of neurons.
Methods: We recorded from hippocampal primary cultured neurons or NG 108–15 cells using patch-clamp technique. Ca^{2+} currents were elicited with voltage steps in the presence of Na^{+} and K^{+} channel blockers TTX and TEA/4-AP, respectively. The contribution of T-Type Ca^{2+} channels was quantified by subtracting the sustained current in the plateau phase from the peak current to isolate the transient component. Miniature EPSPs were recorded in the presence of 50 μM D-APV, 100 nM TTX and 20 μM bicuculline.
Results: Incubation of cultured neurons with TNF-alpha led to an up-regulation of T-type Ca2 + currents. We further observed a transcriptional induction of the T-type Ca^{2+} channel subunit Cav3.2. Our analyses revealed that raising intracellular Zn^{2+} is necessary and sufficient for molecular and functional up-regulation of T-type Ca^{2+} currents. A potential route of entry for Zn^{2+} into cells are GluR2 lacking AMPA receptors. Indeed TNF-alpha induced inward rectification of AMPA receptors indicated increased formation of GluR2 lacking receptors. By application of the AMPA receptor blocker CNQX and the selective antagonist of GluR2-lacking AMPA receptors, IEM 1460, the TNF-alpha-induced increases in ICaT were prevented.
Conclusions: Taken together, these experiments suggest a new mechanism of altered neuronal excitability on a cellular level induced by the neuroinflammatory mediator TNF-alpha.

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Laquinimod's effects on innate immunity directs adaptive T cell modulation

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Objective: Laquinimod is a novel oral agent with immunomodulatory properties for the treatment of relapsing–remitting (RR) multiple sclerosis (MS) and other autoimmune diseases. In two-phase three clinical trials laquinimod has shown to be efficacious in reducing disease activity and disease progression. In this study, we investigated laquinimod's mechanism of action for immune modulation.
Methods: To evaluate laquinimod's capacity for immune modulation we investigated the effect of laquinimod in experimental autoimmune encephalomyelitis (EAE) using C57Bl/6 (H-2^b), DBA/1 (H-2^q) mice, and a transgenic model of spontaneous "opticospinal" EAE. Mice were treated with laquinimod (25 mg/kg) by daily oral gavage.
Results: In vivo laquinimod treatment by daily oral gavage prevented chronic EAE in C57Bl/6 (H-2^b) and in DBA/1 (H-2^q) mice, respectively immunized with MOG35-55 and recombinant MOG1-125. Ex vivo FACS analyses revealed that the beneficial effects of laquinimod are associated with reduced CNS infiltration and decreased Th1/Th17 responses. Rather than a direct influence on T cells, we observed that Laquinimod exerts its

effect by modulating myeloid APC function. In addition to an induction of type II myeloid cells, we observed a profound reduction of a specific DC population (CD11c+CD4+) in spleen and lymph node of mice treated with laquinimod. CD11c+CD4+ cells are potent immunogenic DCs that participate in the generation of BCL-6 expressing T follicular helper cells (TFH). Laquinimod treatment was associated with decrease frequencies of TFH as compared to vehicle treated controls. TFH are highly activated T helper cells that are needed to regulate antigen-specific B cell immunity and antibody class switch. To further investigate laquinimod's effect on B cell function, laquinimod was administered in a model of spontaneous "opticospinal" EAE with ectopic lymphoid structures in the CNS, which was obtained by crossing MOG T cell receptor transgenic mice (2D2) with MOG-specific B cell receptor knock-in (Th) mice.
Conclusions: Laquinimod prevents chronic EAE by its direct function on innate immune cells. Laquinimod also affects frequency of an immunogenic DC population, which may affect TFH cells and B cell immunity.

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Oxidative stress, cytokine/chemokine and disruption of blood–brain barrier in neonate rats after meningitis by Streptococcus agalactiae

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Objective: The aim of this study was to verify the levels of cytokines/chemokine, myeloperoxidase activity and oxidative stress and disruption of BBB in hippocampus and cortex of the neonate Wistar rats after meningitis by *Streptococcus agalactiae*.
Methods: Neonate male Wistar rats (15–20 g body weight), postnatal day 3–4. Rats underwent a cisterna magna tap with a 23-gauge needle. The animals received either 10 μL of sterile saline as a placebo or an equivalent volume of *S. agalactiae* suspension at a concentration of 1×106 cfu/mL and were subsequently returned to their cages. For assessment of TNF-α, IL-1β, IL-6, IL-10 and CINC-1 concentrations and myeloperoxidase activity the animals were killed by decapitation at different times from meningitis induction: at 0, 6, 12, 24, 48 and 96 h. The integrity of the BBB was investigated using Evan's blue dye extravasation, the animals were killed at different times: at 3, 6, 12, 18, 24 and 30 h after induction; for oxidative damage and enzymatic defense activity the animals were killed by decapitation at different times from meningitis induction: 6, 12, 24, 48, 72 and 96 h.
Results: In the hippocampus the levels were increased of CINC-1 at 6 h and 12 h, IL-1β at 6, 12 and 24 h, IL-6 at 6, 24 and 96 h, IL-10 at 24, 48 and 96 h and TNF-α at 24 h and 96 h. In the cortex the CINC-1 and IL-1β levels were found increased at 6 h. The MPO activity was significantly elevated at 24, 48 and 98 h in hippocampus and at 6, 12, 24, 48 and 96 h in the cortex. The breakdown of BBB started at 12 h. TBARS levels were elevated in the hippocampus at 6, 12, 24, 48, 72 and 96 h and cortex at 72 and 96 h. Protein carbonyls were elevated in the hippocampus and cortex at 6, 24, 48, 72 and 96 h. There was a decrease of SOD activity in hippocampus and in cortex. Catalase activity was elevated in hippocampus at 6 h and in the cortex at 12 and 96 h.
Conclusions: Neonatal bacterial infections of the CNS are severe, the interference with the complex network of cytokines/chemokine, other inflammatory mediators and oxidants tend to aggravate the illness and can be involved in the breakdown of the BBB.

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IL-17 signaling in the brain versus spinal cord during CNS autoimmunity

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Objective: Multiple sclerosis (MS) has been studied for decades using an animal model referred to as Experimental Autoimmune Encephalomyelitis (EAE). Our lab has developed an atypical EAE model in which parenchymal inflammation occurs extensively in the brain and the spinal cord. By varying the ratio of myelin-specific TH1 and TH17 cells adoptively transferred into mice, we were able to control whether inflammation occurred in the brain or not. The spinal cord was susceptible to inflammation over a range of TH17:TH1 ratios, while a high TH17:TH1 ratio was required for inflammation in the brain. Therefore, we hypothesize that the brain and spinal cord function as distinct microenvironments that differ in their response to TH1 and TH17 cells, and that this contributes to the varying patterns of inflammation seen in EAE.
Methods: To test this hypothesis, we adoptively transferred wild-type (WT) TH17-skewed cells into either WT or IL-17receptorA-deficient (IL-17RA−/−) recipients. Brain and spinal cord tissue was taken from sick mice; half was analyzed for inflammatory cell infiltration in the brain and spinal cord using flow cytometry, while the other half was frozen down for RT-PCR analysis of mRNA.
Results: We found that the incidence of atypical EAE (reflecting brain inflammation) was decreased in IL-17RA−/− compared to WT recipients. Interestingly, IL-17RA−/− mice that developed EAE had overall similar severity of inflammation in the spinal cord as WT mice. Although inflammatory monocytes and CD4+ T cells were not significantly different in the brains of IL-17RA−/− and WT mice, neutrophil numbers were significantly lower in the brain of IL-17RA−/− mice compared to WT mice. Intriguingly, neutrophil numbers were similar in the spinal cords of WT and IL-17RA−/− mice. Accordingly, we found that CXCL1, CXCL2, CXCL5, IL-1b, and GM-CSF were decreased in the brains of IL-17RA−/− mice, while levels of CXCL2, CXCL5, IL-1b, and GM-CSF were still produced the spinal cords of IL-17RA−/− mice in amounts comparable to WT spinal cords.
Conclusions: My work suggests that IL-17RA signaling preferentially enhances brain inflammation, but is not required for spinal cord inflammation. Thus, the brain and spinal cord function as distinct microenvironments in their response to inflammatory mediators, which may have implications for devising treatment strategies which target inflammation in these locations in the CNS.

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Differential regulation of HLA and IP-10 in adult human glia

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Objective: Human leukocyte antigen (HLA) is widely used as an indicator of glial activation. HLA expression is up-regulated in many neurological disease states, although the functional outcomes of this are not clear. Although most research on neuroinflammatory disorders uses rodent models, there are important differences between rodent glia and their adult human counterparts. This study investigates the effects of cytokines IFNγ, TGFβ and M-CSF on adult human glial inflammatory mechanisms, namely the inducible expression of HLA and production of cytokines by microglia, astrocytes and leptomeningeal fibroblasts.
Methods: Mixed glial cultures were isolated from biopsy tissue obtained with informed consent from patients undergoing temporal lobectomy for intractable epilepsy. HLA-DP, DQ, DR and IP-10 expression were assessed immunocytochemically and quantified using Discovery-1 automated fluorescence microscopy and MetaMorph image analysis software. IP-10 secretion was measured by Cytometric Bead Array.
Results: Human microglial expression of HLA is inducible and highly variable between cases. Expression of HLA on human microglia was increased by exposure to IFNγ (1 ng/ml for 96 h), regardless of the level of basal HLA protein expression. Contrary to data in rodents, the anti-inflammatory cytokine TGFβ (10 ng/ml) did not affect this increase in HLA, nor did TGFβ affect basal microglial HLA expression. However, M-CSF (25 ng/ml) decreased both IFNγ-induced and basal microglial HLA expression.

Leptomeningeal fibroblasts do not basally express HLA but have a marked induction on exposure to IFNγ. Despite TGFβ having no effect on microglial HLA expression, TGFβ blocked the IFNγ-induced expression of HLA by leptomeningeal fibroblasts. Conversely, M-CSF had no effect. Astrocytic expression of HLA was also increased by IFNγ, but was not modulated by TGFβ or M-CSF.

IFNγ also increased adult human glial expression of pro-inflammatory cytokines, particularly IP-10. TGFβ did not block this IFNγ-induced increase in IP-10 as it did for HLA induction in leptomeningeal fibroblasts.
Conclusions: This study highlights species differences, cell type specificity and differential regulation in response to pro- and anti-inflammatory cytokines with major impact on their role in neuroinflammation in the adult human brain. Basal glial expression of HLA is heterogeneous between cases and may be related to variable susceptibility and severity of a range of neurological diseases.

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Sperm-associated antigen 16 (SPAG16): A novel antigenic target that is increased in astrocytes in multiple sclerosis lesions

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In a prior study, we identified a new cerebrospinal autoantibody target for multiple sclerosis (MS): sperm-associated antigen 16 (SPAG16). SPAG16 exists in multiple isoforms (>10), of which isoforms 1 and 2 are experimentally confirmed. Initial literature showed that isoform 1 is exclusively expressed in the axoneme of sperm cells. Yet, more recent data reveals that it is expressed in most tissues including the brain and bone marrow. Protein expression and functional data on isoform 2 are limited; on RNA level it is expressed in multiple healthy tissues, with higher expression in the testis and brain (Pennarun et al., 2002).

Since the function and disease-contributing role of SPAG16 in MS are unknown, we aimed to further characterize SPAG16 as a novel candidate MS autoantigen.

To analyze the humoral anti-SPAG16 response in serum, a recombinant protein ELISA was used to test sera from MS patients (n = 120), patients with non-inflammatory and other inflammatory neurological diseases (NIND: N = 37; OIND: N = 17) and healthy controls (N = 169). Tissue expression of SPAG16 in MS and control brain was analyzed by immunohistochemistry (IHC). Passive transfer experiments with anti-SPAG16 antibodies (Abs) in experimental autoimmune encephalomyelitis (EAE) were performed to investigate their role *in vivo*.

Significantly elevated serum Abs against SPAG16 was detected in MS-patients (21%) compared to healthy controls ($P<0.01$) with a 95% specificity for the disease. IHC analysis of SPAG16 in MS and control brain tissue demonstrated an increased expression in MS lesions (n = 5); more specifically in astrocytes. Less intensive staining was seen in the normal appearing white matter of MS and control brain tissue (n = 5) and in neurons. Three independent EAE experiments revealed that anti-SPAG16 Abs induced disease-exacerbating effects compared to animals injected with isotype control Abs. We are currently investigating how anti-SPAG16 Abs exert their pathogenic effects. We hypothesize that astrocytes in the lesion upregulate SPAG16 as a protective mechanism. We are currently performing *in vitro* experiments on U373 astrocytoma cells to investigate the effect of different stress conditions on SPAG16 expression.

These findings indicate that SPAG16 constitutes a novel and interesting autoantigenic target in MS. Future experiments are aimed at elucidating the protein's function in MS lesions and the role of the autoantibody reactivity in MS.

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A protective role for interferon-gamma in experimental autoimmune encephalomyelitis

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Objective: Multiple sclerosis (MS) is the most common autoimmune disease of the central nervous system (CNS), with over 2.1 million individuals affected worldwide. MS is characterized by demyelination of neuronal axons that is believed to be mediated by CD4+ T cells via secretion of proinflammatory cytokines. Most MS patients progress to chronic disease despite successful treatment of acute episodes, indicating our lack of understanding of the complex contribution of immune cells to pathogenesis and/or protection against tissue damage. In particular, the central role of the Th1 cell-derived cytokine, IFN-γ, as a primary mediator of CNS pathology during experimental autoimmune encephalomyelitis (EAE), a mouse model of MS, has been challenged by the recent characterization of "Th17" cells producing IL-17. Additionally, mice deficient in IFN- develop more severe disease, implicating its role in EAE and MS is protective. In this study, we have compared the CNS of wild-type (Wt) and IFN $^{-/-}$ C57BL/6 mice during EAE to evaluate this potential protective role.
Methods: We have developed a novel quantitative immunofluorescence (qIF) technique enabling us to accurately and reproducibly quantify the amount of three different myelin antigens (Ag), in the CNS. Using the qIF technique, we have modeled EAE lesions in the

CNS of wild-type (Wt) and IFN $^{-/-}$ C57BL/6 mice and analyzed changes in myelin Ag abundance as related to their association with antigen-presenting cells (APCs) or as extracellular debris. Additionally, we have used flow cytometry and qIF on the brain and spinal cords of EAE mice to examine changes in functional cellular phenotype associated with a presence or lack of IFN signaling.
Results: We have observed significantly reduced myelin debris clearance in animals deficient in IFN- as compared to Wt EAE mice. Additionally, APCs deficient in IFN- signaling have lower expression of the phagocyte markers CD172a and CD68, as compared to Wt APCs during the course of EAE.
Conclusions: These results indicate that IFN- plays a role in the activation of myelin debris-clearing phagocytes in EAE.

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Investigating the function of GM-CSF in autoimmune inflammation using a novel reporter/fate-mapping system

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Objective: During autoimmune neuro-inflammation, self-reactive helper T cells (T_H cells) initiate tissue damage and neurological impairment. However, the encephalitogenic factor produced by these cells is still a matter of debate. Recent data by our lab and others, using the animal model experimental autoimmune encephalomyelitis (EAE), suggest T cell-derived GM-CSF as a crucial pathogenic cytokine during CNS autoimmunity.
Methods: The aim of this project is to utilize transgenic mice to reveal the detailed mechanism of GM-CSF in the pathogenesis of autoimmune diseases. Therefore, we recently generated a GM-CSF reporter mouse line, which allows tracing of GM-CSF producing cells by using a fluorescent reporter.
Results: This system simplifies the handling and analysis of GM-CSF expressing cells *in vivo* and *in vitro* and will ultimately be used to examine the function of these cells in autoimmune neuroinflammation. In addition to the usage as a direct reporter, this novel mouse strain allows to perform fate-mapping studies by the use of a GM-CSF specific Cre-recombinase in combination with a floxed-stop ROSA26-YFP allele. This aspect is of particular interest, since the stability of the currently known T_H cell lineages (T_H1, T_H2, T_H17) is a matter of intense debate. The use of this fate-mapping mouse would allow verifying the extent to which GM-CSF producing T cells depict a committed T cell lineage or simply respond to specific stimuli by the local microenvironment.
Conclusions: In summary, this mouse line will clearly broaden our understanding of how GM-CSF can modulate the immune system, in particular during autoimmunity.

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Galectin-9 is up-regulated in astrocytes by TNF and promotes encephalitogenic T-cell apoptosis

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Objective: Galectins are β-galactoside binding lectins, capable of modulating immune responses. While several galectins, including galectin-9 (Gal-9) have been shown to be up-regulated in MS lesions, their role in the pathogenesis of MS has yet to be determined. The aim of this study was to characterize the mechanism whereby Gal-9 is increased in astrocytes as well as the functional role of astrocyte-derived Gal-9 during neuroinflammation.
Methods: Primary astrocyte cultures were stimulated with either anti-inflammatory (IL-6, IL-10, IL-13) or pro-inflammatory (IL-1β, TNF, IFN-γ) cytokines and Gal-9 transcription examined by qRT-PCR. C57BL/6 or TNFR1 $^{-/-}$ mice were used to determine the receptor needed for TNF-induced Gal-9 expression. Pharmacological inhibitors and siRNA-mediated knockdown experiments were used to elucidate the pathway required for TNF-induced upregulation of Gal-9. Conditioned media were used to investigate the effect of astroglial-derived Gal-9 on MBP-specific Tim-3$^+$ encephalitogenic T-cells. Cell death was measured by flow cytometry. Finally, the role of Gal-9 during T-cell mediated demyelination was examined using the MOG-induced EAE model of MS.
Results: While anti-inflammatory cytokines IL-6, IL-10 or IL-13 did not induce Gal-9 expression in astrocytes, proinflammatory cytokines IL-1β, IFN-γ, and especially TNF markedly up-regulated Gal-9. TNF-induced Gal-9 expression was dependent on TNF receptor 1 (TNFR1) as TNF failed to induce Gal-9 in TNFR1 $^{-/-}$ astrocytes. Pretreatment of astrocytes with the JNK inhibitor SP600125 abolished TNF-induced Gal-9, whereas p38 and MEK inhibitors had minimal effects. Consistently, siRNA mediated knockdown of c-Jun in astrocytes prior to TNF treatment suppressed Gal-9 transcription, suggesting that TNF induces astroglial Gal-9 through the TNF/TNFR1/JNK/cJun signaling pathway. In addition, we found that astrocyte conditioned medium from TNF-stimulated Gal-9$^{+/+}$ but not Gal-9$^{-/-}$ cultures increased the percentage of Tim-3$^+$Annexin-V$^+$ apoptotic cells after 72 h of culture. Finally, Gal-9$^{-/-}$ mice were more susceptible to MOG-induced EAE compared to Gal-9$^{+/+}$ mice.
Conclusions: These results demonstrate that gal-9 is potently induced in astrocytes by TNF via the JNK/c-jun pathway and suggest that astrocyte-derived Gal-9 may function as an immunoregulatory protein that has the potential to restrain the number/type of T-cells that have access to the CNS parenchyma.

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Interleukin 17 impedes myelination in the peripheral nervous system

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Objective: Interleukin (IL)-17 is a potent pro-inflammatory cytokine that may exhibit relevant effects during inflammation in the peripheral nervous system (PNS). Therefore, we analysed the role of IL-17 on Schwann cells (SCs), glial cells of the peripheral nerve, focusing on SC's ability to form myelin layers and on putative immunological responses.
Methods: Dorsal root ganglia (DRGs) co-cultures from BL/6 mice as a model for the myelination in the PNS were used. Pure rat SCs were analysed for gene expression on RNA and protein level after IL-17 treatment. In addition, sural nerve biopsy samples of patients with chronic inflammatory demyelinating polyneuropathy (CIDP) and Guillain–Barré syndrome (GBS) were studied immunohistochemically for IL-17 positive cells.
Results: SCs were found to express the IL-17 receptor A and B. In the DRG model, stimulation with IL-17 resulted in reduced myelin synthesis as well as a decrease in length of the internodal segments, while neuronal dendrite growth remained unaffected. In pure SC

cultures, cell viability was not significantly compromised under IL-17 influence, while in SC/DRG co-cultures MMP-2 activity (as an inductor of myelin synthesis) was decreased, and pro-inflammatory MMP-9 activity was mounted. Further immunological markers showed an inflammatory alignment of SCs. In human nerve biopsies IL-17 positive cell populations were detected.
Conclusions: IL-17 may act as an myelin destructive inflammatory mediator in the inflamed peripheral nervous system by directly propagating SC-mediated demyelination.

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Regulation of the expression of growth factors by human microglia

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Objective: Tissue macrophages including brain microglia are crucial cell types in the pathogenesis of a number of neuroinflammatory and neurodegenerative diseases including HIV-associated neurocognitive disorders (HAND). Recently, it has been suggested that macrophages and activated microglia are heterogeneous with respect to their capacity to mediate tissue damage and repair (M1 versus M2). In this study, we examined the role of microglia in the production of neuronal growth factors, insulin-like growth factor 1 (IGF1) and progranulin (PGRN), *in vivo* in human brain sections and *in vitro* in primary human microglial cultures.
Methods: We examined the expression of IGF1 and PGRN in the human brain (HIV encephalitis and controls) by immunohistochemistry. In microglial cultures, IGF1 and PGRN expression was examined by Q-PCR and ELISA following stimulation with Th1 (IL-1, IFNγ) or Th2 (IL-4, IL-13) cytokines or the TLR ligands (LPS or poly IC). The role of IGF1 and PGRN in neuronal survival and differentiation was examined in primary human fetal neuronal cultures employing vital dye exclusion and MAP2 immunocytochemistry.
Results: Microglia were immunoreactive for IGF1 and PGRN in both control and HIV-infected human brains. In microglial cultures, TLR ligands and Th1 cytokines potently suppressed IGF1 and PGRN production, whereas Th2 cytokines increased PGRN. Furthermore, cAMP analogues uniquely increased microglial IGF1 production. Recombinant IGF1 rescued neurons from cytokine-induced killing, whereas rPGRN increased neuronal survival in longer term (weeks) cultures.
Conclusions: Microglia appear to be the primary source of neuronal growth factors in the brain. Microglial expression of neuronal growth factors is suppressed by proinflammatory factors, indicating that local depletion of growth factor might be an underlying mechanism by which neuroinflammation leads to neurodegeneration. In support of this hypothesis, preliminary findings suggest that plasma and cerebrospinal fluid (CSF) IGF1 and PGRN are reduced in individuals with HIV-associated neurocognitive disorder.

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Interleukin-18, involved in Alzheimer's disease, alters protein levels of peroxiredoxins and biliverdin reductase in neuron-like SH-SY5Y cells

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Objective: Alzheimer's disease (AD) brain has signs of chronic inflammation and oxidative stress, sustained by inflammatory cytokines, in addition to classic Amyloid-beta plaques (Abeta) and neurofibrillary tangles. According to our previous findings, expression of pro-inflammatory Interleukin-18 (IL-18) is increased in the AD brain and it affects also Abeta precursor protein cleaving enzymes. Therefore we studied the effects of IL-18 more broadly in differentiated SH-SY5Y cells and found in two-dimensional difference-in-gel-electrophoresis and mass spectrometry analyses that expression of Peroxiredoxins (PRXs) 2, 3, and 6 and Biliverdin reductase A (BLVRA) were altered after the IL-18 treatments. PRXs are a family of multifunctional antioxidant thioredoxin-dependent peroxidases, which comprise cellular protection against oxidative stress. At least PRX3 levels have been shown to be decreased in the AD brain. Another important enzyme is BLVRA which converts biliverdin to potent antioxidant bilirubin. BLVRA levels have been shown to be increased in the hippocampus of AD patients and it may also play a role in amyloidogenesis.
Methods: Differentiated SH-SY5Y cells were treated with IL-18 for varied times and the cell lysates were analysed with Western blots. The protein expression of anti-apoptotic bcl-xL and the activity of lactate dehydrogenase (LDH) were also analyzed.
Results: We found that protein levels of cytosolic PRX2 and mitochondrial PRX3 decrease in 72 h IL-18 treated SH-SY5Y cells compared to untreated controls (mean −23.5%, SEM +/− 7.96; n5, p = .005) and (−26.8 +/− 7.48; 15; .020), respectively. Cytosolic PRX6 decreased at 24 h (−14.5 +/− 6.24; 15; .003) and at 48 h (−23.2 +/− 7.28; 15; .003). BLVRA levels decreased at 48 h (−21.6 +/− 5.9; 16; .010) and at 72 h (−24; +/− 5.8; 16; <.001). Bcl-xL levels reduced during the 6 h IL-18 treatment, but increased at 72 h time-point (23.5 +/− 23.5; 14; .039). LDH activity was not affected significantly after the IL-18 treatments.
Conclusions: As conclusion, IL-18 can reduce antioxidative PRXs and BLVRA levels in SH-SY5Y neuron-like cells in a coordinated manner, suggesting increased oxidative stress in the cells caused by IL-18 treatment. This may be counteracted by neuroprotective and by oxidative stress regulated bcl-xL. However, IL-18 did not trigger apoptosis, observed also in our earlier studies. Further studies are needed to clarify our findings.

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Effects of anti-NMDA receptor antibodies from the patients with NMDA receptor encephalitis on the neurons

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Objective: Anti-NMDA receptor autoantibodies (NMDAR-Abs) are thought to relate closely to the pathogenesis of NMDAR encephalitis. The NMDARs have roles on memory, learning, or cognition and the patients with this disease frequently show impairment of these abilities as their major symptom. It has not been shown clearly that the NMDAR-Abs directly relate to these symptoms. We had reported that the NMDAR-Abs-positive patient's CSF could reduce the size of

long-term potentiation, a model for memory and learning, using mouse hippocampal slices. To understand the variable features of this disease, we examined these antibody-effects on neuronal fate.
Methods: Primary culture of mouse hippocampal neurons were prepared and incubated with the patients' CSF or other controls with or without complement under incubation microscope to see their morphological changes and their network formations for 48 to 72 hour-period. Also the NMDAR-expressed HEK 293 cells co-transfected with NMDAR NR1 and NR2B cDNAs were treated with the same protocol and compared the antibody-effects with or without complement on the fate of these cells.
Results: The NMDAR-expressing HEK 293 cells did not lose their numbers but showed internalization of NMDAR clusters inside the cells when incubated with the patient's CSF at 37 °C but not at 4 °C. When these cells were incubated with the NMDAR-antibody containing CSF with human complement, some cells were disrupted and the cell number was decreased. The hippocampal neurons incubated with NMDAR-Ab-positive CSF showed that the neurons form thicker dendrites and connected with nearby neurons extensively than those incubated with antibody-absorbed CSF or antibody-negative control CSF.
Conclusions: The CSF from NMDAR encephalitis affected the cell-surface NMDARs with internalization of receptor clusters that might protect the cells expressing NMDARs from overactivate their receptor functions by calcium influx through the NMDARs.

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Type-I interferon signaling is pro-inflammatory in models of Parkinson's disease

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Objective: Neuroinflammation has been implicated in the dopaminergic (DA) cell death observed in Parkinson's disease (PD). Key players in the neuroinflammatory cascade are the type-I interferons (IFNs), however the role these cytokines play in PD has not been explored. We propose that type-I IFNs contribute to the progression and exacerbation of neuronal cell death in PD.
Methods: Human post-mortem PD and control brains were obtained from the Australian Brain Bank ($n=10$) and type-I IFN levels were determined by QPCR analysis. mRNA expression of type-I IFNs, TNFα, IL-6 and IL1-β in brains of wildtype mice exposed to MPTP (4x10mg/kg) was also measured. Co-localisation studies with tyrosine hydroxylase (TH), glial fibrillary acidic protein (GFAP) and phospho-Stat3, a marker of type-I IFN signalling were performed on mouse brain sections from MPTP-treated mice. BE(2)M17 neuroblastoma cells lacking the type-I IFN receptor (IFNAR1) were generated (M17IFNAR1KD) and were exposed to the neurotoxin rotenone for up to 72 h. Cell viability was measured by MTT assay, activation of type-I IFN signaling confirmed by western blot analysis and the pro-inflammatory cytokine profiles compared by QPCR analysis.
Results: This study confirmed a 3-fold up-regulation in IFNα in human post mortem PD brains ($p<0.05$). Brains from wildtype mice exposed to MPTP displayed elevated IFNβ levels at day-3 post-MPTP compared to sham control mice with increased immunohistochemical expression of phospho-Stat3 identified in DA neurons of the substantia nigra. Other pro-inflammatory cytokines IL-1β and TNFα were not upregulated until day-7 ($n=6$, $p<0.001$). In vitro, rotenone induced increased Stat-3 phosphorylation and elevated IFNα and IFNβ expression in BE(2)M17 neuroblastoma cells. A protective effect of reduced type-I IFN signalling in response to rotenone (500 nM) was confirmed in vitro with M17IFNAR1KD cells displaying increased cell viability by MTT assay compared to cells expressing a negative control shRNA construct ($83.73\pm4.20\%$ versus $66.19\pm2.91\%$ ($n=5$, $p<0.05$)). This protection against cell death induced by rotenone correlated with reduced mRNA expression of type-I IFNs and IL1-β.
Conclusions: These results have implicated the type-I IFNs in mediating the pro-inflammatory response in both animal and cellular models of PD and in human patients. The type-I IFN pathway may be a novel target in reducing neuroinflammation and thus limiting the neuronal cell damage in PD.

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Upregulation of TNF, TNFRSF1A, TNFRSF1B and NFKB1 genes in temporal lobe epilepsy patients

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Objective: Several animal seizure models have demonstrated that neuroinflammation and neurodegeneration are a preponderant characteristic in epilepsy. Among a number of activated cytokines, TNF emerges as a prominent effector/mediator of both events. Through its two receptors, TNF can play a dichotomous role in animal seizures: programmed cell death activation (via TNFRSF1A) or cell survival actuation (via TNFRSF1B), through the nuclear factor Kappa B activation. Considering the lack of clinical studies, our aim is to analyze the TNF pathway in temporal lobe epilepsy with hippocampal sclerosis [TLE(HS)] patients.
Methods: We evaluated *TNF*, *TNFRSF1A*, *TNFRSF1B* and *NFKB1* relative mRNA expression levels by reverse transcription quantitative PCR in resected hippocampal tissue samples from 14 TLE(HS) and compared them to four *post mortem* controls. Four reference genes were used: *GAPDH*, *HPRT1*, *ENO2* and *TBP*.
Results: Our results showed that *TNF* was markedly upregulated in TLE(HS) patients ($p=0.0035$). *TNF* expression was at least 29.6 times higher in patients than in controls. Similarly, *TNFRSF1A*, *TNFRSF1B* and *NFKB1* were also upregulated in patients ($p<0.02$, $p<0.04$, and $p<0.03$ respectively). Only when using *TBP* as the reference gene, *TNFRSF1A* and *TNFRSF1B* expression in patients was not statistically significant.
Conclusions: Our data clearly suggest that the over activation of *TNF* is associated with the inflammatory component of TLE(HS). Furthermore, since *TNF*, *TNFRSF1A* and *NFKB1* are key genes in the death receptor signaling canonical pathway, we infer that this via plays a crucial role in TLE hippocampal neurodegeneration. There is still some controversy on TNFRSF1B role. We believe that its augmentation is related to a survival mechanism because the concomitant *NFKB1* upregulation; however, some studies indicate that TNFRSF1B may reinforce TNFRSF1A action. Our evidence points the TNF pathway as an important target for pharmacological studies regarding the benefits of an anti-inflammatory therapy in these patients.

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Fractalkine signaling modulates the inflammatory response to alpha-synuclein in models of Parkinson disease

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Objective: Parkinson disease (PD) is a neurodegenerative disorder characterized by loss of dopamine neurons in the substantia nigra (SN) and

aggregates of the protein alpha-synuclein (a-syn). Increasing evidence points to inflammation as a chief mediator, however the role of a-syn in triggering and sustaining inflammation remains unknown. In models of Alzheimer's disease (AD), multiple sclerosis and neurotoxin models of PD, CX3CL1 (fractalkine) and its receptor CX3CR1 have important roles in modulating neuroinflammation. Here, we have examined the role of fractalkine signaling in a-syn-induced neuroinflammation.
Methods: Using an *in vivo* mouse model in which human a-syn is expressed by an AAV2 viral vector, we assessed fractalkine protein concentrations, reactive microgliosis, IgG deposition, and MHCII expression 4 weeks post-transduction in both wild type (WT) and CX3CR1 knockout animals. Additional *in vitro* studies were performed utilizing cultured microglia treated with aggregated a-syn and fractalkine ligand in which phagocytosis was assessed using a fluorescent microsphere assay.
Results: With AAV-induced a-syn expression in WT mice, there was a reduction in SN fractalkine ligand concentrations. Using CX3CR1 −/− mice, we observed a reduction in the inflammatory response to AAV-SYN, with reduced IgG deposition, microglial activation, and MHCII expression at 4 weeks post-transduction. *In vitro* analysis revealed that fractalkine could block the phagocytic activities microglia, but in the presence of exogenous aggregated a-syn, fractalkine was no longer able to inhibit phagocytosis. Using microglia derived from WT and CX3CR1 −/− mice, we found that the receptor knockout mice showed reduced uptake of fluorescent beads, and that exogenous aggregated a-syn did not alter phagocytosis in cells from the CX3CR1 −/− mice.
Conclusions: In WT mice, overexpression of a-syn leads to a loss of fractalkine protein expression which likely contributes to the robust microglial activation observed. In CX3CR1 −/− mice, loss of this receptor attenuates neuro-inflammation, an effect similar to that seen in AD models. The *in vitro* data suggests that aggregated a-syn can directly modulate microglial phagocytosis, overriding the inhibitory effect of fractakine ligand but not the effect of deletion of the CX3CR1 receptor. Together, these data implicate fractalkine signaling as a potent therapeutic target for regulating inflammatory response in synucleinopathies including Parkinson disease.

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Central nervous system responses to systemic inflammation

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Objective: Patients with chronic inflammatory diseases such as rheumatoid arthritis, inflammatory bowel disease or psoriasis are often further burdened with neuropsychiatric symptoms such as depression, anxiety and fatigue. Despite the recent advances in our understanding of neuroimmune communication pathways, the molecular mechanisms behind these co-morbidities remain unclear. Utilising transcriptomics in a well-characterised animal model of systemic inflammation, we have started to investigate the molecular mechanisms by which inflammation originating in the periphery can induce neurological transcriptional modulation and resulting behavioural changes.
Methods: Systemic inflammation was induced in male C57BL6 mice via intraperitoneal injection of lipopolysaccharide (LPS). After 48 h, the transcriptional profiles of the brains of these mice were compared to vehicle-injected controls using Affymetrix GeneChip® microarrays. Data was validated independently using QPCR.
Results: Microarray analysis of whole brains collected 48 h after challenge revealed increased transcription of a range of interferon-response genes in the central nervous system (CNS). Unlike peripheral blood leukocytes, the response in the brain included a significant upregulation of interferon regulatory factor 7 and the classic interferon-inducible chemokine, CXCL10.
Conclusions: This transcriptional response is indicative of peripherally triggered, interferon-mediated CNS inflammation and the induction of CXCL10 suggests that the brain may be being primed for T cell infiltration. As considerable evidence links type I interferons to psychiatric disorders, interferon production in the brain may represent a crucial mechanism linking systemic LPS-induced inflammation with behavioural changes.

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Increased severity of EAE disease in MK2-deficient mice

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Objective: In models for Parkinson's disease and cerebral ischemia mitogen-activated protein kinase-activated protein kinase 2 (MK2)-deficient mice showed reduced neurotoxicity and neuroinflammation. Moreover, after spinal cord injury reduced neuronal cell loss and myelin loss was seen in MK2 knockout mice. The findings in these models suggest that a lack of MK2 reduces inflammation and protects against destruction of the brain and other tissues. The aim of the present study was to investigate the role of MK2 in MOG_{35-55}-induced experimental autoimmune encephalomyelitis (EAE).
Methods: After induction of EAE by $MOG_{35-55,}$ MK2 knockout (−/−) and wildtype (WT) mice were studied over a period of 24 days. Infiltrated leukocytes were isolated from the CNS and counted at different stages of the disease (day 8, day 16 and day 24). mRNA expression of the CD95/Fas receptor was analyzed by real-time PCR in these cells. In addition, serum level of TNFα was measured using the Luminex® system at day 8, day 16 and day 24 after EAE induction.
Results: $MK2^{-/-}$ mice showed more severe disease parameters than control mice. Although there were no differences in both the onset of the disease and the maximum clinical score between MK2-deficient mice and controls, MK2-deficient mice had a significant more severe disease course starting at day 18. While WT mice went into remission, the $MK2^{-/-}$ mice showed prolonged disease activity. The severe course in $MK2^{-/-}$ was associated with an increased number of leukocytes at day 24 ($p<0.05$), while there was no difference in the number of cerebral leukocyte at day 16. Moreover, the expression of CD95/Fas receptor was decreased at day 16 in $MK2^{-/-}$ mice compared to WT controls ($p<0.01$). In addition, the serum level of TNFα was upregulated in WT controls at day 16 but not in $MK2^{-/-}$ mice.
Conclusions: In contrast to our hypothesis, $MK2^{-/-}$ mice display a more severe disease course in MOG_{35-55}-induced EAE, in particular they did not go into remission. The lack of remission was associated by a consistently high number of leukocytes in the CNS suggesting that a lack of apoptosis in these cells may be the underlying course. The prevention of an upregulation in CD95/Fas death receptor may be associated with the lack of TNFα expression and may cause the lack in apoptosis of cerebral leukocytes and therefore persistent disease signs in $MK2^{-/-}$ mice.

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CCL2 in the hippocampus of multiple sclerosis patients and its cuprizone model

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Objective: Impaired cognition is present in 40–65 % of Multiple Sclerosis (MS) patients, at least partly reflecting hippocampal dysfunction. Histopathological studies indicate a profound hippocampal demyelination together with microglial activation, which were more often found in cases with cognitive decline. We hypothesize that in MS glial-derived factors can directly or indirectly determine hippocampal neuronal functioning and fate. The chemokine CCL2 (MCP-1), produced by glial cells in the brain, is known to play a role in leukocyte infiltration, and has recently been described to be associated with increased cognitive decline in early Alzheimer's disease (Westing et al., PLoS One, 2012). We question whether CCL2 is present in the hippocampus of MS patients and in its cuprizone animal model.
Methods: First, we had to validate our cuprizone animal model with respect to hippocampus-related behavioural changes as can be observed in MS patients. Hippocampus-related behaviour was assessed using the modified Barnes Maze (mBM; Youn et al., Brain Behav. Res., 2012). Semi-quantitative RT-PCR was performed on cDNA transcribed from RNA isolated from hippocampi of MS patients and control subjects and from hippocampi of cuprizone or vehicle-treated mice. Moreover, immunohistochemical CCL2 analysis was performed on post-mortem hippocampi from MS patients and control subjects.
Results: Already after 1 week of cuprizone treatment the mice showed poorer performance on the mBM. From 3 weeks of treatment onwards this effect became even more pronounced. CCL2 mRNA was significantly enhanced in demyelinated MS hippocampi compared to non-myelinated and control hippocampi. Also in cuprizone-treated mice an increase in CCL2 expression was found compared to control hippocampi.

Finally, CCL2 immunoreactivity was present in both control and MS hippocampi, but was clearly enhanced in MS hippocampal lesions. Fluorescent double-labeling indicated that CCL2 is mostly present in astrocytes.
Conclusions: The cuprizone model mimics the demyelinating aspects of MS, and is of particular interest to be used for testing hippocampal cognitive function. The chemokine CCL2 is enhanced in hippocampal MS lesions and in the cuprizone model. All together we propose that CCL2 is a promising glial-derived factor upregulated in the MS hippocampus that may contribute to cognitive dysfunction as seen in MS patients. Future studies will elaborate on this hypothesis.

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Aquaporin-4-specific T cells in neuromyelitis optica exhibit a Th17 bias and recognize Clostridium ABC transporter

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Objective: Aquaporin-4 (AQP4)-specific autoantibodies in neuromyelitis optica (NMO) are IgG1, a T cell-dependent Ig subclass, indicating AQP4-specific T cells participate in NMO pathogenesis. Our goal was to identify and characterize AQP4-specific T cells in NMO patients and healthy controls (HC).
Methods: Peripheral blood T cells from NMO patients and HC were examined for recognition of AQP4 and production of proinflammatory cytokines. Monocytes were evaluated for production of T cell-polarizing cytokines and expression of costimulatory molecules.
Results: T cells from NMO patients and HC proliferated to intact AQP4 or AQP4 peptides (p11–30, p21–40, p61–80, p131–150, p156–170, p211–230 and p261–280). T cells from NMO patients demonstrated greater proliferation to AQP4 than HC, and responded most vigorously to p61–80, a naturally processed immunodominant determinant of intact AQP4. T cells were $CD4^+$, and corresponding to association of NMO with HLA-DRB1*0301 and DRB3, AQP4 p61–80-specific T cells were HLA-DR-restricted. The T cell epitope within AQP4 p61–80 was mapped to 63–76, which contains ten residues with 90% homology to a sequence within *Clostridium perfringens* ABC transporter permease. T cells from NMO patients proliferated to this homologous bacterial sequence and cross-reactivity between it and self-AQP4 was observed, supporting molecular mimicry. In NMO, AQP4 p61–80-specific T cells exhibited Th17 polarization, and furthermore, monocytes produced more IL-6, a Th17-polarizing cytokine, and expressed elevated CD40 and CD80 costimulatory molecules, suggesting innate immunologic dysfunction.
Conclusions: AQP4-specific T cell responses are amplified in NMO, exhibit a Th17 bias and display cross-reactivity to a protein of an indigenous intestinal bacterium, providing new perspectives for investigating NMO pathogenesis.

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Astrocyte elevated gene-1, a novel modulator of neuroinflammation: Implications for HIV-1 associated neurodegeneration

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Objective: Astrocyte Elevated Gene-1 (AEG-1), a novel human immunodeficiency virus (HIV-1)- and tumor necrosis factor (TNF)-α-inducible transcript, has engendered tremendous interest in the field of cancer research as a therapeutic target for many metastatic aggressive tumors. However, little is known of its role in astrocytes during HIV-1 infection in the human brain, and whether it contributes towards the development of HIV-1-associated neurocognitive disorders (HAND). Astrocyte inflammatory responses are crucial regulators of central nervous system pathologies and neurodegeneration. Therefore, in this study, we investigate AEG-1 expression in the context of neuroinflammation and elucidate the mechanism of AEG-1 regulation of astrocyte inflammatory responses during HIV-1-associated neuroinflammation.
Methods: AEG-1 expression in HIV + human brain tissues was quantified by real time-PCR and colocalization analysis was performed by immunohistochemistry. Change in AEG-1 expression in cultured human astrocytes following treatment with HAND-relevant stimuli was further assayed using RT-PCR and immunoblotting. AEG-1 intra-cellular localization was monitored by immunoblotting and immunocytochemistry. Interaction of AEG-1 with Nuclear factor κB (NFκB) was analyzed using immunoprecipitation.
Results: Elevated AEG-1 mRNA levels were detected in HIV + human brain tissues, which positively co-localized with astrocytes in HIV-1-infected brain tissue specimens. Our preliminary *in vitro* studies demonstrated that HAND-relevant stimuli, tumor necrosis factor (TNF)-α, interleukin (IL)-1β and HIV-1, significantly induced AEG-1 expression at both mRNA and protein levels. A dramatic change in intra-cellular localization of AEG-1 in human astrocytes was detected following treatment with HAND-relevant stimuli. Inflammation-induced phosphorylation of AEG-1 at serine 568 was detected following treatment with HAND-relevant inflammatory stimuli. Also, AEG-1 showed enhanced physical interaction with NFκB, a crucial mediator of astrocyte inflammatory responses, in response to IL-1β and TNF-α treatment.

AEG-1 over-expression in cultured human astrocytes lead to elevated levels of CXCL8 and suppressed levels of EAAT2 at both mRNA and protein level, a hallmark feature of HAND and neuroinflammation.
Conclusions: In conclusion, this study implicates AEG-1 in HAND neuroinflammation and identifies a novel mechanism of AEG-1-mediated regulation of astrocyte inflammatory responses.

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Upregulation of substance P and its receptor in human NT2 neurons via functional IL-17 receptor

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Introduction: There is some suggestion that IL-17 pleiotropic effects may include direct effects on neurons in neuroinflammatory conditions such as multiple sclerosis. In this study we investigated the effects of IL-17A (IL-17) on the expression of pro-inflammatory neuropeptide substance P (SP) and its NK1 receptor (NK1R) in human NT2 neurons (NT2N).
Objective: To investigate the expression of SP and NK1R in human NT2N in response to IL-17 and IFNγ, as part of Th17 and Th1 pathways respectively.
Methods: Terminally differentiated NT2N were cultured with Th17 and Th1 cytokines and the effects on SP and NK1R expression was investigated by real-time-PCR. Western blotting and nuclear fractionation were additionally used to study IL-17 receptor subtype A (IL-17RA) expression and signaling.
Results: IL-17 had direct effects on neurons via its functionally expressed IL-17RA. Neuronal NK1R mRNA-level expression was subject to regulation by IL-17, whereas SP precursor was considerably less upregulated by IL-17. As an established stimulus in NT2N, the effects of IFN-γ were comparable with the effects of IL-17. IL-17 effects were prevalent earlier at 24 h as compared to IFN-γ effects prevailing at 48 h. NFκB does not signal IL-17RA response in neurons; other pathways may be involved, e.g. Erk1/2.
Conclusions: IL-17RA is functionally expressed on NT2N with IL-17 direct effects on NT2N. The findings support neuronal involvement in immune interactions involving Th17 pathway and SP. The above effects may have important implications in neuroinflammatory conditions.

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Astrocyte-targeted IL-10 production has a beneficial effect on short-term neuronal survival after facial nerve axotomy

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Interleukin-10 (IL-10) is a cytokine that plays a crucial role in counteracting the inflammatory response and immune reactions. In the central nervous system (CNS), IL-10 is mainly produced by astrocytes and microglia and its upregulation has been widely demonstrated after several insults, such as experimental autoimmune encephalomyelitis, middle cerebral artery occlusion, excitotoxicity and traumatic brain injury. The objective of the present study was to investigate the effects of local astrocyte-targeted IL-10 production on glial reactivity and neuronal degeneration after experimental axotomy. To accomplish that, facial nerve transection was performed on GFAP-IL10 transgenic (Tg) mice and their corresponding wild-type (WT) littermates. After 3, 7, 14, 21 and 28 days post injury (dpi), animals were perfused intracardially with 4% paraformaldehyde, coronal sections of brainstem containing the facial nucleus (FN) were obtained using a cryostat and free-floating sections were processed for immunohistochemical analysis. In order to analyze neuronal survival in the FN, additional WT and GFAP-IL10Tg axotomized animals were examined at 21 dpi and serial cryostat cut sections were counterstained with toluidine blue. Our results showed that motoneuron survival was higher in GFAP-IL10Tg animals at this time-point, suggesting a putative role of this IL-10 in the rescue of motoneurons from neuronal death. Microglia in axotomized GFAP-IL10Tg animals also had decreased Iba1 expression suggestive of lower microglial activation in the FN at 3 and 21 dpi. Surprisingly at 14 dpi, there was a significant increase in MHC-II in cells with a ramified morphology in GFAP-IL10Tg animals. In conclusion, our findings indicate that, in this paradigm, astrocyte-targeted IL-10 has a direct impact on short-term neuronal survival and the pattern of microglial activation. Further studies are however necessary to elucidate the impact of IL-10 over immune cell populations, especially T cells, the third key player involved on motoneuron survival in this model.

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The effect of classical or alternative activation on macrophage motility in the CNS

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Objective: Multiple sclerosis (MS) is a demyelinating and neurodegenerative disease in which inflammation plays an important role. Characteristic of MS pathology is the activation of microglia and infiltration of macrophages. The role of macrophages in MS can be detrimental, causing tissue damage, or beneficial, promoting regeneration and repair. The effects of macrophages in the CNS depend on their activation. The polar ends of these activation states are classically activated, pro-inflammatory macrophages (M1), and alternatively activated, anti-inflammatory macrophages (M2). We investigate the functional properties of human macrophage subsets in the CNS. Here we focus on their migratory properties.

The aim of this study is to investigate if human M1 and M2 macrophages differ in terms of morphology, motility, adhesion to extra cellular matrix and migration towards chemokines (CCL2, CCL5, CXCL10 and CXCL12) and complement factors (C1q and C5a) present in the CNS, known to be highly expressed in MS lesions.
Methods: Peripheral blood derived monocytes were isolated from buffy coats and cultured for seven days. Macrophages were then skewed either into M1 by classical activation with interferon gamma and lipopolysaccharide or into M2 by alternative activation with interleukin-4. To assess the morphology, the cytoskeleton was

stained with rhodamine phalloidin. Adhesion to extra cellular matrix molecules was assessed using plates precoated with fibronectin and collagen. After culturing for two hours the percentage of adherent cells was measured using a fluorimeter. The migration towards different chemokines was determined using a TaxiScan migration chamber. The chemokine receptor expression of the subsets was measured using FACS analysis.
Results: The cytoskeleton of M1 macrophages is elongated in contrast to the more spherical M2 cells. The adherence assays reveals that there is no difference in adhering capacity between the subsets. M2 macrophages migrate faster towards C1q, C5a, CCL2, CCL5, CXCL10 and CXCL12 than M1 macrophages. No differences were found in M1and M2 chemokine receptor expression compared to M0.
Conclusions: Marked differences were observed between M1 and M2 macrophages in migration towards CNS associated chemokines. Further studies are required to investgate if this is due to an effect of the activation method on cytoskeletal rearrangement.

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The role of tau-tubulin kinase 1 in inflammatory mononuclear phagocyte polarization

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Objective: The CNS immune response involves activation of mononuclear phagocytes (brain resident microglia, peripheral macrophages), classified into classical (M1) and alternative (M2) phenotypes. Tau-tubulin kinase 1 (TTBK1) directly phosphorylates tau at known sites. JNPL3 mice expressing mutant human P30IL tau, and TTBK1/JNPL3 double transgenic mice show neuroinflammatory pathology including forebrain and spinal cord accumulation of tau and spinal cord reduction of motoneurons (MNs). However, the role of neuroinflammation in this degeneration is unknown. We characterized mononuclear phagocyte activation in JNPL3, TTBK1, TTBK1/JNPL3, and non-Tg spinal cord, and investigated involvement of TTBK1 in the induction of MN degeneration *in vivo* and *in vitro*.
Methods: TTBK1-Tg mice (line 141) harboring human TTBK1 DNA were crossed with JNPL3 mice expressing P301L mutant tau (4R0N20) to generate TTBK1, JNPL3, TTBK1/JNPL3, and non-Tg mice. Animals were assessed with rotarod and grip strength tests and MN counts. Immunohistochemistry (IHC) was performed using IBA1, CD169, NOS2, YM1, CD11c, and CD206 antibodies. E13 mouse primary cultured MNs were treated with mouse primary microglia conditioned media (MCM; with LPS (M1), IL-4 (M2a), or PBS), shRNA against TTBK1, and DNA plasmids expressing TTBK1 and/or human 2 N/4R WT tau.
Results: In accord with motor deficits, all Tg mice showed reduced spinal cord MNs, with JNPL3 least severe and TTBK1/JNPL3 most severe, suggesting that TTBK1 expression causes motor deficits and MN death. IHC revealed mainly M2 microglia in JNPL3 mice as evidenced by ramified $IBA1^+$, $CD11c^+$, $YM1^+$, $CD206^+$ cells, and in contrast, M1 macrophages in TTBK1 and TTBK1/JNPL3 mice shown by amoeboid $IBA1^+$ cells with increased $NOS2^+$ and decreased/absent $YM1^+$, $CD206^+$ and $CD11c^+$ cells. MNs treated with M1-skewed MCM showed reduced neurite density and neuronal death, which was partially reversed by silencing of endogenous TTBK1. TTBK1 and/or tau expression resulted in axonal collapse.
Conclusions: Our study reveals that TTBK1 prominently alters mononuclear phagocyte population and activation phenotype from neuroprotective M2 microglia in JNPL3 mice to M1 macrophages in TTBK1 and TTBK1/JNPL3 mice, leading to enhanced MN degeneration. These data suggest that TTBK1 plays a pivotal role in accelerating neurodegeneration through mononuclear phagocyte activation switching, and highlight the value of these mice as models of M1/M2 skewing in neurodegeneration.

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Blocking of NMDA receptors inhibits inflammatory activation of microglia

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Objective: During the last decade it has been reported, that treatment of animals suffering from EAE, the prototypical animal model for MS, with antagonists of both the NMDA as well as the AMPA/Kainate family of glutamate receptors significantly ameliorates the clinical signs of EAE and results in reduced inflammatory infiltrates and less blood brain barrier disruption. Microglia are the innate immune cell population within the CNS. They have been shown to be highly activated once EAE is induced, leading to upregulation of MHCII and secretion of inflammatory mediators. By MHC II expression and cytokine secretion they are thought to contribute as local APCs in the reactivation of the CNS-infiltrating lymphocytes and an overall proinflammatory microenvironment.
Methods: For the *In vitro* analysis of microglia activation and the role of NMDA receptors in this process we used the microglia cell line BV2 and cortical slice cultures.

The *in vivo* analysis was performed in the oDTR mice that express the diphtheria toxin receptor specifically in oligodendrocytes (ODCs) and by that allow induced death of ODCs and in wild type mice that have been immunized with MOG/CFA to induce EAE.
Results: We could show that *in vitro* microglia activation could be achieved by the proinflammatory cytokine IL-17A, which plays a pivotal role in the pathogenesis of MS and its animal model EAE. Therefore we treated the microglia cell line BV2 and cortical slice cultures with IL-17A to achieve activation and subsequently treated the cultures with the NMDA receptor antagonists MK801 and AP5 to block NMDA receptor signaling. By that we have been able to inhibit IL-17A mediated activation of microglia and in particular ROS production, proliferation and migration of the BV2 cells. Additionally we could detect increased secretion of IL-6 and G-CSF. Furthermore, IL-17A leads to a higher activation of the NMDA receptor through phosphorylation and in consequence to higher influx of calcium after ligand binding.

To further analyze the NMDA receptor depended microglia activation we made use of two different mouse models that provoke different mechanisms of microglia activation. Whereas in EAE a strong inflammatory process activates microglia, in the oDTR mice, activation occurs due to induced ODC death (Locatelli et al., 2012). Interestingly it revealed that in the context of EAE microglia/macrophage 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 mice. 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: Taken together these findings indicate that NMDA receptors are not involved in general microglia activation but rather play a role in microglia activation in an inflammatory context.

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Effects of daily in vitro FTY720 exposures on human astrocytes signaling and neuroinflammation-relevant functional responses

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Objective: to define the neuroinflammatory-relevant influences of daily FTY720 exposures on primary human astrocytes. FTY720 (Fingolimod, Gilenya™), a daily oral therapy for Multiple Sclerosis (MS), readily accesses the central nervous system (CNS). FTY720 activates sphingosine-1-phosphate (S1P) receptors and induces extracellular signal-regulated kinases phosphorylation (pERK) in astrocytes. S1P receptors engaged by FTY720 are internalized, resulting in functional antagonism, but these internalized receptors can have a persisted effect on astrocytes and influence their cellular responses to inflammatory activations.
Methods: We exposed astrocytes isolated from the fetal human CNS to a single or repeated daily (3–5 days) doses of FTY720, and measured astrocyte signaling via ERK phosphorylation. Furthermore, we studied functional responses relevant to neuroinflammation such as cell proliferation (by Ki67 labeling) and IL-1β-evoked calcium (Ca^{2+}) efflux.
Results: a single FTY720 exposure (without wash-out) desensitized pERK signaling for >24 hours, and this response correlated with the loss of S1P-induced proliferation. Signal recovery was observed by 72 h and repeated (3–5 days) FTY720 treatments maintained receptor desensitization. Daily FTY720 inhibited IL-1β-evoked intracellular Ca^{2+} release but did not inhibit serum-induced pERK activation, or IL-6 and CXCL10 secretions in response to IL-1β.
Conclusions: Our in vitro studies indicate that daily FY720 exposures can regulate neuro-inflammation relevant responses in astrocytes by acting as a functional antagonist for external stimuli (natural ligand S1P) while sustaining internalized receptor-dependent functions (inhibit IL-1β evoked Ca^{2+} mobilzation).

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Characterization of the neuroprotective aspects of the immune response after peripheral nerve injury

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Objective: The activation of the immune system in neurodegeneration has been shown to have both detrimental as well as beneficial effects. However, which aspects of the immune response determine the protective versus degenerative balance in neurodegenerative diseases remains an open question. Injury to the peripheral nervous system (PNS) has been shown to induce a permissive environment for neuronal regrowth and functional improvement. To unravel the neuroprotective aspects of the immune system we focused on Wallerian neurodegeneration, in which the immune system has been shown to be protective.
Methods: To determine which type of immune response is triggered after peripheral nerve injury, we evaluated markers representing the two extremes of a type I and type II immune response (classical versus alternative) using RT-qPCR, western blot and immunohistochemistry. To characterize the cellular response in more depth, the injured nerves were analyzed using FACS.
Results: We found that, after injury to the PNS, a transient immune response is induced, accompanied by the induction of several negative regulators. Limiting tissue damage mediated by inflammatory components, could be essential in restricting immune mediated damage to the nerve by turning off the immune system. Furthermore, peripheral nerve injury sets off an anti-inflammatory, immunosuppressive type II immune response, as reflected by the strong induction of type II markers like arginase-1 and IL-10. On the other hand, proinflammatory type I markers like iNOS or IL12p40 were completely absent. As type II immune responses are generally associated with tissue repair and tissue protection, we suggest that PNS injury elicits an inherent protective environment by inducing the M2 phenotype of macrophages and arginase-1 expression. To fully understand the neuroprotective capacity of the immune response in the PNS, the phenotype of the macrophage was characterized in detail. Additionally, a complete temporal profile of immune cell infiltration in the injured peripheral nerves was analyzed to determine the interplay between immune cells. The neuroinflammatory role of Schwann cells was also addressed.
Conclusions: In conclusion, these data lead to a more complete view of the neuroprotective function of the immune response in the PNS and will allow us to reorient the immune system in such a way that the protective aspect is accentuated and tissue damage is limited.

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Th17 cells are a neuritogenic T helper cell subset in Guillain-Barré syndrome

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Introduction: The role of T helper (Th)17 cells, a newly found Th cell subset, has been scarcely studied in human Guillain–Barré syndrome (GBS) and its animal model experimental autoimmune neuritis (EAN). Emerging evidence points to a pathogenetic role of Th17 cells in GBS and EAN.
Methods: The EAN model was induced on IFN-γ knockout (KO) mice and its wild type (WT) counterparts, C57BL/6 mice by immunizing animals with P0 protein peptide 180–199 together with complete Freund's adjuvant and pertussis toxin. Flow cytometry and ELISA were used to detect Th17 cells and interleukin (IL)-17 in GBS and EAN. RORgammaT, a critical transcription factor responsible for the production of Th17 cells, was inhibited to corroborate the pathogenetic role of Th17 cells and to explore the therapeutic potential of Th17 blockade.
Results: The clinical signs of IFN-γ KO mice were significantly more severe than those of WT controls, along with higher proliferation of splenic mononuclear cells in KO mice. At the peak of EAN, the proportion of IL-17A-expressing cells in cauda equina (CE) infiltrating cells, and the levels of IL-17A in sera were elevated in IFN-γ KO mice; the proportions of MHC II, ED1, and IL-12/IL-23p40 expressing cells, relative to total CE infiltrating cells were correspondingly increased. However, IFN-γ deficiency reduced the production of nitric oxide (NO) by cultured macrophages in response to proinflammatory stimuli and induced a systemic Th2-oriented immune response. We further investigated Th17 cells and IL-17A in 29 GBS patients, 15 relapsing-remitting multiple sclerosis (RRMS), 17 viral encephalitis or meningitis (VEM) and 20 healthy controls (HC). Circulating Th1 ($CD4^{+}IFN\text{-}\gamma^{+}$), Th17

($CD4^+IL\text{-}17A^+$) and Th22 ($CD4^+IL\text{-}22^+$) cells were significantly increased in GBS patients during the acute phase compared with HC. Moreover, CSF and plasma levels of IL-17A and IL-22 were also elevated in GBS patients. Furthermore, IL-17 and IL-22 levels in CSF, respectively, were correlated with GBS disability scale scores. Interestingly, intravenous immunoglobulin not only down-regulated circulating Th1, Th17 and Th22 cells, but also reduced concentrations of IL-17 and IL-22 in the plasma of GBS patients during the plateau phase Further dynamic detection of Th17 cells in CE infiltrating cells and splenocytes from mice with EAN revealed that Th17 cells were increased with the disease progression and their dynamic changes roughly paralleled the clinical course of EAN. GSK-1, a synthetic compound that can specifically block the production of Th17 cells, suppressed the severity of EAN symptoms when it was administered from the immunization day. However, GSK-1 was incapable of obviously affecting the clinical signs of EAN when administered after the onset of EAN.
Conclusion: Th17 cells are a neuritogenic T helper cell subset in GBS and EAN

Key words: Interferon gamma; Guillain–Barré syndrome; experimental autoimmune neuritis; T helper 17 cell; interleukin 17; RORgammaT

Mechanisms of CNS disease

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A Forebrain Immune Cellular Pathway for the Prevention of CNS Autoimmunity

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Objective: The parenchymal tissue of the CNS is known to be "immune privileged". While it was thought that physical exclusion of systemic immune cells maintained this situation, subsequent data showing immune cell access to this CNS site suggests active mechanisms maintain immune tolerance. However, unlike most, if not all, organs, the CNS has no well-recognized cellular immune pathway that directly modulates anti-CNS immunity in the secondary lymphoid organs (lymph nodes) to restrain organ specific immune activation. We wished to see if this type of pathway was present in the brain.
Methods: Using IHC, CNS injury, CFSE labeling and Fingolimod administration, combined with quantitative CNS FACS, we determine if immune cells were present and exit the CNS. We then interrupt this putative cell traffic with CNS directed Fingolimod treatment in 2D2 TCR transgenic mice and assess T-regulatory function in their cervical lymph nodes. Finally, we use delayed type hypersensitivity to assess the role of dendritic cells in regulating anti-CNS immunity in the cervical lymph nodes
Results: We identify dendritic cell traffic from the brain to the cervical lymph nodes, via the rostral migratory stream, a neuronal stem cell pathway. This pathway may be interrupted by systemic Fingolimod treatment. Localized CNS Fingolimod treatment of 2D2 mice leads to a significant increased in EAE disease incidence from 0 to 60%. EAE induction is associated with reduced T-regulatory function in the cervical lymph nodes. Dendritic cells isolated from the cervical lymph nodes of mice undergoing CNS Fingolimod treatment can induce similar T-regulatory defects in the cervical lymph nodes of recipient animals.
Conclusions: Dendritic cell traffic from the CNS to the cervical lymph nodes regulates anti-CNS immunity by modulating T-regulatory function in the peripheral immune system. The cervical lymph node represents an attractive site for topical therapy to modulate anti-CNS immunity.

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Mitochondrial fragmentation and loss of function at the onset of neurological deficit in a neuroinflammatory model of multiple sclerosis (MS) studied by in vivo imaging

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Reversible neurological deficits in MS have traditionally been attributed to demyelination, but increasing clinical, imaging and biopsy evidence implicates inflammation as playing a major role, although the mechanisms remain unknown.
Objective: To assess the potential role of mitochondria in causing temporary loss of function in an animal model of MS.
Methods: Experimental autoimmune encephalomyelitis (EAE) was induced using $MOG_{35\text{-}55}$ in complete Freund's adjuvant followed by pertussis toxin, in adult female mice transgenic for expression of cyan fluorescent protein (CFP) in axonal mitochondria. Mice were assessed daily for neurological deficits and time-matched pairs with EAE were anesthetized for spinal imaging such that symptomatic and asymptomatic animals were examined at the onset of neurological deficit, or at 6 weeks post-immunization (p.i.). Fluorescent potentiometric dye (TMRM) was applied to the exposed spinal cord to reveal mitochondrial function, and the issue imaged confocally prior to fixation and blind image analysis.
Results: At the onset of neurological deficits, symptomatic animals were distinguished from time-matched asymptomatic and naive controls by fragmentation and loss of function of spinal cord axonal mitochondria. The number of small mitochondria (1.5–3 μm) increased by ~54% ($p = <0.01$), and longer mitochondria (6–9 μm) decreased by ~49% ($p = <0.01$) compared with asymptomatic and naive animals. Approx. 40% of the total mitochondrial mass was non-functional (i.e. $TMRM^-$) in symptomatic animals, vs. only ~28% of mass in asymptomatic ($p = <0.01$). Symptomatic animals also showed greater inflammation (perivascular CD3 + T cells and $CD45^+$ and $iNOS^+$ microglia/macrophages), and damage to astrocytes (loss of GFAP and aquaporin 4 labelling) specifically in the vicinity of axons with damaged mitochondria. Activated astrocytes became positive for phosphofructokinase 2.3, indicating increased glycolysis. In more chronic animals (6 weeks p.i.) the deficits were more severe, and ~51% of the mitochondrial mass was non-functional, although the inflammation had subsided: function remained unchanged in asymptomatic animals. No demyelination was observed.
Conclusions: Mitochondrial bioenergetic failure is implicated as a cause of neurological deficits at the onset of EAE expression, in association with increased glycolysis in astrocytes consistent with a mitochondrial energy insufficiency. Mitochondrial dysfunction persists despite the resolution of inflammation.

Neurodegeneration

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Molecular mechanisms of immune-mediated neurodegeneration — An in vivo two-photon imaging approach

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Objective: Neuronal damage in autoimmune neuroinflammation is the correlate for long-term disability in patients suffering from multiple sclerosis (MS). Here we investigated the role of immune cells in early neuronal damage processes by intravital imaging of damage mechanisms. Our recent findings revealed that long-lasting contacts of Th17 cells led to severe, localized and partially reversible fluctuation in neuronal intracellular Ca^{2+} concentrations in living animals and that CD4 + T cells were capable of inducing cell death in cultured neurons in an antigen unspecific manner.
Methods: We now analyzed relevant mechanisms for T cell-neuronal interaction to understand the relevant mechanisms, which underlie (i) the establishment of T cell neuronal contact formation, (ii) the molecular interaction partners and (iii) the downstream events in the neuron upon T cell interaction. We employed murine T cell-neuronal co-culture systems for identification of cell death by Annexin-V-7AAD by flow cytometry. In vivo imaging relied on semi-quantitative measurement of neuronal Ca^{2+} fluxes in EAE lesions of living anaesthetized transgenic mice, which carry a Ca^{2+} sensor protein (TN-XXL mice).
Results: The mode of cell death (necrosis vs. apoptosis) was clarified in this work by morphologic analysis and detection of Caspase-3 as a downstream mediator of Th17 induced neuronal cell death under these conditions. Furthermore – on the T cells' side – a direct contribution of the effector cytokines IL-17A and IL-17 F (further IFN-γ) to the cytotoxic properties of Th17 could be ruled out stressing other components of the Th17 phenotype to be crucial. In addition – on the neurons' side – the role of NMDA receptors for the observed Ca^{2+}-associated neuronal dysfunctions after Th17 cell contact has been analyzed.
Conclusions: Here we investigated in depth the mode of immune-mediated neuroinflammation in acute neuroinflammation in vivo. We identified the mode of cell death and characterized the T cells' properties and propensity to induce neurodegeneration in a reductionist in vitro co-culture model.

Distinct glutamate receptor blockers have been found to be partially effective in rescuing neurons.

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TRPM4 cation channel mediates neuro-axonal degeneration in central nervous system inflammation

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Objective: In multiple sclerosis (MS), an inflammatory disease of the central nervous system (CNS), axonal and neuronal loss is the major cause for irreversible neurological disability. However, which CNS molecules contribute to neuro-axonal injury under inflammatory insults remains largely unknown. Here, we investigated the contribution of the transient receptor potential melastatin 4 (TRPM4) cation channel to the pathogenesis of CNS autoimmune disease.
Methods: After induction of experimental autoimmune encephalomyelitis (EAE) in *Trpm4*$^{-/-}$ and wild-type mice we analyzed in addition to the disease course the axonal and neuronal injury and degeneration as well as T cell proliferation, cytokine production and CNS immune cell infiltration. Furthermore we created bone marrow chimeras to separate immune cell and neuronal effects of the TRPM4 depletion. We performed expression analysis of TRPM4 mRNA and protein in healthy and diseased mice (EAE) and humans (MS). Additionally, patch clamp recordings, calcium imaging, immunocytochemistry and cytotoxicity assays were used to investigate the role of neuronal TRPM4 in response to an inflammation mimicking environment *in vitro*. Finally we confirmed our results collected from *Trpm4*$^{-/-}$ mice by pharmacological inhibition of the ion channel.
Results: We now report that the TRPM4 cation channel is critically involved in the process of inflammation induced axonal and neuronal injury. We show for the first time that the calcium-activated nonselective cation channel TRPM4 is expressed in mouse and human neuronal somata, but also in axons in inflammatory CNS lesions in EAE and human MS tissue. TRPM4 deficiency or pharmacological inhibition resulted in reduced neuro-axonal degeneration and ameliorated clinical disease course in EAE, yet without altering immune cell functions. Furthermore, *Trpm4*$^{-/-}$ neurons were protected against inflammatory effector mechanisms, such as excitotoxic stress and energy deficiency *in vitro*. Electrophysiological recordings revealed TRPM4-dependent neuronal ion influx and oncotic cell swelling upon excitotoxic stimulation.
Conclusions: Therefore, interference with TRPM4 could translate into a novel neuroprotective treatment strategy.

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Kif21b and kif5a expression is associated with accelerated neurodegeneration in multiple sclerosis and Alzheimer's disease

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Objective: Kif21b is one of the few multiple sclerosis (MS) risk genes with a presumed function within the CNS. Kif21b belongs to the family of kinesins, proteins which are involved in intracellular transport of proteins and organelles. Currently, very little is known about the function of kif21b. We hypothesised that kif21b might be involved in the neurodegenerative component of MS pathology.
Methods: Postmortem expression of kinesins was assessed in 54 MS patients, 59 age and gender matched non-demented controls (NDC) and 50 Alzheimer's patients (AD). Kif21b expression was correlated to clinical progression from disease onset to EDSS 6. Moreover, it was correlated with clinical pathology (Braak score for AD and grey matter demyelination in MS). Kif21b rs12122721 genotype was determined using Taqman technology.
Results: Kif21b expression was five-fold increased in AD compared to MS and NDC aged below 62 years ($p<0.001$), three-fold between 62 and 72 years of age ($p<0.01$) and similar above 72 years of age. No significant differences were observed between MS and NDC. Next we assessed whether kif21b expression correlated with neuronal

pathology. In AD, kif21b expression did not correlate with the amount of amyloid-beta deposition. However, the expression was two-fold increased in Braak stage 6 (scoring for density of neurofibrillary tangles) compared with stage 5 ($p = 0.003$). In MS patients, kif21b correlated with the extensiveness of grey matter demyelination (Spearman's rho = 0.41, $p = 0.005$). Next, we determined whether rapid development of neurological disability in MS patients (defined as time to EDSS 6.0) correlated with kif21b. High expression of kif21b was associated with a two-fold accelerated development of EDSS 6 (median time to EDSS 6 in low kif21b group 16 year vs high kif21b 7.5 year, log-rank test $p = 0.02$). Given the genetic association of kif21b in MS, we stratified our results according to the kif21b rs12122721 [A] SNP and found no association between kif21b expression or with the time to develop EDSS 6 in kif21b risk carriers compared to non-risk carriers. To assess whether the observed differences are kinesin specific or that the findings reflects a general pathological mechanism, we also assessed six other kinesins. Interestingly, only kif5a gave similar results, indicating that the observed findings are kinesin specific. Kif5a has previously been associated with MS in a candidate gene study.
Conclusions: We showed that abundant kif21b and kif5a expression is associated with more severe pathology in MS and AD and with a more accelerated neurodegeneration independent of the kif21b risk SNP.

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Targeting granzyme B: A potentially novel neuroprotective strategy for inflammatory-mediated neurodegenerative diseases

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Objective: The serine-protease granzyme B (GrB) is the main weapon used by cytotoxic T cells to induce target cell death. We have previously shown that GrB is expressed within active lesions of multiple sclerosis (MS), and activated T cells release GrB and induce neurodegeneration. However, the mechanisms of GrB-mediated neuronal injury/death are still unclear. Here, we report the detailed mechanisms of GrB-mediated neuronal killing and a potential neuroprotective strategy blocking this pathway of neuronal death.
Methods: Human fetal neurons (HFNs) were cultured alone or co-cultured with unactivated or activated T cells or treated with purified human GrB. Neuronal viability was assessed by immunocytochemistry for MAP-2. Expression and cleavage of alpha-tubulin and caspases were evaluated by western blotting. GrB was labeled with Alexafluor488 and its colocalization with early endosomes was evaluated. An animal model of MS, experimental autoimmune encephalomyelitis (EAE) was induced and the mice were treated with GrB-inhibitor serpina3n before and/or at the onset as well as at the peak of the disease. Clinical score and motor coordination of the mice were assessed.
Results: Our *in vitro* results show that the 27 kDa purified granule-derived human GrB, but not the 32 kDa constitutively secreted, induces severe neuronal death to the same extent of activated T cells. Our data further show that unlike the constitutively secreted GrB, granule-derived GrB enters neuronal cells via mannose-6-phosphate receptor. Within 15 minutes, it is taken up by the early endosomes, and within half an hour it diffuses out of the endosomes possibly through a perforin-independent mechanism. When free in the cytoplasm, GrB induces activation of caspases, cleavage of α-tubulin and subsequent neuronal apoptosis. Inhibition of caspase-3, a well-known substrate for GrB, significantly reduces GrB-mediated neurotoxicity. Furthermore, we demonstrate that treatment of neurons with mannose-6-phosphate prevents GrB entry and inhibits GrB-mediated neuronal death, suggesting mannose-6-phosphate receptor-dependent endocytosis. In addition, pretreatment of activated T cells with the GrB-inhibitor serpina3n significantly reduces the cell-cell contact mediated neurotoxcicty. *In vivo,* treatment of EAE mice with serpina3n significantly decreases the severity of the disease.
Conclusions: Altogether, our data unveil a novel mechanism by which granule derived GrB induces selective neuronal injury and suggest GrB as a potential new target for the treatment of inflammatory-mediated neurodegenerative diseases such as MS.

Stem cells

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Ablation of endogenous neural precursor cells of the periventricular zone worsens acute cerebral ischemia

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Objective: Experimental studies show that focal cerebral ischemia promotes neurogenesis in the subventricular zone (SVZ) leading to migration of neural progenitor cells (NPCs) from the SVZ to the peri-ischemic area. However, until now, it has not been possible to clearly identify the functional role attributed to endogenous NPCs in stroke. Aim of this project is to study the specific role of NPCs after acute stroke by using a transgenic mouse in which NPCs of the SVZ can be selectively killed by administration of ganciclovir (GCV).
Methods: We used a transgenic mouse that expresses the herpes simplex virus thymidine kinase (TK) under control of the 2nd intron enhancer of Nestin (2ndI-Nestin-Tk). Transient, 45 min long, middle cerebral artery occlusion was induced in 2ndI-Nestin-Tk and control mice after 28 days of subcutaneous gancyclovir (GCV) or saline treatment. Neurological disability, ischemic volume as well as alterations of the blood brain barrier were assessed.
Results: Post-ischemic functional deficits, measured using the modified neurological severity score were greater in the GCV-treated 2ndI-Nestin-Tk than in saline treated 2ndI-Nestin-Tk or saline or GCV-treated WT mice. GCV treatment of 2ndI-Nestin-Tk determined an increased infarct lesion volume and an increased number of hemorrhages compared with that observed after saline treatment of 2ndI-Nestin-Tk or saline or GCV treatment of WT mice. Survival of GCV treated 2ndI-Nestin-Tk mice at seven days was found to be significantly inferior to the other treatment groups. No difference regarding brain edema was found, but a reduced blood brain barrier permeability to medium and high molecular weight compounds was noted.
Conclusions: Ablation of neural precursor cells in acute stroke results in increased lesion volume, worsening of neurological disability, and decreased overall survival. Current experiments are ongoing to unravel the protective mechanisms exerted by endogenous neural progenitor cells.

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Neural stem cells derived from iPSCs represent a safe and effective source for stem cell therapy in experimental autoimmune encephalomyelitis

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Objective: Neural stem cell (NSCs) transplantation is a promising therapy for Multiple Sclerosis (MS). The clinical translation of such approach is limited by the lack of expandable autologous precursors. Induced pluripotent stem cells (iPSCs) may overcome this limitation. In our work we investigated whether the transplantation of NSCs derived from iPSCs (NS iPSCs) could represent a safe and effective therapeutic strategy in a mouse model of MS, namely experimental autoimmune encephalomyelitis (EAE).
Methods: NS iPSCs were derived from iPSCs obtained by lentiviral reprogramming of mouse fibroblasts. EAE was induced in C57/BL6 mice by subcutaneous immunization with myelin oligodendrocyte glycoprotein (MOG)35-55. At 25 days post immunization (dpi), EAE mice were intrathecally transplanted with GFP-labelled NS iPSCs. Neuropathology was assessed at 40 and 80 dpi while the influence of NS iPSCs on remyelination was further evaluated in vitro on primary oligodendrocyte precursor cell (OPC) cultures.
Results: Upon transplantation in EAE mice, NS iPSCs did not induce any tumour formation and significantly reduced clinical severity, demyelination, axonal loss and neuroinflammation when compared to sham treatment. Since transplanted NS iPSCs remained undifferentiated in close contact with perivascular inflammatory infiltrates, we investigated whether the inflammatory environment could induce NS iPSCs to promote endogenous repair mechanisms. Indeed we observed that, in vitro, the conditioned medium of NS iPSCs – challenged with inflammatory cytokines (IFNγ and TNFα) – markedly increased survival and differentiation of OPC primary cultures. This effect was not dependent on IFNγ or TNFα, as these cytokines alone were not able to sustain OPC survival.
Conclusions: Our work provides the first evidence of the safety and efficacy of NS iPSCs in EAE. We showed that transplanted NS iPSCs exert their therapeutic bystander effect by persisting undifferentiated near the perivascular infiltrate. In vitro experiments suggest that the inflammatory environment could induce NS iPSC to secrete a variety of molecules that might promote endogenous remyelination.

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Neural stem cells sort protein and RNA cargoes for export with exosomes in response to inflammation

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Objective: Accumulating evidences shown that transplanted neural stem/precursor cells (NPCs) are able to protect the damaged CNS through mechanisms other than the anticipated cell replacement. This therapeutic plasticity of NPC may be the result of sophisticated programmes of intercellular communication at the level of the microenvironment including the transfer of secreted extracellular membrane vesicles (EMVs) from donor to recipient cells.

The overall objective of this work was to assess whether horizontal communication through EMVs exists for NPCs and if this may play a role in NPCs therapeutic plasticity.
Methods: NPCs derived EMVs have been characterized by several different techniques spanning from electron microscopy, flow cytometry, nanoparticle tracking analysis and western blot. Here we report on the quantitative study of proteins from NPCs derived EMVs using stable-isotope labeled amino acids in cell culture (SILAC) combined with mass spectrometry and the application of next generation deep sequencing of small and long RNAs to understand the profile and expression of RNAs in EMVs and donor NPC.
Results: NPCs secrete large numbers of EMVs comprising a mixed population of exosomes and larger particles, similar to previously described shedding vesicles. EMVs derived from NPC possess a unique repertoire of molecules that are preferentially enriched in EMVs respect to the donor cells. We identified ~900 proteins that are specifically associated with exosomes, of which 11–14% were differentially expressed in response to pro-inflammatory and anti-inflammatory cytokines, with a suppression of proteins involved in RNA translation and processing. Deep RNA sequencing revealed 35 miRs that were highly enriched within exosomes, compared to NPCs, including mmu-miR-9 and mmu-miR-181a that have well characterized pro-neurogenic and immune-regulatory potential, respectively. We have shown that EMVs contents can be transferred to recipient fibroblasts, with consequent RNA and protein expression changes in recipient cell, and effects on cell cycle regulation, induction of stress response and MHC-class I dependent antigen presentation pathways.
Conclusions: NPCs secrete EMVs containing RNAs and proteins dynamically regulated by cytokines. These molecules may be trafficked regulators to be employed for the treatment of neuroinflammatory diseases as new classes of cell-derived therapeutics.

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Subventricular zone neural progenitors protect striatal neurons from glutamatergic excitotoxicity

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Objective: Adult neurogenesis occurs mainly in two regions of the brain: the subventricular zone (SVZ) of the lateral ventricles and the subgranular zone (SGZ) of the dentate gyrus in the hippocampus. aNPCs in the SVZ are known to migrate to the olfactory bulb, but it is less clear if they exert other processes in physiological and in pathological conditions. Aim of this study was to investigate whether

endogenous aNPCs of the SVZ exert an homeostatic regulatory function in response to glutamatergic excitoxicity as in induced experimental epilepsy.
Methods: We generated a *NestfloxGFPfloxTK-IRES-LacZ* transgenic mouse line in which aNPCs can be selectively ablated upon ganciclovir (GCV) administration. These mice have the green fluorescent protein (GFP) and thymidine kinase (TK) gene expression controlled by the 2nd intron enhancer of the nestin gene. In these transgenic mice we induced, using a potassium channel blocker (4-aminopyridine), experimental epilepsy and evaluated the functional outcome as well as histological and gene expression alterations. Moreover by whole cell ex-vivo patch clamp we evaluated the excitability of striatal neurons.
Results: GCV treatment of our *Nestin-TK* mice (NestinTK-GCV$^+$) selectively ablated aNPCs in the SVZ but not in the hippocampus. Neurophysiological analysis revealed that SVZ aNPCs ablation induces an increase of frequency and duration of spontaneous excitatory post synaptic currents of nearby striatal medium spiny neurons. Moreover we found that induction of experimental epilepsy in NestinTK-GCV$^+$ resulted in an increased number and severity of epileptic seizures and in a significant reduced survival compared to control mice. Interestingly LC-ESI-MS analysis revealed that NestinTK-GCV$^+$ had reduced levels of the endogenous cannabinoid anandamide (AEA) in the striatum, as SVZ aNPCs are able to produce AEA. Thus intrastriatal administration of CB1 receptor agonists or of the inhibitor (URB597) of the principal catabolic enzyme fatty acid amide hydrolase of cannabinoids was able, in NestinTK-GCV$^+$ mice to revert the increased epilepsy related morbidity and mortality.
Conclusions: Our data show that SVZ aNPCs contribute to protect neurons from glutamate-mediated excitotoxicity occurring mainly during CNS-compartmentalized inflammatory events via cannabinoid secretion.

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Therapeutic potential of amnion epithelial cells for the treatment of autoimmune-mediated demyelination

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Objective: Multiple sclerosis (MS) is an autoimmune inflammatory disease of the central nervous system (CNS). The cause of MS is as yet unknown, but is believed to involve both cell-mediated and humoral immune responses directed against components of the CNS. Current treatments available for MS patients are predominantly non-specific immuno-modulators, which are only effective in 30% of RR-MS patients, have no long-term beneficial effects and are often associated with significant side effects. In recent years it has been found that stem cells, such as mesenchymal stem cells (MSC), and more recently human amnion epithelial cells (hAEC), have the ability to suppress immune responses in vitro and in vivo and can differentiate into many cell lineages in vitro. These properties make them very attractive potential therapies for MS in that they may dampen CNS inflammation, reduce damage and may have neuro-regenerative potential. Accordingly, we have investigated the potential immuno-regulatory effects of hAECs as a possible therapy for an MS-like disease, EAE (Experimental Autoimmune Encephalomyelitis) in mice.
Methods: The immunosuppressive properties of freshly isolated hAECs were assessed in *in vitro* proliferation assays with PMA and Ionomycin stimulation of naïve spleen cells and MOG stimulated 2D2 spleen cells. Immunological profiling of hAECs was also performed using FACS and the markers assessed included MHC class I and II antigens, co-stimulatory markers and HLA-G. RR-EAE was induced in 10-week old female NOD/Lt mice with 75 μg rMOG and 350 ng pertusis was given on day 0 and 2. Either 1 million or 5 million hAECs were injected on days 8, 10 and 12 and mice were monitored daily for their clinical signs of disease. CNS was collected for histopathological analysis. Proliferation and cytokine assays were performed with spleens from these animals. T-regulatory and T-helper cell phenotyping was performed on spleens, lymph nodes and CNS.
Results: We found that *in vitro* hAECs can suppress both specific and non-specific T-cell proliferation and decrease the amount of pro-inflammatory cytokines produced by T-cells. *In vivo* studies have also revealed that hAECs not only suppress the development of RR-EAE but also prevent relapse in these mice. Mice protected from EAE by hAEC administration had reduced MOG-specific T-cell responses and produced less pro-inflammatory cytokines upon antigen specific and non-specific stimulation. Further investigations also revealed that hAEC treated mice had increased percentages of Th2 cells in the peripheral lymphoid organs and, most importantly, within the CNS.
Conclusion: These results imply that hAECs produce their immunoregulatory effect in part by initiating an anti-inflammatory response directly within the CNS. Collectively this work suggests that hAECs hold promise for the treatment of autoimmune diseases, like MS.

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The small heat shock protein Alpha B-Crystallin rescues oligodendrocyte progenitors from cuprizone-induced demyelination and promotes remyelination

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Objective: In this study, we investigated the role of cryab in oligodendrocyte and oligodendrocyte progenitor cell (OPC) fate in *in vivo* de- and remyelination.
Methods: Demyelination was induced in 129/SV wild type (wt) and cryab −/− mice by feeding 0.2% cuprizone in chow for up to 8 weeks, after which remyelination was induced by returning mice to a normal diet for 2–3 weeks. Disease outcome was assessed by rotarod motor skill testing. Size and cellular composition of demyelinating lesions was assessed by immunohistochemistry. In addition, the role of cryab in oligodendrocyte progenitor cell (OPC) survival was studied using the oligodendrocyte progenitor cell line mOP and siRNA technology to transiently knock down cryab expression.
Results: In a parallel study, we found that cuprizone-induced lesions are significantly larger and more severe in the presence of cryab. We hypothesize that this severe pathology is mainly due to increased reactive astrogliosis, facilitated by cryab, which has been shown to be anti-apoptotic in astrocytes.

Conversely however, although more severe, demyelinated areas in wildtype mice contain more PDGF-R alpha positive OPCs than in cryab −/− mice. In addition, our data suggest that remyelination is more efficient in wildtype controls than in cryab −/− mice. Moreover, remyelinated areas in wildtype mice contain more CC1 positive oligodendrocytes than remyelinated areas in cryab −/− mice. Additional *in vitro* experiments using the mOP oligodendrocyte progenitor cell line suggest that cryab protects OPC and oligodendrocytes from cytokine-induced apoptosis.
Conclusions: Together, we hypothesize that cryab, similar to its function in astrocytes, mediates protection of OPCs and oligodendrocytes from apoptosis, facilitating remyelination *in vivo*. This notion supports the role of cryab as a possible target in a regenerative therapeutic approach for MS. However, considering its contrary role in reactive

astrogliosis and the subsequent consequences for demyelination, the possible benefit of cryab as a treatment target needs further investigation.

T cells

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Bridging effector and regulatory T cells via resolving macrophages: The beneficial cross-talk of the innate and adaptive immune system following spinal cord injury

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Objective: Trauma to the central nervous system (CNS) results in immune activation/entrance into the injured tissue, assumed to be neurotoxic and contributing to secondary tissue damage. Recent studies have shown that certain immune responses can be beneficial and stimulate axonal regeneration. A subset of monocyte-derived macrophages was shown to be endowed with a unique anti-inflammatory phenotype essential for repair. The recruitment of such critical macrophages is limited but was found to be improved using T cell-based vaccination. It remains unclear how T cells contribute to macrophage recruitment to the traumatized CNS and whether T cells support a neuroprotective role by other ways beyond facilitation of the macrophage recruitment.

Methods: Knockout mice, adoptive transfer as well as depletion of immune cell populations were used to determine the role of specific immune cell subsets. Functional recovery of the hind limbs after contusion was assessed using the Basso Mouse Scale.

Results: Here, we found that both effector T cells and regulatory T (Treg) cells are important in the wound healing response following the insult, and are part of a network that involves the beneficial resolving macrophages. The early effector response, characterized here as T helper (Th) 1, is beneficial to recovery by promoting the recruitment of resolving macrophages to the injured parenchyma. In turn, such monocyte-derived macrophages recruit Treg cells via specific signalling molecules. These Treg cells are endowed with a specific phenotype allowing them to efficiently regulate the immune cell effector response, when it is no longer needed and potentially detrimental for recovery.

Conclusions: These results demonstrate the intricate cross-talk between infiltrating macrophages and Treg cells, a feedback loop that is compromized in the absence of Th1 cells. In this crosstalk, where the infiltrating monocyte-derived macrophages are central players, the effector T cells, although critical in the early phase following the insult, later must be counterbalanced by Treg cells , which can properly limit the type 1 inflammation associated with tissue destruction and immunopathology. Altogether, this network of immune cells warrants an appropriate immune response following the insult that favors wound healing and regeneration of the tissue. This physiological response can be fine-tuned to improve recovery, thereby having important therapeutic implications for CNS trauma.

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Interferon gamma signaling in T cells attenuates autoimmunity, but is not essential for Th17 plasticity in vivo

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Objective: 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 naïve mice. Additionally it was additionally shown that IFNγ synergizes with IL-12 to induce effective IFNγ expression from *in vivo* generated Th17 cells, after *in vitro* reactivation. These findings led us to investigate the role of IFNγ signaling in $CD4^+$ T cells in regard to Th17 plasticity and EAE development, by using an IFNγ receptor knock-out model.

Methods: We crossed mice with a floxed *Ifngr2* gene to CD4-Cre mice (CD4/IFNγR2 KO). $CD4^+$ T cells were analyzed *in vitro* for Th1 differentiation, as well as Th17 plasticity and we performed an adoptive transfer of *in vivo* generated Th17 cells into RAG1 KO mice to check for passive EAE and Th17 plasticity.

Results: We found that *in vitro* differentiated Th17 cells from CD4/IFNγR2 KO mice could not be shifted as effectively as control T cells, when reactivated with IFNγ and IL-12. Additionally $CD4^+$ T cells were less potent in Th1 differentiation under these conditions. We found IL-27 to be a strong inducer of Th1 differentiation in both control and CD4/IFNγR2 KO T cells, especially when combined with IL-12. This combination additionally shifted differentiated Th17 cells towards IFNγ expression almost as effectively as IFNγ with IL-12. Transfer of *in vivo* generated Th17 cells from CD4/IFNγR2 KO mice led to a more severe outcome of EAE, with significantly more IL-$17A^+$, RORγt^+ as well as IL-$17A^+$ IFNγ^+ T cells in the CNS of these mice.

Conclusions: Our adoptive transfer experiments demonstrate, 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 supported by our *in vitro* data.

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Inhibition of CD4 + T cell-mediated tissue inflammation by Podoplanin

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$CD4^+$ T cells orchestrate the activation and recruitment of a coordinated immune response, largely due to their ability to differentiate into subsets that produce distinct sets of effector cytokines. Th17 cells are a recently identified subset of $CD4^+$ T cells that promote acute and chronic tissue inflammation and have been implicated in the pathogenesis of a variety of human chronic autoimmune and inflammatory diseases. Understanding the cellular and molecular mechanisms that regulate Th17-mediated inflammation is therefore an important area of investigation. Podoplanin (Pdp) is a transmembrane sialomucin-like glycoprotein that we have previously shown to be preferentially expressed by Th17 cells. In order to investigate the role of Pdp in T cell biology *in vivo*, we studied the immunologic phenotype of Pdp-deficient mice. While most Pdp-deficient mice die perinatally due to respiratory failure, we found that Pdp-deficient mice that survive to adulthood suffer from multi-organ tissue inflammation with lymphocytic infiltrates in multiple non-lymphoid tissues. $CD4^+$ T

cells isolated from Pdp-deficient mice produce increased amounts of effector cytokines *ex vivo* and are hyperproliferative *in vitro*. Furthermore, Pdp-deficient mice that also express the myelin oligodendrocyte glycoprotein (MOG)-specific 2D2 transgenic T cell receptor rapidly develop spontaneous experimental autoimmune encephalomyelitis (EAE). We also generated mice that transgenically overexpress Pdp under the control of the CD2 promoter. In contrast to Pdp-deficient mice, the transgenic mice exhibit a profound reduction in peripheral T cells, despite normal numbers of major thymocyte subsets. Moreover, the remaining Pdp-transgenic $CD4^+$ T cells are hypoproliferative *in vitro* and following immunization with MOG peptide Pdp-transgenic mice exhibit more rapid resolution of EAE. Taken together, these data suggest that Pdp acts as a negative regulator of $CD4^+$ T cell-mediated tissue inflammation.

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Role of ATF3/Id1 axis in the resistance of memory CD4 T cells to suppression in autoimmune encephalomyelitis

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Objective: T cell-mediated autoimmune diseases are thought to be driven by persisting memory CD4+ T-cells. Emerging approaches for the treatment of autoimmune diseases involve induction of CD4+FOXP3+CD25+ regulatory T cells (Tregs). We investigated whether memory CD4+ T-cells can be tolerized by Treg cells in experimental autoimmune encephalomyelitis (EAE), a mouse model of multiple sclerosis.
Methods: C57BL/6 WT mice and ATF3−/− and Id1−/− mice were used. Cytokine production was measured by intracellular FACS staining and by Luminex. T cell proliferation was analyzed by 3H-thymidine incorporation.
Results: We show that EAE induced by transfer of myelin-specific memory CD4+ T-cells generated is resistant to suppression by CD4+FoxP3 Tregs (isolated from Foxp3-GFP reporter mice) while EAE induced by primed $CD4^+$ T cells was suppressed by Tregs. Using gene microarray, we identified two key regulators of memory T-cell resistance to suppression: (i) Activating transcription factor 3 (ATF3), a transcriptional repressor and member of the mammalian activation transcription factor/cAMP responsive element-binding (CREB) protein family of transcription factors, and (ii) inhibitor of DNA-binding (Id1), a helix-loop-helix protein that plays a role in the progression of a variety of cancers and is negatively regulated by ATF3. ATF3 was downregulated in memory T-cells compared to their effector counterparts (10.6 fold) while Id1 was induced in memory T-cells (15.5 fold). This was confirmed by Taqman PCR and by Western blot.

Furthermore, we found that ATF3−/− CD4+ T-cells cultured with titrated doses of recombinant TGF-beta1, are resistant to suppression, while Id1−/− CD4+ T cells were more susceptible. Mechanistically, we demonstrate that ATF3 interacts physically with Smad3, a molecule that mediates the immunosuppressive properties of TGF-beta, and that ectopic expression of ATF3 leads to increased Smad3 protein stability as shown by [35S]-methionine-mediated pulse chase assay.

We confirmed these findings in human cells, where CD4+ CD45RO+ memory T-cells isolated from PBMCs of healthy subjects express low ATF3 and high Id1 compared to naive T-cells and are resistant to TGF-beta1-mediated suppression. This resistance was reversed by ectopic expression of ATF3.
Conclusions: Our investigations elucidate the mechanism of memory T-cells resistance to Tregs. These findings have implications for the therapeutic use of Tregs in multiple sclerosis and other autoimmune diseases.

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Polyclonal autoreactive T cells that co-recognize a myelin and a neuronal antigen: A natural component of the T-cell compartment of C57BL6 mice

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Objective: Pathogenic T cells in autoimmunity might recognize multiple self-antigens. We observed that in mice expressing an I-Ab-restricted transgenic TcR specific for the myelin oligodendrocyte glycoprotein (MOG)-derived peptide MOG_{35-55}, CD4 T cells also recognize an epitope within the axonal protein Neurofilament medium (NF-M). This feature enhanced the pathogenicity of bi-specific T cells. Here we assess the frequency of these highly pathogenic T cells in the polyclonal T-cell compartment of C57BL/6 mice, and the molecular origin of the bi-specificity.
Methods: 18 MOG_{35-55}-specific T-cell hybridomas generated from MOG_{35-55} immunized BL/6 and expressing distinct TcRs were selected for this study. T-cell clones were classified based on their response to MOG_{38-50} and NF-M_{18-30}, the minimal epitopes that share partial sequence homology. To establish the molecular nature of bispecificity, we performed an alanine scan of the NF-M_{18-30} epitope so as to identify the contribution of each amino acid in I-Ab binding and TcR engagement.
Results: The analysis of 18 independent I-Ab restricted T-cell hybridomas revealed 6 bispecific T cell clones that reacted to both MOG_{38-50} and NF-M_{18-30}. This proves that multiple T cell clones in the polyclonal T cell compartment recognize both MOG_{35-55} and NF-M_{18-30}. To analyze if this is a public feature we assessed the antigen recall responses of individual MOG_{35-55}-immunized mice: CD4 T cells from each individual mouse responded to both MOG_{35-55} and NF-M_{15-35}. The presence of T cells that co-recognize MOG and NF-M is therefore a public property of BL/6 mice. Previous studies have identified the TcR contact residues of MOG_{38-50} when presented in the context of I-Ab. These residues are conserved in the NF-M_{18-30} epitope, and mutating these residues in an alanine scan of NF-M_{18-30} showed that such linear homology underlies the observed bispecificity.
Conclusions: We show that the CD4 T cell compartment of BL/6 mice comprises multiple T cell clones specific for both MOG and NF-M. Bispecificity for myelin and neuronal antigens is a public feature of the CD4 T cell compartment of BL/6 mice and results from the preservation of the TcR contact residues in the minimal epitopes MOG_{38-50} and NF-M_{18-30}. These observations indicate that bispecific CD4 T cells that recognize multiple self-antigens are a natural component of the T cell compartment. The pathogenic contribution of these polyclonal bispecific T cells is being investigated.

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T cell invasion of the CNS: the lung paths the way

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A crucial question in CNS autoimmune diseases such as MS is how autoreactive T cells transmigrate through an intact blood–brain barrier and build up inflammation in the CNS. According to the current view,

myelin-reactive T cell blasts are thought to perform this initial step and to create a pro-inflammatory milieu in the CNS, thereby opening the way for a second autoimmune attack. By combining two-photon imaging and functional characterization in a Lewis rat model of EAE we could demonstrate that T cell blasts do not efficiently enter the CNS and are not required for priming the CNS milieu. Instead, after being injected intravenously, these cells home into the lung. Here they use the airways as roads to move to bronchus-associated lymphoid tissues and lung-draining mediastinal lymph nodes before entering the blood circulation, from where they then reach the CNS. During this sojourn in the periphery the autoreactive T cells undergo a global change in their motility and functional profile by down-regulating their activation and proliferation program and up-regulating their cellular locomotion molecules and their chemokine and adhesion receptors. The latter include ninjurin-1 which plays a role in T cell intravascular crawling on cerebral blood vessels. This migratory phenotype is different from the blast phenotype in that it allows the effector T cells to enter the CNS and induce disease there very efficiently. Interestingly the general reprogramming is not limited to effector T cells: resting myelin-reactive memory T cells could also be stimulated in the lung, and after strong proliferation they assumed a migratory phenotype which enabled them to enter the CNS. We therefore propose that the lung plays a major role in the activation of potentially autoaggressive T cells and their transition to a migratory mode prerequisite to entering the intact blood–brain barrier and inducing autoimmune disease.

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Direct imaging of T cell activation during experimental autoimmune encephalomyelitis

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Objective: Multiple sclerosis is initiated when brain-reactive T cells enter the CNS and become locally reactivated upon encountering their cognate antigen. When and where the disease-relevant activation processes occur in the target tissue and which cells present the antigens to the invading effector T cells is currently unclear**.** Here we developed a fluorescent sensor for real-time imaging of T cell activation. We used this tool to analyze the interactions between antigen presenting cells and encephalitogenic T cells during experimental autoimmune encephalomyelitis (EAE) of Lewis rats**.**
Methods: A fluorescently labeled nuclear factor of activated T cells (NFAT) and histone H2B were used in combination as molecular sensors to visualize antigen-driven T cell activation. Using intravital 2-photon microscopy the activation state of T cells specific for myelin basic protein retrovirally engineered to express the NFAT-H2B sensors was tracked within incipient EAE lesions. These imaging studies were complemented with functional pharmacological and genetic intervention, making it possible to evaluate the relevance of the observed T cell activation events for the clinical outcome.
Results: Nuclear translocation of the NFAT/H2B reporter upon stimulation of T cell receptors was fast, reversible and antigen-dependent and it closely correlated with expression of pro-inflammatory cytokines and surface activation markers. The utility of the NFAT/H2B reporter was validated by intravital imaging of T cell activation after antigen encounter within lymph nodes. In EAE, myelin-specific effector T cells entering the CNS during the preclinical phase became activated after short and transient contacts with leptomeningeal phagocytes. During established disease, the T cell activation process was extended to the depth of the CNS parenchyma. There, the T cells formed contacts with resident microglia and recruited phagocytes. The later activation processes included long-lasting interactions with antigen-presenting cells. Inhibition of T cell activation significantly ameliorated EAE when applied early in the preclinical phase but failed to do so in the acute phase.
Conclusions: The established NFAT/H2B reporter allows sensitive and reliable detection of T cell activation *in vivo*. Our data indicate that it is the activation processes during the preclinical phase rather than during the established disease that are essential for the intensity and duration of the subsequent disease bout.

Brain tumors and parneoplastic

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Characterization of Yo antibodies

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Objective: Yo antibodies are associated with paraneoplastic cerebellar degeneration (PCD) and ovarian or breast cancer. Yo antibodies are mainly referred to as CDR2 antibodies. The objective was to characterize Yo antibodies by determining both CDR2 and CDR2L antibodies and their structural localization.
Methods: 335 patient sera were screened for CDR2 and CDR2L by radioimmune assay (RIA). The sera included 42 Yo positive sera from our routine laboratory, 179 sera from ovarian cancer patients and 114 sera from breast cancer patients. Hospital records were reviewed retrospectively. We also used HeLa cells transfected with CDR2 or CDR2L fused to GFP and co-stained the cells with patient serum. Absorption experiments were done to determine which of the CDR antibodies that stained Purkinje cells. We also used confocal microscopy to determine the subcellular localization of the CDR2 and CDR2L proteins.
Results: We found using RIA that 36 of the 42 sera from the patients with PNS contained both CDR2 and CDR2L antibodies, whereas only CDR2 antibodies were present in the remaining 6 sera. Of the 179 ovarian cancer sera, 5 were positive for only CDR2L antibodies. Of the 114 breast cancer sera, 2 were positive for CDR2 antibodies and 2 for CDR2L antibodies. Only patients with both CDR2 and CDR2L antibodies had PCD. We found no CDR2 or CDR2L antibodies in 100 healthy blood donor sera used as control. The CDR2 or CDR2L antibodies could also be determined in the GFP transfected cells. Absorption experiments of Yo positive sera showed that CDR2L antibodies mediate the primary staining of rat Purkinje cells. We found by confocal microscopy that CDR2 and CDR2L are localized to the cytoplasm, whereas CDR2L is also present in the cell membrane.
Conclusions: We found that Yo antibodies usually harbor both CDR2 and CDR2L antibodies. However, Yo sera can also contain only one of these antibodies. The presence of both CDR2 and CDR2L antibodies seem to be necessary for the development of PCD. CDR2 and CDR2L are both localized to the cytoplasm, whereas CDR2L is also membrane-bound. CDR2L and CDR2 antibodies may be of pathogenic importance for the development of PCD.

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On the role of nucleolin in glioma progression

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Objective: Gliomas are intrinsic brain tumors and its most malignant form, glioblastoma, has a dismal prognosis despite multimodality treatment approaches. This is based on invasive growth, relative resistance to radiotherapy and chemotherapy, and tumor-associated immunosuppression. Thus, it is imperative to develop new treatment modalities.

Nucleolin is a major nucleolar protein involved in ribosome biogenesis, regulation of cell proliferation and growth, but is also expressed on the cell surface where it serves as a binding protein for a variety of ligands implicated in tumorigenesis and angiogenesis. In this project we focused on the role of nucleolin during the progression of gliomas.
Methods: We investigated the expression and localization of nucleolin in a tissue microarray (TMA) of low and high grade gliomas by immunohistochemistry and performed statistical correlation analysis. Freshly isolated glioblastoma tumor and cancer stem cells were cultured and the impact of nucleolin silencing by siRNA was evaluated on their proliferation potential. In addition the ability of AS1411, a GC-rich oligonucleotide which interacts with nucleolin, to inhibit proliferation and induce cytotoxicity was assessed.
Results: Western blot analysis demonstrated a different nucleolin pattern in glioblastoma tumor and cancer stem cells. In vitro experiments have shown that small interfering RNA (siRNA)-mediated silencing of nucleolin inhibited the growth of glioblastoma cells. In addition, AS1411 inhibited the proliferation and induced apoptosis of glioblastoma tumor and cancer stem cells. TMA data revealed an increase in nucleolin expression in glioma progression and a negative correlation with survival.
Conclusions: Our findings suggest strategies to target nucleolin (e.g. by AS1411) as a new therapeutic treatment for gliobastomas.

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Modulation of innate immunity in glioblastoma

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Objective: Glioblastoma (GBM) remains an aggressive and incurable brain tumor despite the well-characterized molecular biology of cancer cells that comprise this malignancy. The immune system is increasingly being recognized as a key factor in carcinogenesis and immune modulation is being actively investigated to ameliorate tumor growth. We aimed to identify alterations in gene profile of tumor-associated myeloid cells in order to reveal targets for their future modulation.
Methods: We used an intracranial GBM mouse model to study the involvement of innate immunity in gliomagenesis. By flow cytometry we analyzed different populations of myeloid cells from brain and spleen of GBM-bearing mice. To reveal tumor-specific profiles, we sorted various subsets of myeloid cells from brain and spleen and performed mRNA and miRNA expression analysis.
Results: We identified profound changes in the immune profile of myeloid cells in GBM-bearing mice, both in brain and the periphery. A population of monocytic myeloid-derived suppressor cells (MDSCs) was reduced while granulocytic MDSCs subset was expanded. Together with changes in immune cell composition, we found an anti-inflammatory and pro-tumorigenic gene profile in glioma-infiltrating myeloid (GIMs) cells. These alterations were accompanied by changes in the expression of several microRNAs (miRNAs), which may simultaneously regulate number of biological pathways.
Conclusions: Given the high plasticity of myeloid cells, we propose that directed modulation of miRNA levels in monocytes of GBM-bearing mice and ultimately humans reprogram these cells and shift their phenotype to pro-inflammatory/tumoricidal phenotype.

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A set of target antigens of autoantibodies associated with paraneoplastic neurological syndromes is expressed in small-cell lung cancer cell lines

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Objective: Autoantibodies against neuronal antigens are markers of small-cell lung cancer (SCLC) in a proportion of patients with paraneoplastic neurological syndromes (PNS). The trigger mechanism leading to their induction remains elusive. SCLC cells often show characteristics of neuronal cells, e.g. they express neuropeptides as well their receptors. Possibly they erroneously present these antigens to the immune system. We have analyzed, if different defined PNS-associated autoantigens are expressed in three SCLC cell lines.
Methods: Total lysates of NCI-H69, NCI-H146, and NCI-H209 cells, established from three individual Caucasian male SCLC patients, and HEp2 cells as well as porcine cerebellum and liver tissue were analyzed by Western blots. Selected sera from human patients with PNS as well as polyclonal sera from rabbits immunized with bacterially over-expressed PNS-associated autoantigens or mitochondrial protein PDC-E2 (control) were used as probes. Specificities of the individual reactions were controlled by using sera from healthy controls, rabbit sera prior to immunization, and neutralization of antibody reactivities with purified recombinant proteins.
Results: Expression of neuronal antigens Ma1 (40 kDa), Ma2/Ta (42 kDa), HuD (42 kDa), Ri (52 kDa), CV2/CRMP5 (61 kDa), and amphiphysin (76 kDa) could be demonstrated unequivocally in all three SCLC cell lines and cerebellum but not in HEp2 cells and liver whereas a band corresponding to PDC-E2 was obtained in each lysate. An 38 kDa band was exclusively obtained with the three SCLC cell lines and cerebellum after incubation with sera containing anti-recoverin (25 kDa). A 62 kDa band was produced with all substrates but liver tissue by sera containing anti-Yo.
Conclusions: Although the three cell lines, NCI-H69, NCI-H146, and NCI-H209, originated from different patients, they express the same set of PNS-associated autoantigens. Expression analyses, including mRNA, of further SCLC cell lines, non-SCLC cell lines, primary tumor cells, and alveolar lavage fluid might therefore define them as markers for the early diagnosis of SCLC. Such experiments might also help to understand the pathomechanism of PNS and how anti-neuronal autoantibodies are induced.

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Paraneoplastic neurological manifestations in patients with lung cancer without onconeural antibodies

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Objective: Paraneoplastic neurological syndromes (PNS) are immune-mediated neurological disorders that occur in less than 1% of patients with cancer. PNS may affect the central and/or peripheral nervous system. About 50% of the patients with PNS have onconeural antibodies. Small-cell lung cancer (SCLC) is the far most common cancer associated with PNS and the relationship between SCLC, PNS and onconeural antibodies is well known. The aim of this study was to analyse a group of patients with PNS and lung cancer, seronegative for onconeural antibodies. Previous data on the clinical spectrum of seronegative PNS are sparse.
Methods: We reviewed the medical records of 68 patients with confirmed lung cancer and neurological symptoms that were referred for analysis of onconeural antibodies in the period 1995–2005. All patients were seronegative for the well-characterized onconeural antibodies. Data from the Norwegian Cancer Registry and follow-up medical data were analysed. We used well-established diagnostic criteria for PNS to diagnose the patients retrospectively.
Results: 14 of the 68 patients with lung cancer had a PNS. 9 of these patients had SCLC, 4 non-small cell lung cancer (NSCLC) and 1 a poorly differentiated carcinoma. 3 patients had paraneoplastic encephalomyelitis, 1 paraneoplastic limbic encephalitis, 1 paraneoplastic cerebellar degeneration, 3 subacute sensory neuronopathy, 2 Lambert-Eaton myasthenic syndrome, 1 multiple sensory mononeuropathy, 1 lower motor neuron disease, 1 myelopathy and 1 unilateral chorea. Among the patients with SCLC, 6 out of 9 had PNS affecting the central nervous system. 3 out of 4 patients with NSCLC had PNS affecting the peripheral nervous system. Median survival time after debut of neurological symptoms was 8 months. In 5 of the patients there was a clear association between recurrence of lung cancer and debut/worsening of PNS. The most common causes of neurological symptoms in the remaining 54 patients were metastases to the nervous system and local tumour infiltration.

Conclusions: About 1/5 of our patients with lung cancer and no detectable onconeural antibodies had PNS. CNS manifestations were most associated with SCLC, whereas NSCLC was associated with peripheral neuropathy. The absence of detectable onconeural antibodies does not exclude PNS in patients with lung cancer.

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Antibody repertoire in paraneoplastic cerebellar degeneration associated with small cell lung cancer

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Background: Patients with paraneoplastic cerebellar degeneration (PCD) often have small cell lung cancer (SCLC) and about 50% of them develop antibodies against voltage-gated calcium channels (VGCC-ab). VGCC-ab are the cause of Lambert Eaton myasthenic syndrome (LEMS). When LEMS associates with SCLC, 70% of patients also have SOX1-ab, a surrogate marker of SCLC. It is unknown whether a similar antibody association occurs in patients with PCD and SCLC. It is also unknown whether patients with PCD develop relevant antibodies against cell surface or synaptic antigens as recently demonstrated in limbic encephalitis.
Aims of the work: To determine the association between SOX1-ab and VGCC-ab in patients with PCD and to investigate whether these patients also develop antibodies against several known cerebellar antigens or unknown neuronal cell surface proteins.
Patients and methods: Serum and CSF of 34 patients with PCD and SCLC were examined by immunohistochemistry on rat brain and cultures of live hippocampal neurons. Ten sera were used to probe cDNA expression libraries of human fetal brain and cerebellum. VGCC-ab were measured by RIA, and SOX1-ab, ZIC-ab, and ELKS1 by immunoblot. Antibodies against β4 and γ2 subunits of VGCC, highly expressed in cerebellum, were examined by a cell-based assay.
Results: SOX1-ab were detected in 50% and VGCC-ab in 47% of the patients. Only 23% of patients were Hu-ab-positive. At least one of these three antibodies was positive in 73% of patients. SOX1-ab occurred more frequently in patients who had VGCC-ab (75% vs 28%, $p = 0.014$). No antibodies were detected against β4 and γ2 subunits of VGCC. Screening of cDNA libraries identified known onconeuronal antigens (ZIC1, ZIC2, ZIC4), and a novel autoantigen, ELSK1, which is expressed in cerebellum and coprecipitates with the β4 subunit of VGCC. Frequency of ZIC1/ZIC2/ZIC4-ab was 11.8%, 14.7%,14.7% respectively and 2.9% for ELSK1-ab. Five patients (14.3%) had antibodies against cell surface antigens. Three of these 5 cases also had VGCC-ab; none of them improved with immunotherapy.
Conclusion: At least one of the following antibodies: Hu, VGCC, and SOX1 are detected in 73.5% of patients with PCD and SCLC. There is a significant association between VGCC-ab and SOX1-ab; detection of these antibodies indicates an underlying SCLC in patients with cerebellar ataxia of unknown etiology. Antibodies to cell surface antigens are rare in PCD, suggesting that neuronal damage may be T-cell mediated.

Infections

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Impaired influenza immunity in spinal cord injured mice

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Objective: Individuals suffering from spinal cord injury (SCI) are at higher risk for infection. Our objective is to better understand the mechanisms of spinal cord injury induced immune-depression with the goal of developing more effective therapies and reduce mortality due to complications from influenza and other infections.
Methods: To study how the immune system is affected by SCI we used a well established influenza virus model. Briefly, spinal cord injured (6–7 weeks post-SCI) and control mice were infected intra-nasally with H3N2 influenza-A virus (1×10^4 PFU) and analyzed at different times post-infection for virus titer in the lungs, cytokine/chemokine production, virus-specific $CD8^+$ T cell response in the lung and lymphoid organs, and production of neutralizing antibodies against the virus.
Results: While all the control mice cleared the virus from the lungs 10 days post-infection, a significant number of SCI mice did not clear the virus. In addition, these SCI mice which did not clear the virus showed a significant lower number of virus-specific $CD8^+$ T cells in both the spleen and the BAL. Furthermore the sera isolated from those mice were not able to neutralize the virus suggesting an impaired adaptive immune response. We are currently investigating whether the innate immune response in the lungs of SCI mice is deficient compared to control mice.
Conclusions: Taken together our data demonstrate that the immune system of chronically spinal cord injured mice is compromised.

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Herpes simplex encephalitis leads to acute lateral ventricular enlargement in mice and man

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Objective: To compare the neuropathology associated with herpes simplex virus-induced encephalitis (HSE) in mice and humans and determine the contributing factors associated with controlling infection and inducing disease.
Methods: MRI T2-weighted images were obtained using a Bruker Biospec 5.0 imaging system from wild type, type I IFN receptor deficient mice ($CD118^{-/-}$), and mouse chimeras. Human patient records (19 each of control and HSE patients) were retrieved and examined in masked fashion by a neuroradiologist. Inflammation and virus spread/replication (HSV-1 strain McKrae) in the brain in vivo or in organotypic slice cultures were evaluated using $CD118^{-/-}$ mouse chimeras and wild type mice or mice deficient in the TLR adaptor proteins, MyD88 and TRIF. Confocal microscopy, flow cytometry, ELISAs/suspension arrays, Western blot analysis, and real time RT-PCR were employed to measure various parameters. Data was analyzed for significance ($p<.05$) using ANOVA and post-hoc Tukey's t-test for comparison of more than two groups whereas Student's t-test was used to compare two variables.
Results: $CD118^{-/-}$ mice were highly susceptible to encephalitis with observable features of encephalitis (motor dysfunction) as early as day 4 post infection (pi). MRI analysis revealed ventricular enlargement restricted to the lateral ventricles by day 5 pi. By comparison, MRI scans of HSE patients identified a gross dilatation of the lateral ventricles of which 26% of patients had a CT Evan's index > 0.3. The virus was found to localize to the ependymal region of the brain with RBCs and significantly elevated CXCL10 protein in the CSF and loss of cilia by ependymal cells in the encephalitic mice. $CD118^{-/-}$ bone marrow (BM) chimeras revealed a CNS resident population was responsible for viral surveillance. Organotypic brain slice cultures revealed microglia as the primary resident cell responsible for control of viral replication through a TRIF-dependent pathway.
Conclusions: We have found herpes simplex virus type 1 infection of the CNS displays specific tropism for the ependymal region resulting in lateral ventricle enlargement consistent with human encephalitic findings. Once lytic infection is established, a potent TRIF-dependent, anti-viral, microglial-centric, BM-independent immune response is activated to contain further viral spread.

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Non-redundant in vivo functions of IFN-beta protect against virus infections within the central nervous system

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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 $IFN\text{-}\beta^{-/-}$ 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 $IFN\text{-}\beta^{-/-}$ 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, 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, was detected in the serum. Analysis of IFN-β reporter mice revealed that within the central nervous system protective IFN-β was induced primarily in the olfactory bulb, presumably by neuroectodermal cells. 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 $IFN\text{-}\beta^{-/-}$ did not show local IFNAR signaling within the CNS. 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.

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Dynamics of memory T cells clearing a persistent CNS infection in the absence of severe tissue Injury

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Objective: Many viruses can infect the mammalian central nervous system (CNS), some with devastating consequences, and others with little or no overt pathology. Immunotherapy is a successful approach to treat persistent infections that has shown promise in humans. Our laboratory studies a murine model of persistent lymphocytic choriomeningitis virus (LCMV) infection to understand the mechanism(s) by which therapeutically administered virus-specific memory T cells cleanse the infected CNS without causing cellular damage or blood brain barrier breakdown.
Methods: In this study, advanced imaging techniques, such as intra-vital two photon microscopy, were used in combination with cellular and molecular approaches to provide a comprehensive understanding of how innate and adaptive immune cells coordinate a response with the CNS to locate and engage virally infected cells.
Results: Using two-photon microscopy, we observed that therapeutic memory T cells invade the meninges and brain parenchyma, scanning both areas systemically for viral antigen. Interestingly, T cell surveillance of the persistently infected brain was not associated with massive recruitment of peripheral myelomonocytic cells — innate cells known to cause severe vascular pathology during LCMV meningitis. Instead, we revealed using CD11c-YFP reporter mice that immunotherapeutic T cells directly engaged brain resident microglia and transformed them into a cell population resembling dendritic cells. Interestingly, CD11c + microglia were protected from T cell mediated apoptosis following engagement and also directly participated in the immunotherapeutic process by releasing chemokines such as CCL5.
Conclusions: We propose that successful clearance of a persistently infected brain can be achieved without induction of severe tissue injury by tailoring chemokine release to limit myelomonocytic cell

recruitment and by converting microglia into local antigen presenting cells.

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A NK complex-linked locus restricts the spread of herpes simplex virus type 1 (HSV-1) in the central nervous system (CNS)

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Objective: Natural resistance to mortality in mice infected with HSV-1 is mouse strain dependent and an autosomal dominant trait. In mice infected with virus via the oral mucosa, HSV-1 spreads throughout the CNS of susceptible strains but is restricted to the brainstem of resistant mice. In resistant BL/6 mice, viral restriction is mediated by NK and $CD8^+$ T-lymphocytes. The objective of this study is to further define the role of NK cells.
Methods: We combined Mendelian analysis, studies of congenic and intra-NKC recombinant mice, along with antibody depleted mice to further examine the restriction of viral spread in the brains of BL/6 mice.
Results: We report a NK complex-linked genetic locus, *Hrl2*, whose alleles determine the restriction of viral spread in the CNS. Mendelian analysis determined that restriction of viral spread is a dominant trait and consistent with a single gene effect. Studies with congenic mice determined that the locus maps to the NKC on chromosome 6 but is separate from previously defined loci: *Hrl1* and *Rhs1*. Studies with intra-NKC recombinants determined that the locus maps to a segment between Cd69 and D6Wum16. Studies with antibody depleted mice determined the effect of this locus is mediated by $NK1.1^+$ expressing cells.
Conclusions: A NK complex-linked locus (*Hrl2*) restricts the spread of HSV-1 in the CNS of BL/6 mice however the precise mode of action of the locus remains unknown.

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The role of IL-10 in modulating fatal Alphavirus encephalomyelitis

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Objective: Neuroadapted Sindbis virus (NSV) infects neurons and induces fatal, paralytic disease in C57Bl/6 mice as a consequence of inflammation. Tight regulation of the immune response to promote efficient viral clearance while limiting immunopathology is critical, particularly in the central nervous system (CNS). The mechanisms of immunoregulation and immunopathology during NSV infection are poorly understood. IL-10 is a key regulator of immune responses, thus we wanted to determine its role during NSV-induced encepalomyelitis.
Methods: To determine the effect of IL-10 on the course of NSV infection, we compared wild-type B6 and IL-10 −/− mice infected with NSV. Morbidity and mortality as well as various parameters of viral replication and clearance including infectious virus titers, viral RNA, and viral antigen distribution in the CNS were assessed. We used qRT-PCR on brain and spinal cord tissue to determine the effects IL-10 deficiency on pro-inflammatory cytokine and chemokine mRNA expression.
Results: IL-10 −/− mice died earlier with a mean day of death of 8 days compared to 10 days for WT mice ($p<0.0001$). Viral RNA levels from whole brain and spinal cord were similar between both groups. Infectious virus titers reached similar levels in whole brain; however clearance was delayed in the absence of IL-10. In the spinal cord, peak virus titers were higher in IL-10 −/− mice compared to WT animals. Viral antigen distribution at 5dpi was also different with more viral protein in the cerebellum, brain stem, and spinal cord of IL-10 −/− mice than WT mice; however, antigen staining was similar in the cortex, hippocampus, and thalamus. Baseline levels of pro-inflammatory cytokine and chemokine mRNAs were significantly higher ($p<0.001$) in the spinal cords of IL-10 −/− mice relative to WT mice, whereas baseline levels in the brain were similar. Production of inflammatory cytokine and chemokine mRNAs reached similar levels in IL-10 −/− and WT mice in both the brain and spinal cord during the course of infection.
Conclusions: These data suggest that IL-10 is important in maintaining a neuroprotective and/or anti-inflammatory environment, particularly in the spinal cord. The data also suggest that IL-10 influences viral clearance and has a region-specific role in inhibiting viral replication in some populations of neurons. In the absence of IL-10, NSV-induced encephalomyelitis is exacerbated and/or accelerated.

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Absence of a robust innate immune response in rat neurons facilitates persistent infection of Borna Disease Virus in the central nervous system

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Objective: Borna disease virus (BDV) is a negative-stranded RNA virus preferentially infecting neurons of the limbic system. BDV is known to be an IFN sensitive virus, however, microarray analysis of rat organotypic hippocampal cultures revealed a pronounced up-regulation of IFN-induced genes (ISGs) upon BDV infection. The objective of this work is to determine the strategy BDV utilizes to overcome the IFN response during establishment of a persistent infection.
Methods: Rat or mouse hippocampal slice cultures or dissociated primary neuron cultures were prepared for BDV infection in the presence or absence IFN. Viral load or IFN responses were determined by western blotting, staining of Mx, a specific marker for the induction of IFN-inducing genes (ISGs) or by using recombinant vesicular stomatitis virus (VSV), harboring a green fluorescent protein (VSV-GFP).
Results: In hippocampal slice cultures BDV replicates and spreads almost exclusively in neurons, however, IFN-induced Mx proteins could only be identified in microglia and astrocytes. Treatment of uninfected cultures with IFN revealed similar results, suggesting that neurons are not able to mount a robust immune response and thus represent a preferential site for BDV replication. In support of this assumption, we found that continuous IFN treatment of BDV-infected rat hippocampal cultures or dissociated neuronal cell cultures did not affect BDV persistence. However, a delay of viral dissemination was observed. This could be related to a weak IFN response in neurons as judged by the clearance of VSV-GFP from neuronal tissues. A BDV mutant virus (BDV-LL), which is known to replicate efficiently within a cell but only inefficiently spreads from cell to cell, revealed a significantly increased IFN-sensitivity

compared to wild type BDV. In contrast to rat neurons, neurons of mice responded to IFN treatment and inhibited BDV dissemination efficiently, indicating species-specific differences in the IFN response. Infection of hippocampal cultures of wild type and type I/III IFN receptor knock-out mice with BDV-LL confirmed the pronounced IFN-sensitivity of this mutant virus.
Conclusions: Compared to the mouse model, which has more robust IFN responses upon infection, rat neurons can only mount weak antiviral responses and thus represent an ideal cell type for establishing a persistent infection. Moreover, the IFN-mediated antiviral response affects viral dissemination rather the intracellular replication.

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Treatment of *Helicobacter pylori* infection and risk of Parkinson's disease in Denmark

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Objective: Parkinson's disease (PD) is characterized by a progressive loss of dopaminergic substantia nigra cells leading to a broad spectrum of motor and non-motor features like gastrointestinal dysfunction, which may precede the motor symptoms.

Helicobacter pylori (HP), the well-known causative agent for chronic gastritis, peptic ulcer disease, and gastric cancer, has been suggested to play a role in the development of PD, due to similar epidemiological features, such as familial aggregation and association with water sources. Antibodies against HP have also been found to be elevated in PD patients and in patients with parkinsonian features. Eradication of HP-infections has even been shown to ameliorate symptoms of PD.

Peripheral HP infection can trigger microglia activation through the humeral pathway when circulating proinflammatory or monocytes cross the blood–brain barrier or noxious chemicals produced by HP can be transmitted through vagal afferent pathways and affects neurons in the brainstem. HP may also access the brain via oral-nasal-olfactory pathways or by infected monocytes leading to neurodegeneration by inducing apoptosis through mitochondrial apoptotic pathways or through inducible nitric oxide.

In the present study, we linked unique databases, i.e., the Danish National Patient Register (DNPR) and the National Prescription Registry (NPR), as part of a large population-based case control study to explore whether HP contributes to development of PD.
Methods: We identified 4484 patients with a first time PD diagnosis between 2001 and 2008 from the DNPR and 22,416 population controls from the Danish CRS. Information on drug use was obtained from the NPR. We used logistic regression to compute odds ratios for the association between treatment for HP and risk of PD.
Results: Prescriptions for HP eradication drugs and proton pump inhibitors five or more years prior to the diagnosis of PD were associated with a 45% and 23% increase in PD risk, respectively. Hospitalizations and outpatient visits for gastritis and peptic/duodenal ulcers, however, were not associated with PD.
Conclusions: Our population-based study suggests that chronic HP-infections may increase the risk of PD. Whether this association is the result of a chronic infection leading to aggravated inflammatory pathophysiological insults or merely related to the disease due to degeneration of the autonomous and enteric nervous systems remains to be determined.

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Lipocalin 2 expression and function in the central nervous system host immune response to West Nile Virus infection

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Objective: Lipocalin 2 (Lcn2) is an iron-siderophore binding bacteriostatic factor produced during the host innate immune response to bacterial infection. Further, Lcn2 may also regulate cellular iron metabolism and apoptosis. Whether Lcn2 has a function in the host response to viral infection is unknown. Here, we investigated the regulation and function of Lcn2 in the central nervous system (CNS) of mice during West Nile Virus (WNV) encephalitis.
Methods: Wild type (WT) and Lcn2 knockout (KO) mice (8–10 weeks old) were inoculated intranasally with 6×10^2–10^4 PFU WNV in sterile PBS or PBS alone as control. Animals were 1) either monitored for survival rates, or 2) euthanized on different days post-infection and brains collected. From brain lysates plaque assay, RNase protection assay, or immunoblot were performed to analyze viral titers, expression of host immune response genes, as well as glial and iron markers, respectively.
Results: Both Lcn2 mRNA and protein levels were highly induced in the brain by day 5 following WNV infection. Lcn2 mRNA and protein were found at high levels in the choroid plexus, vascular endothelium and astrocytes. Neuronal subsets contained Lcn2 protein but no detectable mRNA suggesting uptake and accumulation of Lcn2 in these cells. The kinetics of Lcn2 gene expression in the brain following WNV infection overlapped with the expression of a number of genes involved in the antiviral response including IFN-beta, TLR3, MDA5 and RIG-I. With the exception of CXCL5, which was significantly downregulated in Lcn2 KO mice compared with WT mice on day 7 post-infection, expression of host immune genes was not significantly different between the different genotypes. Examination of the susceptibility of Lcn2 KO versus WT mice to infection with different doses of WNV revealed no significant difference in survival. Furthermore, viral loads based on NS-1 RNA levels were similar in WT and Lcn2 KO mice at day 7 post-infection.
Conclusions: We conclude that Lcn2 is induced to high levels in the CNS during WNV infection. Whereas microglia are known as strong immune-mediating cells in the CNS, Lcn2 producing astrocytes may play a more dominant role in WNV-encephalitis. Further, Lcn2 protein distribution after viral infection suggests that neurons, which are the dominantly infected cells in WNV encephalitis, may be a key target for Lcn2 action. Finally, the function of Lcn2 in the host response to WNV infection remains largely unknown. Supported by NNMRC grant 512408.

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Characterization of Epstein-Barr virus genotypes in multiple sclerosis

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Objective: Converging epidemiological, clinical and laboratory studies support an etiologic role for Epstein Barr virus (EBV) in MS. Nonetheless, it is difficult to understand how a virus that ubiquitously infects humans can be associated with a disease of relative limited

prevalence. The possibility that only a particular viral genotype associates with the disease has been proposed and investigated by many groups, with conflicting results.

With the aim to shed light on this controversial scenery we carried out a study aimed at characterizing of Epstein-Barr virus genotypes in MS patients and matched controls from continental Italy.

Methods: Genomic DNA was extracted from CD19 + B cells or peripheral blood mononuclear cells (PBMCs). All samples were analyzed by nested PCR approach using EBNA2 type specific primers and PCR products were assayed by a standard sequence analysis. We addressed to high-throughput sequencing by the Roche 454 platform the PCR amplicons obtained from two large genomic regions of OriP and EBNA1.

Results: We characterized 39 MS patients and 33 HD for EBNA2 genotypes in a region of about 500 bp length. A significant bias in the distribution of EBV subtypes was observed.

In 4 MS and 4 HD we performed experiments in which we attempted to sequence longer stretches (about 1000 bp) of the viral genome within the OriP and EBNA1 regions. The shotgun sequencing of the two amplicons generated a total of about 800,000 reads of high quality, most of them having a length consistent with the expected one of about 400 bases.

Conclusions: We detected several variants with respect to the known EBV genomes and will present data on their differential representation in MS and normal samples.

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alpha-Proteobacteria predominate in the human brain microbiome independent of host immune status

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Objective: Brain infections often occur during diseases such as HIV/AIDS without apparent neurological disease. However, the composition of the human brain's microbial population(s) during systemic diseases together with the associated neuroimmune responses is unknown. Herein, we investigated the profile of infectious agents with accompanying host responses in human brains.

Methods: The profile of infectious agents and associated host responses in brain tissues from persons with HIV/AIDS (HIV; n = 10), other disease controls (ODC; n = 10) and surgical cerebral resections for epilepsy (n = 6) were investigated by massively parallel (deep) sequencing of cerebral white matter-derived RNA, which were confirmed by RT-PCR, *in situ* hybridization, immunocytochemistry and in animal models. Human brain homogenates were stereotactically implanted into the striatum of *RAG1*$^{-/-}$ mice and bacterial genome in brain was subsequently analysed.

Results: Deep sequencing of human cerebral white matter-derived RNA revealed bacterially-encoded RNA sequences in brain specimens from all clinical groups with α-proteobacteria representing over 70% of detected bacterial sequences. Bacteriophage-encoded RNA sequences associated with proteobacteria-tropic phage also predominated in human brain tissues. Bacterial rRNA was detected by *in situ* hybridization in astrocytes and microglia in brains from both HIV and ODC groups. The bacterial wall molecule, peptidoglycan, was also evident by immunocytochemistry in brains from each clinical group. Analyses of conventionally amplified and sequenced bacterial 16s rRNA sequences verified that the Proteobacteria was the principal bacterial phylum in human brain samples from all clinical groups with similar levels of bacteria rRNA detection in both the HIV and ODC groups. Host immune responses in the brain were increased in the HIV group, defined by elevated levels of MHC-class II, CD3, IL-23, IL-12 and other immune gene transcripts although anti-bacterial host genes did not differ between groups. Bacterial burden in human brain was inversely correlated with the expression of host gene transcripts implicated in cell division, morphology and maintenance but were not associated with antibacterial gene regulation. Bacterial sequences were not detected in brains from *RAG1*$^{-/-}$ mice but brains from SIV- and SHIV-infected macaques displayed a profile of bacterial phyla similar to that observed in the human brains. Implantation of human brain homogenates into *RAG1*$^{-/-}$ mice by intracerebral injection revealed a preponderance of α-proteobacteria 16s RNA sequences in the brains of recipient mice at 7 weeks post-implantation, which was abrogated by heat-treatment of the homogenate prior to implantation.

Conclusions: α-proteobacteria represented the major component of the primate brain's microbiome. Bacterial abundance and type in the human brain were independent of systemic immune status and failed to elicit a specific a neuroimmune response, perhaps permitting bacterial persistence in an immune-privileged organ. The capacity for certain bacteria to produce essential neurotransmitters might underlie the presence of microbes in the human brain.

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Th17 cells mediate protection against acute viral infection of the CNS

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Objective: Immunosurveillance and protective immunity in solid organs like the CNS are mediated by different T cell subsets. However, the role of Th1 and Th17 cells during viral infections remains unclear. We have shown that Th1 cells depend on α4 integrins in order to cross the blood brain barrier whereas Th17 cells are able to enter the CNS independently of α4 integrins. Here, we describe a novel T helper cell dependent model of vaccinia virus (VV) induced encephalitis suitable to study the differential contribution of Th1 and Th17 cells to virus clearance in the CNS.

Methods: Wild type C57BL/6 mice were immunized with a replication deficient strain of Vaccinia Virus (MVA) in complete Freund's adjuvant and subsequently challenged by intrathecal injection of a replication competent strain of VV. Starting one day before infection, animals were treated with intraperitoneal injections of a blocking antibody to α4 integrin or isotype control every other day.

Results: Whereas mock immunized mice displayed clinical signs of encephalitis and finally died on day 6 after infection, MVA immunized mice exhibited an initial weight loss but recovered completely within 6 days after infection irrespective of anti-α4 integrin antibody treatment. Consistent with the clinical outcome, a massive infiltrate of VV specific CD4 + T cells was detected in the CNS of immunized mice. In the IgG control treated subgroup, infiltrating CD4 + T cells produced IL-17, IFN-γ or both. In animals treated with blocking anti-α4 antibodies, the number of IFN-γ producing T helper cell in the CNS was significantly reduced. In contrast, the number of IL-17 producers was unchanged suggesting that the Th17 phenotype prevailed in VV specific T helper cells under α4 blockade.

Conclusions: We have generated a novel model of T helper cell dependent immunoprotection against acute viral infection of the CNS

involving both Th1 and Th17 cells. α4 blockade did not affect the clinical recovery of infected mice but led to a relative enrichment of Th17 cells in the CNS. Therefore, in acute viral infection of the CNS, blockade of α4 integrins does not impair virus clearance and both Th1 and Th17 cells are capable of mediating protective immunity. We will further exploit this model to better understand the conditions of T helper cell driven immunity vs immunopathology in viral infections of the CNS in order to prevent the break-down of immunosurveillance when therapeutically targeting immunopathology in chronic inflammation and autoimmunity of the CNS.

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Antiviral priming during differentiation of neurons restricts alphavirus replication

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Objective: The neurotropic nature of many encephalitic arboviruses poses a unique challenge to virus clearance. Due to the nonrenewable nature of neurons, preservation of neuronal function following infection is paramount for survival. Susceptibility to alphavirus infection is age-dependent and host maturation is associated with decreased virus replication and less severe encephalitis. Our studies aim to identify inherent host maturation traits of neurons.
Methods: We have compared the rate of virus replication and host antiviral gene expression during alphavirus infection of cycling and differentiated rat olfactory bulb neurons (AP-7 neurons) using molecular techniques.
Results: Neuronal differentiation is associated with decreased replication of both Old (Sindbis) and New (Venezuelan equine encephalitis) World alphaviruses by 150 to 1000-fold. Restriction affects virus replication, but not the susceptibility of neurons to infection, as both dsRNA (replication complexes) and viral glycoprotein synthesis are present in similar numbers of differentiated and undifferentiated neurons. Virus-induced shutoff of host protein synthesis and cell death is decreased with differentiation. This suggests that a mature neuron is intrinsically less amenable to virus replication compared to an immature neuron. Transcriptional profiling showed increased levels of mRNAs for several interferon (IFN)-regulated genes with differentiation. Uninfected, differentiated neurons produce low, but detectable levels of IFN. However, differentiated, but not undifferentiated, neurons produce IFN rapidly after infection. Expression of interferon regulatory factor-7 (*Irf-7*), a critical transcriptional regulator of IFN synthesis, increased with differentiation and only differentiated neurons produced the full-length isoform.
Conclusions: We propose that neuronal differentiation confers antiviral "priming" to neurons by up-regulating important antiviral factors, thus reducing virus replication in mature neurons.

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Varicella zoster virus IgG titers correlate with multiple sclerosis

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Objective: Potential links between multiple sclerosis (MS) and common viral infections have been extensively studied. To date, Epstein-Barr virus, EBV, is the most established infectious risk factor for MS. Our aim was to analyse the association between varicella zoster virus (VZV) specific IgG antibody titers and MS, while adjusting for *HLA-DRB1*15, A*02*, and EBNA1 IgG, an EBV-related antigen.
Methods: The Epidemiological Investigations of MS (EIMS) consists of incident MS cases and population based controls matched for age, gender and area of residence, recruited from over 40 clinics in Sweden, and blood samples are collected. Anti-VZV antibody titers and anti-EBNA IgG titers were measured with ELISA in 870 individuals, 410 MS and 460 controls. We used imputed *HLA* genotypes from the recent IMSGC/WTCCC2 MS-GWAS. 5 MS cases (1.2%) and 5 controls (1.1%) were VZV IgG negative according to ELISA. The study includes 805 VZV IgG positive MS cases and 855 controls. VZV IgG and EBNA1 IgG titers were normalized using rank based transformation (GenABEL package for R). We used logistic regression to assess association to MS, and adjusted for index age and gender in all analyses.
Results: MS cases had a higher median than controls, 2.14, and 1.67, respectively. First we tested the association of VZV IgG to MS as a dichotomized variable, based on the median among controls, $p = 8\times10^{-5}$, OR = 1.8 (1.3–2.3 95% CI), and $p = 3\times10^{-4}$, OR = 1.8 (1.3–2.4 95% CI) when we adjusted for DRB1*15, A*02 and EBNA1 IgG. We also analysed VZV IgG as a continuous variable after transformation and it was associated with MS, $p = 7\times10^{-9}$, beta = 0.432. Adjusted for the other risk factors, the p-value was 2×10^{-8}, beta 0.459. Women (n = 644) had lower titer levels than men (n = 216), median 1.78 and 2.16, respectively, $p = 2\times10^{-4}$, after adjusting for MS status and age at sampling.
Conclusions: MS cases have higher titers of anti-VZV IgG antibodies, seen by both analyzing titers as a transformed continuous and as a dichotomized variable. The association remains after adjustment for the strongest genetic MS risk factors, HLA-DRB1*15 and A*02, as well as adjusted for antibodies directed towards an EBV-related antigen, and we observed a gender difference. EBV and VZV antibody titers seem to correlate positively with MS, while others, such as CMV, show a negative correlation with MS. The role of antibodies against common viruses in MS pathogenesis is unknown, and needs to be studied further.

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IFN-g response against measles virus peptides in subacute sclerosing panencephalitis patients

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Objective: Subacute sclerosing panencephalitis (SSPE) is a rare, persistent slow virus infection of the central nervous system caused by measles virus (MV). Impaired immune response to measles infection may play a role in MV persistence and reactivation of the virus. To investigate antigen specific T cell response, we analyzed the IFN-γ secretion of peripheral blood mononuclear cells (PBMC) against selected MV peptides restricted to HLA-A2.

Methods: Patients with SSPE had clinical and laboratory findings supporting the diagnosis. IFN-γ secreting cells of 22 SSPE patients (11 girls and 11 boys, mean age: 12.0 ± 5.3 years) and 14 controls (CON) (5 girls and 9 boys, mean age: 11.4 ± 4.8 years) were counted against MV peptides in ELISPOT. HLA-A2 was present in 6/19 patients and 6/14 CON. PBMCs were stimulated with MV peptides from hemagglutinin (H30-38), matrix (M211-219), core (C84-92), nucleoprotein (N1 210–218 and N2 340–348) and with the pool of these peptides (MVp). A peptide pool (CEF) for HLA class I response and PHA were used as controls. The numbers of IFN-γ secreting cells between stimulated and un-stimulated cultures per $2x10^5$ were evaluated by non-parametric statistics.
Results: IFN-γ secreting cell numbers after stimulation with H and N1 peptides were significantly higher in SSPE patients than in CON (4 vs. 0, $p = 0.034$ and 2 vs. 0, $p = 0.028$, respectively). MVp has also stimulated higher number of IFN-γ secreting cells in SSPE patients without reaching statistical significance (111 vs. 92). However, IFN-γ response of SSPE patients to CEF peptides was lower than CON (12 vs. 48), these differences being not statistically significant either. The SSPE patients had similar numbers of $CD4^+$ cells in the PBMC, whereas $CD8^+$ T cells were lower than CON (28.3 vs. 40.5), but the difference was not significant.
Conclusions: Given the importance of $CD8^+$ T cells in clearing the virus, a difference in the immune response against MV peptides in patients may help to understand the pathogenesis of SSPE. The higher IFN-γ responses against H and N1 MV peptides in SSPE suggest that virus specific response is not impaired as the non-specific response to CEF peptides in SSPE. As the MV specific response was measured only by some HLA class I restricted peptides of the virus, these findings in individuals with different genetic backgrounds may only account for the part of the immune response in these children.

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Mechanisms of CNS disease

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Role of oligodendrocytes in excitotoxic axonal injury

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Background: Glutamate receptor overactivation (excitotoxicity) plays an important role in white matter injury in a wide range of neurological diseases, including multiple sclerosis. White matter elements, in particular oligodendrocytes are highly vulnerable to excitotoxicity, mediated through the activation of oligodendroglial AMPA/Kainate receptors. Axons are also vulnerable to glutamate toxicity, but the mechanisms leading to excitotoxic damage in axons are poorly understood. Recent studies suggest that glutamate receptors are functionally expressed on axons and that their activation may play a role in axonal injury. Interestingly, isolated axons in culture are resistant to glutamate toxicity and become vulnerable only when co-cultured with oligodendrocytes, suggesting that excitotoxic injury to axons may require interaction with oligodendrocytes.
Objectives: We examined the hypothesis, that excitotoxic axonal damage is transmitted through white matter oligodendrocytes.
Methods: In an *in vivo* approach, we made use of transgenic mice that express diphtheria toxin receptors under the control of the MOG promoter (MOGi-Cre/iDTR), also expressing yellow fluorescent protein (YFP) under control of the *thy1* promoter. Treatment with diphtheria toxin (0.4 μg/ml–1.1 μl) via intraspinal injections resulted in the local ablation of circa 95% of mature oligodendrocytes. After 9 days we injected a glutamate receptor agonist, AMPA (30 mM, 0.07 μl) into lumbar dorsal columns in DT- and vehicle-treated MOGi-Cre/iDTR mice. Axonal and neuronal morphology were examined in both groups after 24 h.
Results: We found that injection of AMPA into the lumbar dorsal columns induced similar axonal and neuronal degeneration in oligodendrocyte-depleted and the control mice.
Conclusions: In our *in vivo* model, axons are damaged by excitotoxicity independent of presence or absence of oligodendrocytes. This suggests that different mechanisms may apply to excitotoxic axonal damage *in vitro* and *in vivo*, e.g. axons *in vivo* but not *in vitro* may be directly susceptible to excitotoxicity. Elucidating the mechanisms for glutamate-mediated axonal injury may reveal new targets for neuroprotective therapy.

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Anti-CD20 therapy reduces microglial activation and lesion volume in focal models of pattern I and pattern II multiple sclerosis

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Objective: An important conceptual challenge concerning CD20 and MS is to discover whether CD20 targeting is effective in treating both pattern I and pattern II type MS-lesions. Pattern II lesions are associated with deposition of antibody and complement, but pattern I lesions are thought to be mediated principally by $CD4^+$ myelin-reactive Th1/Th17 cells and macrophages in a B-cell independent fashion. However, there is growing evidence to suggest that B cells play a role in antigen presentation in an antibody independent fashion. The aim of the present study was to determine whether anti-CD20 would inhibit lesion development and microglial activation in the focal (pattern I) Th1-type delayed type hypersensitivity (BCG-DTH) model and in an antibody-mediated focal (pattern II) fMOG-EAE of MS.
Methods: To induce pattern I MS lesions, Lewis rats were injected stereotaxically into the left striatum with heat-killed BCG (1 μl volume). Four weeks after the intrastriatal injection, the focal DTH lesion was initiated by an intradermal injection of BCG in CFA. To induce pattern II lesion, Lewis rats were injected subcutaneously at the base of the tail with 100 μl of MOG (35–55) peptide (25 μg) emulsified in IFA. To target the lesions animals were stereotaxically injected with a mixture of 1.45 μg TNF and 1 μg IFNg into the corpus callosum 21 days after MOG in a modification of our focal spinal EAE model. Animals ($n = 6$) received anti-CD20 (clone 5D2) at 5 mg/kg/iv on d1 and d7, and another set received vehicle ($n = 9$) or FTY720. The animals were killed on d12. Microglial activation as assessed by the binding of the TSPO ligand [^{125}I]-DPA-713.
Results: The anti-CD20-treated rats displayed significantly reduced lesion volume relative to the vehicle-treated rats in both the pattern I (65.7%, $p \leq 0.001$) and pattern II (56.8%, $p \leq 0.013$) models. In vivo SPECT imaging with [^{125}I]-DPA-713 revealed that binding was significantly reduced in the anti-CD20 treated rats. The anti-CD20 therapy reduced 68-IB3 + ve B cell in the spleen by 55% at day 12. Our results show that the anti-CD20 treatment was able to block the development of both an aggressive Th1 pattern I-type MS lesion, and the pattern II fMOG-EAE lesion.
Conclusions: The suppression B cell function by anti-CD20 in pattern I MS-like lesions, which hitherto would not have been considered B-cell dependent, suggests that B cells play an important role in lesion development that is independent of local CNS antibody production.

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Oligodendrocyte cell death in progressive multifocal leukoencephalopathy: Virus-induced rather than cytotoxic-T cell mediated

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Objective: Progressive multifocal leukoencephalopathy (PML) is a demyelinating and often fatal disease caused by infection of oligodendrocytes and astrocytes in human brain. Despite the severity of the disease, surprisingly little is known about the cause of oligodendrocyte cell death. Generally it is assumed that the infected oligodendrocytes are eliminated by cytotoxic T Cells. Nevertheless, at present there is no clear evidence that these cytotoxic T cells are actively engaged in elimination of infected cells. Here we analysed cytotoxic T cells and mechanisms of cell death in AIDS-associated PML.
Methods: This study was performed on paraffin-embedded, formalin-fixed AIDS-associated material. The samples consisted of 8 PML, 5 HSVE and 7 CMVE cases. We quantified CD3+, CD8+ and GrB+T cells and analysed the presence of single or multiple appositions of these cytotoxic T cells to infected brain cells. Furthermore we analysed caspase-dependent and independent cell death mechanisms of infected cells with immunohistochemical staining for caspase-3, or apoptosis inducing factor (AIF) and poly(ADP-ribose) (PAR).
Results: CD8+ and GrB+cytotoxic T cells were present in all encephalitides and we found no statistical difference in the absolute numbers of these cells. However in PML we found only 0,5% of all virus-infected cells in apposition to single GrB+T cells and, even less, only 0.02% of infected cells in apposition to multiple (2 or more) GrB+T cells. In HSVE we found 3,2% in apposition to single GrB+cells and 0,45% of the infected cells in apposition to multiple cytotoxic T cells. In CMV these percentages were 1,65% and 0,15% respectively. Also the death mechanisms differed between PML and the other encephalitides. In JC-infected cells we found that AIF translocated from the cytosol to the nucleus whereas PAR was seen to translocate to the cytoplasm. JC-infected cells did not show upregulation of activated caspase-3 in the cytoplasm. Activated caspase-3 however could be found in HSV and CMV-infected cells.
Conclusions: Our data suggest differences in cell death between PML and HSVE or CMVE. In the latter two, infected cells are seen to be attacked by GrB+T cells which induce capsase-3 mediated apoptosis. In PML however such T cell cytotoxicity was not detected. Instead, infected cells harbored nuclear presence of AIF and cytoplasmic localization of PAR suggestive of a cell death called parthanatos.

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The MAO inhibitor phenelzine improves motor and non-motor outcomes in experimental autoimmune encephalomyletis (EAE)

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Objective: Many multiple sclerosis (MS) symptoms can be related to changes in the levels of key neurotransmitters. These neurotransmitters have a direct role in the maintenance of healthy neurons and have immune modulatory properties. Previously we have shown daily treatment with the monoamine oxidase (MAO) inhibitor phenelzine (PLZ) caused substantial behavioural improvements, using the animal model experimental autoimmune encephalomyelitis (EAE). However, not all of the improvements were sustained. To determine whether modifying the treatment schedule of PLZ could better limit immune infiltration and sustain behavioural outcomes, PLZ treatment was given on alternating days.
Methods: EAE was induced in female C57/BL6 using of 50 μg of MOG_{35-55}. PLZ treatment (15 mg/kg) was started at the onset of symptoms and given either *daily* or on *alternating* days for 14 days. EAE mice were tested using the rotorod, an open field test and the novel object recognition assay (NOR). Spinal cord sections were analyzed with rat anti-CD4 and rat anti-Iba1. Human PBMCs activated in-vitro with anti-CD3 and PMA differentiated U937 cells were treated with PLZ and analyzed for IFN-γ and TNF-α. Brain and spinal cord homogenates were assayed for neurotransmitter levels using HPLC and assayed with ^{14}C radio-labeling MAO to determine MAO activity.
Results: Alternating PLZ treatment reduced the severity of EAE clinical signs, improved exploratory behaviours and reversed EAE-induced deficits in an assay of learning and memory, the novel object recognition test. In contrast to daily PLZ treatment providing PLZ, on alternating days lead to higher levels of GABA upon completion of the experiment. MAO enzyme activity showed that with alternating treatment there was less inhibition of MAO compared to mice treated with daily PLZ, accounting for the difference in GABA levels. To determine whether the PLZ had any direct influence on inflammation, human peripheral blood mononuclear cells (PBMCs) and U937 cells were treated in-vitro with PLZ. PBMCs treated with PLZ produced significantly less IFN-γ, which is not accounted for by increased cell death due to PLZ treatment.
Conclusions: These results indicate that providing alternating PLZ treatment is able to sustain GABA increases and better maintain positive behavioral outcomes compared to daily PLZ treatment. Furthermore, PLZ is able to directly modulate immune cell activity and demonstrates another possible mechanism for the action of PLZ in EAE.

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Reduced hippocampal NOS activity and behavioral changes in restraint stressed rats

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Chronic stress leads to activation of the hypothalamo–pituitary–adrenal axis (HPA) and changes in different parameters in limbic areas associated with stress. The hippocampus is one of the limbic brain structures implicated in the pathophysiology of mood disorders and it has been demonstrated that nitric oxide (NO), a free radical produced by nitric oxide syntase (NOS), participates in these processes.

The aim of the present work was to study the effects of chronic restraint stress on behavior and the participation of hippocampal NO. Restraint stress was applied to adult Sprague–Dawley male rats 2 h daily for 7 consecutive days. Inhibitory avoidance and elevated plus maze tests, oxytocin and corticosterone plasma levels, constitutive NOS isoforms (eNOS and nNOS) mRNA expression and total NOS activity in hippocampus were evaluated.

A significant decrease ($p<0.001$) in the activity of hippocampal total NOS was found in stressed animals during 7 days respect to control

animals. The expression of eNOS mRNA was significantly reduced ($p<0.05$) after 7 days of stress however no changes on nNOS mRNA were found. Oxytocin and corticosterone plasma levels were significantly increased ($p<0.01$ and $p<0.05$ respectively) in stressed rats. Moreover, we found a significant decrease ($p<0.05$) in the latency to enter into the dark compartment in the inhibitory avoidance test and a significant increase ($p<0.05$) in the percentage of time spent in the open arms in the elevated plus maze test after 7 days of chronic restraint stress respect to control animals.

In conclusion, in our model of stress the increase in corticosterone levels could account for the decrease in NOS activity and eNOS mRNA expression in hippocampus. Also, the increase in oxytocin levels elicited by stress could be related to the deficit in associative memory and an anxiolytic-like behavior observed in this stress model. (PICT-06-0258; PIP 02546 CONICET).

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Nkx2.2 transcription factor promotes expression of genes involved in myelin production in murine central nervous system

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Objective: Previous studies suggest that remyelination of demyelinated lesions in patients with multiple sclerosis (MS) frequently fails in the process of oligodendrocyte progenitor cell (OPC) differentiation. The OPC differentiation and the formation of myelin by mature oligodendrocytes are orchestrated by transcription factors. The essential role of the Nkx2.2 transcription factor in OPC differentiation has been clearly demonstrated in perinatal Nkx2.2 deficient mice suffering from severely impaired oligodendrocyte formation. However, only three Nkx2.2 target genes, Ermn, Mbp, and Plp1, have so far been identified in oligodendrocyte-lineage cells.
Methods: To further elucidate the function of Nkx2.2 during oligodendrocyte formation, we have investigated the mRNA profile in the brainstem and rostral spinal cord of postnatal Nkx2.2$^{-/-}$ and wild type mice by mRNA expression microarray. The oligodendrocyte expression of the differently expressed, putative Nkx2.2 target genes, were confirmed by *in situ* mRNA detection combined with MBP-immunostaining on *in vivo* material.
Results: The mRNA profiling discovered 29 genes, including the known Nkx2.2 target genes Mbp, Plp1, and Sirt2, which were significantly differently expressed in Nkx2.2$^{-/-}$ and wild type mice. Most of them contained a predicted Nkx2.2 binding site in their cis-regulatory element, and we considered them as potential Nkx2.2 target genes. All the potential Nkx2.2 target genes were down-regulated in Nkx2.2$^{-/-}$ mice, and 22 of them encoded for proteins expressed by oligodendrocyte-lineage cells and most with known or possible roles in myelination indicating that Nkx2.2 activates the expression of several genes involved in the myelination of developing CNS. Higher NG2 and PDGFαR mRNA levels and an apparently higher number of NG2^{+} and PDGFαR^{+} OPC in Nkx2.2$^{-/-}$ mice suggested that Nkx2.2 is important for the terminal oligodendrocyte differentiation and not for cell fate specification of progenitor cells in developing murine CNS. We also found that several of the potential Nkx2.2 target genes were up-regulated in MBP^{+} oligodendrocytes in the corpus callosum of adult mice during cuprizone-induced remyelination, thereby suggesting that Nkx2.2 may have similar role during remyelination.
Conclusions: We currently investigate whether the identified genes are real Nkx2.2 target genes by performing immunoprecipitation and subsequent sequencing of the Nkx2.2 binding chromatin from OPC and *in vitro* differentiated oligodendrocytes. Identification of the Nkx2.2 target genes may be relevant to develop new ways to promote remyelination in MS.

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Consistent patterns of clinical and laboratory findings in patients with co-existent autoimmune thyroid disease and autoimmune demyelinating disease in the central nervous system

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Objective: Preliminary observations suggested that there were many common features relating to disease symptoms and signs among patients who had a combination of autoimmune thyroid disease (AITD) and autoimmune demyelination in the CNS, either multiple sclerosis (MS) or neuromyelitis optica (NMO). The aim of the current study was to systematically compare the clinical and laboratory features of patients with co-existent AITD and MS/NMO with those of patients with MS alone.
Methods: Thirty-five patients with co-existent autoimmune CNS demyelinating disease (either MS ($n=31$) or NMO ($n=4$)) and AITD were identified and their clinical and radiological features were recorded. Only individuals who had developed AITD prior to the use of IFN-β were included in this study. Blood and DNA were collected and tested for HLA type, specificity of T cells and antibodies, and polymorphisms in CTLA-4 genes. Patients with MS alone, healthy individuals, and individuals with AITD alone were included as controls.
Results: Patients with co-existent AITD and MS/NMO are almost exclusively female (34/35) and have prominent spinal cord involvement. They are also more likely to carry HLA molecules DQB1*0501 and DPB1*0401 and to show elevated levels of reactivity to peptides of LGR4, a homologue of the thyroid-stimulating hormone receptor that is found in the spinal cord, compared to the other groups of individuals.
Conclusions: The consistent patterns in the subgroup of patients with co-existent AITD and MS/NMO suggests that combinations of autoimmune diseases may arise from common pathogenic mechanisms. Cross reactivity of autoreactive immune cells in these patients with antigens present in both the thyroid and the spinal cord might be a possible mechanism underlying the pattern of demyelinating lesions in the CNS.

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Interleukin-6 class cytokines regulate the development of inflammatory CNS lesions

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Objective: The interleukin-6 class cytokines leukemia inhibitory factor (LIF) and oncostatin M (OSM) are upregulated in multiple sclerosis (MS) lesions, but their effects on CNS lesion development are far

from understood. LIF and OSM potentially influence immune responses as well as CNS resident cells during neuroinflammatory conditions. Importantly, since they activate different receptors, LIF and OSM can influence these parameters to different extents. This study was designed to elucidate the role of OSM and LIF in MS lesion development.
Methods: Stereotactic application of lentiviral vectors was performed to achieve a stable expression and secretion of LIF or OSM in the CNS of adult mice. Experimental autoimmune encephalomyelitis (EAE) was induced in C57BL6J mice with MOG-peptide. Receptor expression on human immune cell subsets was determined by flow cytometry. Primary cultures of oligodendrocytes and macrophages were used to study direct effects on these cell types. Immunohistochemistry and real time PCR were applied to elucidate the in vivo effects of local cytokine expression.
Results: Our previous study showed that gene therapeutic expression of LIF in the CNS significantly reduced immune-mediated demyelination. Our *in vitro* studies show that LIF protects oligodendrocytes from inflammatory insults. Now, we demonstrated that even treatment after disease onset ameliorates EAE symptoms. To define which immune cells play a crucial role in the immunomodulatory actions of LIF, expression of the LIF receptor was determined on human immune cells. Monocytes express the LIF receptor subunits at high levels, while there is only a limited population of T cells and B cells that express the receptor. LIF reduces production of toxic mediators by macrophages. In contrast to LIF, local expression of OSM did not simply alleviate symptoms but suppressed the incidence of EAE. While we did find a local inflammatory response at the site of OSM expression, infiltration of typical EAE-related immune cells in the CNS was blocked. CNS-targeted OSM production was necessary, since systemic administration of the cytokine did not affect development of autoimmune induced CNS lesions.
Conclusions: Our study demonstrates that OSM and LIF play an important but distinct role in lesion development in neuroinflammatory disease. These cytokines and their downstream signalling molecules are potential candidates for therapy.

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Expressional regulation of Na^+, K^+-ATPases in the mouse brain and the association to neurological disorders

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Objective: The Na^+, K^+-ATPases drive the electrochemical gradient across the plasma membrane that is essential for all mammalian cells and use approximately ¾ of the energy available to the CNS. Consequently, dysregulated pump activity is associated with a number of CNS diseases. Mutations in specific Na^+, K^+-ATPase alpha isoforms are associated with Familial Hemiplegic Migraine type 2 (FHM2) and Rapid onset of Dystonia Parkinson (RDP). Furthermore, reduced activity has been shown in the CNS of patients with Alzheimer's disease as well as in several rodent stress and depression models.

The core ATPase heterodimer consists of an alpha (α) and a beta (β) subunit. The α subunit contains catalytic activity and the β subunit is mainly responsible for chaperoning the heterodimer to the plasma membrane. A third type of subunit may interact with the $\alpha\beta$ subunits, in a cell- and development-specific manner. This influences the enzyme kinetics by modifying e.g. affinities towards K^+, Na^+ and ATP.

Mammals express 4 alpha isoforms (α_{1-4}) of which α_{1-3} have been identified in the CNS. Despite structural similarity, each α isoform has distinct properties and display different kinetic properties towards Na^+ and K^+, the substrate ATP, and the inhibitors Ca^{2+} and ouabain. In the CNS, the α_1 isoform is expressed in most cell types whereas the α_2 isoform is expressed mainly in astrocytes and is important for e.g. neurotransmitter clearance. The α_3 isoform has an important role in action potential formation and is expressed in neurons. These subtle differences mean that a reduction in the functionality of one isoform may not be rescued by increased activity of another isoform. Consequently, homozygous α_{1-3} knockout mice are nonviable.
Methods: In order to understand the interplay between the Na^+, K^+-ATPase subunits in more details, we are mapping the expression levels of Na^+, K^+-ATPase subunits in various brain regions of C57BL/6J mice using qPCR at different stages in development.
Results: Preliminary results show that the expression of Na^+, K^+-ATPase subunits is regulated during development.
Conclusions: The correlation between dysregulated pump activity and CNS diseases suggest that a better knowledge of the regulation of the ATPase subunits will provide the basis for a better understanding of the etiology and progression of CNS diseases.

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Changes and functions of chondroitin sulfate proteoglycans following focal demyelination and remyelination

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Objective: Central nervous system demyelination results in marked changes in the composition of lesion associated extracellular matrix components. We recently reported that chondroitin sulfate proteoglycans (CSPGs) are upregulated in the murine lysolecithin demyelination model (Lau et al., Annals Neurol, in press). In vitro, CSPGs reduce the adherence and process outgrowth of cultured oligodendrocyte precursor cells (OPCs), the requisite cells for myelin repair. The current study is aimed at understanding the contribution of individual CSPG members in demyelinating conditions.
Methods: Lysolecithin was injected into the T3/T4 spinal cord dorsal column of C57BL/6 mice, producing a demyelinated lesion that undergoes endogenous repair over several weeks. Injured and control mice were tested for functional deficits though beam walking. In vitro, primary OPC cultures were seeded onto various CSPG substrates. Cell adhesion and process outgrowth, necessary steps for myelin regeneration, were quantified.
Results: Animals showed a persistent functional deficit following lysolecithin injection with partial recovery over 4 weeks. The CSPGs aggrecan and neurocan were unaltered from controls in the dorsal column. Interestingly, there were opposite expression patterns of two splice variants of the CSPG versican, with V2 predominating in early "demyelinating" time points, and V1 predominating in later "remyelinating" time points. Iba1+ macrophages/microglia were present at early and late time points, and associate with areas of versican staining. In culture, a CSPG mixture purified from biological sources consisting of aggrecan, versicans, neurocan and phosphacan collectively inhibited OPC adherence and process extension. Despite playing a minor role in the dorsal column, aggrecan and neurocan show antagonistic effects on OPCs, with neurocan surprisingly acting as a permissive substrate for adherence and process extension while aggrecan is inhibitory. We are currently purifying V1 and V2 isoforms from bovine CNS to determine how these variants will interact with OPCs in culture.
Conclusions: A complicated relationship of versican isoform expression takes place following lysolecithin injury. The nature of CSPGs

that accumulates in demyelinating injury affects remyelination; understanding their dynamics may allow the shift of the extracellular matrix to a composition that is more conducive for myelin repair.

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Interleukin-1 beta: A driver of the lesion formation in neuromyelitis optica?

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Objective: Neuromyelitis optica (NMO) is a severe demyelinating, inflammatory disease of the central nervous system (CNS). Since the presence of NMO-specific antibodies (NMO-IgG) in the serum is not sufficient to trigger astrocyte-destructive lesions, we investigated inflammatory mechanisms potentially involved in this process. More specifically, we analyzed the role of pro-inflammatory cytokines in the formation of NMO-like lesions in the CNS of Lewis rats.
Methods: Cytokines were injected into the striatum of wild-type Lewis rats, along with i.p injections of patient-derived NMO-IgG or control human IgG. The animals were then sacrificed, and the brain tissue was further processed for immunohistochemical analysis. The effects of those cytokines *in vitro* on the rat brain endothelial cells (a crucial point for recruitment of immune cells and serum components into the CNS parenchyma) were tested at RNA level by semi-quantitative PCR analyses, and at protein level by immunocytochemical and antibody array analyses. Additionally, human CNS tissue of various pathology was tested for the presence of interleukin-1 (IL-1β) by immunohistochemistry.
Results: Among the different cytokines tested, only IL-1β induced the formation of perivascular lesions with aquaporin 4 (AQP4) loss and varying degree of astrocyte destruction and complement deposition, when NMO-IgG was present in the peripheral circulation. Those NMO-like lesions were also rich in granulocytic infiltrates and activated microglia/macrophages. Furthermore, when endothelial cells were challenged with IL-1β, profound up-regulation of expression of molecules involved in granulocyte differentiation, survival and recruitment was observed. Finally, immunohistochemical analysis revealed the presence of IL-1β reactivity in granulocyte-rich lesions of NMO patients.
Conclusions: IL-1β, which is in the CNS produced mainly by activated microglia cells, is able to provide inflammatory conditions that lead to breakdown of the blood–brain barrier and to granulocyte-mediated formation of NMO lesions in the CNS.

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Cerebrospinal fluid cytokine levels in narcolepsy with cataplexy patients close to onset

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Objective: Narcolepsy with cataplexy (NC) is caused by a loss of hypothalamic hypocretin producing neurons. Autoimmunity due to environmental triggers e.g. infections is considered the most likely pathogenesis, recently highlighted by increased numbers of NC cases after H1N1-vaccination/infection.
We here report an analysis of cerebrospinal fluid (CSF) cytokine levels in NC patients close to onset.
Methods: CSF samples were collected in a clinical setting when the patient had the first sleep examination. CSF cytokine levels from 9 patients and 9 controls were analyzed using a 51-plex Luminex platform. The dataset was tested using SAxCyB (Significance Analysis of xMap Cytokine Beads) statistics. Relevant hits were then tested using ELISA in a larger independent cohort (30 patients, and 15 age matched controls). The patients were all within 9 months of cataplexy onset, 100% were DQB1*06:02 positive, and all had CSF hypocretin <110 pg/ml. The controls were matched for age, 53% were DQB1*06:02 positive, and all had CSF hypocretin >110 pg/ml.
Results: Our Luminex data showed a significant increase in CSF IL-1B, CCL2/MCP-1, CCL4/MIP-1B, leptin, LIF, and PAI-1 in NC patients close to disease onset. No tested cytokines were significantly decreased. We further saw that CCL5/RANTES was significantly increased in the NC patients closest to onset of the disease. We confirmed the finding of increased CCL4 in CSF from patients close to onset, but while CCL5 was indeed high in a subgroup of patients, there was no significant difference from the control group.
Conclusions: Here we show that CCL4 is significantly increased in CSF from NC patients close to onset. CCL4/MIP-1B is a strong chemotaxic factor for lymphocytes, and so is CCL2 and CCL5. This suggests an immune mediated pathogenesis in NC patients. We further show that the general marker of inflammation IL-1B is higher. Leptin, LIF, and PAI-1 are all involved in metabolism and obesity, and levels of all three correlate with body mass index. Leptin has previously been shown to be increased in CSF from NC patients. We suggest that the changes in these are secondary to the metabolic disturbances in NC. Overall our finding shows an increase in inflammatory markers in CSF close to narcolepsy with cataplexy onset.

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Role of microglial/macrophage IKKbeta in experimental autoimmune encephalomyelitis

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Multiple sclerosis (MS) is the most common inflammatory demyelinating disease in the Central Nervous System (CNS). Studies of gene-targeted deficient and transgenic mice have unraveled the function of peripheral immune system and of multiple chemokines and cytokines in pathology of Experimental Autoimmune Encephalomyelits (EAE)[1,3], a well established experimental model for autoimmune demyelination in CNS. Nuclear factor (NF-) kappaB (NF-κB) is a ubiquitously expressed transcription factor that is involved in many inflammatory processes. NF-κB activation induces the expression of many cytokines, chemokines and adhesion molecules that mediate the recruitment and activation of immune cells, but also protects cells from cytokine-induced death by inducing the expression of antiapoptotic proteins[4]. Upon stimulation, NF-κB is activated by IkB kinase (IKK) complex, in a manner dependent mainly on the IKKβ subunit. It was previously documented that besides T-cell proliferation and activation, NF-κB plays a crucial role in coordinating the expression of a wide variety of genes involved in monocyte/macrophage activation. In

particular, the macrophage-derived pro-inflammatory cytokines IL-1β and TNF-α are potent activators of NF-κB, which in turn controls their expression resulting in a positive feedback loop. Hence, NF-κB signaling pathways may play a pivotal role in activating myeloid cell function during autoimmune inflammation. In our study, we investigate the role of NF-κB in myeloid cells during myelin-oligodendrocyte-glycoprotein induced experimental autoimmune encephalomyelitis (MOG-EAE). For that reason, we use conditional knockout mice for IKKβ in myeloid cells (Mac1CreIKKβF/F mice)[5,6,7]. These mice display a constitutive deletion of NF-κB proteins in cells of myeloid origin including macrophages and microglia. Our results demonstrate that NF-κB-dependent pro-inflammatory gene expression in myeloid cells is important for CNS pathology in MOG-EAE. Furthermore, the Mac1CreIKKβF/F mice are being examined for their response to an in vivo model of excitotoxic brain injury induced by kainic acid in order to study specifically the role of the microglial IKKβ in excitotoxin-induced neuronal cell death.

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The influence of interleukin-22 receptor alpha 2 on experimental autoimmune encephalomyelitis

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Objective: The Interleukin-22 receptor alpha 2 (IL22RA2) gene has been strongly implicated as a multiple sclerosis (MS) risk gene. The primary objective of this study is to experimentally confirm the involvement of this gene in MS-like pathogenesis and secondly to gain more knowledge about the nature of its influence on neuroinflammation.
Methods: Myelin oligodendrocyte glycoprotein (MOG) induced experimental autoimmune encephalomyelitis (EAE), in both mice and rats, was used as a disease model for MS. *IL22ra2*$^{-/-}$-mice were used in the study as well as a congenic rat strain with a chromosomal fragment that includes a protective natural variant of *IL22ra2* bred onto a susceptible genetic background. *IL22ra2* gene expression was studied by qPCR. The immunopathology was characterized by flow cytometry, histology, qPCR and ELISA. Furthermore, gene expression in MS patient peripheral blood mononuclear cells has been correlated to presence of *IL22RA2* risk allele.
Results: We show that *IL22ra2* gene expression in rodents is primarily confined to secondary lymphoid tissue, the intestines and skin. *IL22ra2* gene expression in draining lymph nodes is highest in the naïve state and quickly is reduced upon immunization with MOG.

In studies of secondary lymphoid tissue from the knockout mice we show a significantly higher level of proliferation in several immune cell types compared to wild type controls.

The EAE disease course is distinctly different in *IL22ra2*$^{-/-}$ mice compared to controls. The knockout mice consistently get a slightly earlier disease peak but then recover significantly faster than control mice.
Conclusions: IL22ra2 is involved in the pathogenesis of EAE. A possible mechanism relies on the modulation of proliferation and activation of different cell types.

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Dynamics and roles of monocytoid cells in an animal model of MS

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Monocytoid cells (monocytes, macrophages and microglia) are important in the pathogenesis of multiple sclerosis (MS) and its animal model experimental autoimmune encephalomyelitis (EAE). The excessive accumulation of monocytoid cells in the CNS is thought to contribute to the neuropathology of MS. Despite this, much remains unknown about when pro-inflammatory monocytes are generated during disease course, the dynamics of their accumulation in the circulation, and the mechanisms by which they penetrate the CNS to become macrophages. We have used the EAE model to address these issues. In mice immunized for EAE with a peptide of myelin oligodendrocyte glycoprotein (MOG), CD11b + CCR2 + Ly6C^{high} pro-inflammatory monocytes increase and accumulate in blood prior to the development of clinical signs. Indeed, their accumulation in blood occurred by the second day of MOG immunization, and was not observed in mice with control immunizations. At onset of clinical signs, these cells enter into the CNS correspondent with a drop of counts in blood. Immunohistochemistry for Iba1 and flow cytometry (CD45$^{hi/low}$CD11b+) indicated the elevation of activated macrophage/microglia in EAE spinal cord. The transmigration of pro-inflammatory monocytes is correspondent with their increased levels of matrix metalloproteinase (MMP)-9, their increased capacity to traverse a fibronectin-coated barrier, and is correlated with CD62L expression. Of interest, the emerging MS medication, laquinimod, reduced the migration of pro-inflammatory monocytes into the CNS (Mishra et al., Am J Pathol, in press). To address the impact of pro-inflammatory monocytes on neuronal health when they enter the CNS, we have resorted to tissue culture studies by polarizing bone marrow derived macrophages to pro-inflammatory M1 or regulatory/anti-inflammatory M2 phenotypes. M1 cells expressed iNOS, TNF-α and IL-12, while M2 cells were characterized by ariginase-1, YM-1, IL-5 and IL10. Ongoing experiments seek to determine the capacity of these subtypes to differentially regulate neuronal survival.

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The role of chemokine receptors CCR6/CCR4 in the pathogenesis of experimental autoimmune encephalomyelitis

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Purpose: Chemokines play an important role in immune regulation by controlling leukocyte migration and tissue infiltration. Thus, chemokine receptors (CCRs) have been identified as therapeutic targets. Further, treatment for T-cell leukemia/lymphoma in adults, using anti-CCR4 antibody has been initiated. Th17 and Th1 cells play important roles in the pathogenesis of experimental autoimmune encephalomyelitis (EAE), an animal model of multiple sclerosis. CCR6 is expressed only on Th17 cells, whereas CCR4 is expressed on both Th17 and Th2 cells. Together, these CCRs have modified the pathogenesis of EAE by disruption of the blood–brain barrier, which promotes immigration of pathogenic T cells and cytokine balance. Here, we analyzed the roles of CCR6 and CCR4 in EAE, using knockout mice.

Method: CCR6 and CCR4 double-deficient mice (DKO) were created by crossing CCR6-deficient mice (CCR6-KO) and CCR4-deficient mice (CCR4-KO). These mice were backcrossed to C57BL/6 mice for more than eight generations. After creating the required mice models, EAE was induced by active immunization of DKO (n = 18), CCR6-KO (n = 17), CCR4-KO (n = 22), and wild-type mice (Wt; n = 27) with MOG (35–55). The disease severity was evaluated by clinical symptoms (EAE score) and pathological findings. Recall response of MOG-reactive T cells was evaluated by cell proliferation (uptake of [3H]) and cytokine production in the culture supernatant. Cell surface marker of infiltrated lymphocytes in the brain was analyzed using fluorescence-activated cell sorting (FACS).
Results: There were no significant changes in the appearance and growth of DKO and Wt. EAE score in DKO was significantly lower than that in Wt. Furthermore, EAE score was milder in DKO than that in CCR6-KO and CCR4-KO. The EAE cumulative scores were DKO: CCR6-KO:CCR4-KO:Wt = 23.0:29.9:34.1:43.5 ($P<0.05$). There was no difference in the incidence rate and date of onset. In the pathological findings, inflammatory cell infiltration in DKO was the mildest. The recall response with higher IL-17 production was observed in DKO than in Wt. No significant differences were observed in the production of other cytokines, including IL-2, IL-4, IL-6, IFN-γ, TNF-α, and IL-10 among the groups. The number of infiltrated CD4+ lymphocytes in the central nervous system in DKO was lower than that in Wt.
Conclusion: CCR4 and CCR6 play important roles in disease aggravation in EAE, and these CCRs could be considered as new therapeutic targets.

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Acute mechanisms underlying antibody effects in anti-NMDA receptor encephalitis

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Objective: Patients with anti-NMDAR encephalitis, characterized by autoantibodies against the NR1 subunit of the NMDA receptor in cerebrospinal fluid (CSF), present with severe neurological and psychiatric symptoms that often improve as antibody titer is reduced. Previous studies showed that treatment of cultured hippocampal neurons with patient CSF causes a specific decrease in the synaptic localization and function of NMDA receptors (Hughes et al., 2010). We have now extended these studies to further understand the detailed mechanisms and time course of antibody-mediated pathogenesis, and to develop an animal model of the disease to further our understanding of the functional effects of patient antibodies in vivo.
Methods: Dissociated hippocampal neuron cultures were used for immunocytochemical, physiological, and molecular studies of the effects of patient antibodies. In addition, we have developed an animal model of anti-NMDAR encephalitis by infusing patient antibodies into mice hippocampi. We are currently performing behavioral analyses and electrophysiological recordings from hippocampal slices from these animals to monitor the effects of patient antibodies on plasticity and excitatory-inhibitory balance.
Results: Patient antibodies rapidly increase the internalization rate of surface NMDAR clusters, independent of the activity of the NMDARs themselves. NMDAR internalization accounts for the decrease in NMDAR-mediated currents, as we could detect no evidence of acute functional NMDAR blockade. Internalized, antibody-bound NMDAR clusters traffic through early and recycling endosomes, as well as lysosomes. Depletion of IgG from patient CSF eliminates staining of surface NMDAR clusters and abrogates their loss. Interestingly, we find that anti-NMDAR antibodies do not induce measurable homeostatic plasticity, either at the level of NMDAR insertion into synapses or receptor gene transcription.
Conclusions: Together, these data suggest an antibody-mediated mechanism of disease pathogenesis in this form of autoimmune synaptopathy. Antibody-mediated down-regulation of surface NMDARs is time-dependent and activity-independent, and engages multiple trafficking pathways. This NMDAR downregulation does not stimulate homeostatic synaptic plasticity mechanisms, which may further contribute to disease pathogenesis and influence the time course of patient recovery.

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Fetal microchimerism and the response to ischemic injury in the maternal mouse brain

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Objective: Clinical and experimental data have shown that stroke is a sexually dimorphic disease. Females, in particular, have a varying degree of ischemic sensitivity across the lifespan. The inflammatory response to stroke has been recognized as a major contributor to tissue injury. A growing body of work suggests that fetal progenitor cells persist in the blood of parous females for years and aggregate at sites of tissue injury, conferring protection in models of myocardial ischemia. Whether this occurs in the injured brain or contributes to stroke sensitivity is unknown. We hypothesize that fetal progenitors are actively recruited to the maternal brain as part of the inflammatory response to ischemic stroke.
Methods: Wildtype C57Bl/6 female mice were bred with $GFP^{+/+}$ transgenic male mice to yield $GFP^{+/-}$ offspring. Parous females (10–12 weeks old) were subject to 90 min right middle cerebral artery occlusion 4 weeks after delivery followed by either 24- or 72 h of reperfusion. The blood and brain of each mouse was examined using immunohistochemistry and flow cytometry and compared to GFP-negative controls.
Results: Preliminary data revealed the presence of GFP^+ cells in the blood and brain of parous mice, increasing significantly in number at 24- and 72 h post-reperfusion, respectively. Further characterization of GFP^+ cells after stroke using flow analysis revealed a subpopulation of $CD45^{hi}CD3^-CD19^-CD11b^+$ cells of myeloid origin in the blood and ischemic brain compared to sham. The ischemic hemisphere contained clusters of GFP^+ cells located adjacent to blood vessels and more sparsely within the parenchyma. Immunohistochemical analysis at various locations along anterior-posterior axis within the brain show GFP^+ cells increasing with larger areas of infarct tissue damage as evidenced by cresyl violet staining.
Conclusions: Fetal progenitor cells are highly responsive to ischemic brain injury in parous females. At 72 h post-reperfusion, the number of GFP^+ cells of fetal origin in the maternal brain correlates with infarct size and is comprised of $CD11b^+$ peripheral myeloid cells suggesting direct involvement in mediating the cascade of inflammatory events following stroke injury. Although the precise contribution of these cells is unknown, further studies in this area may enhance our understanding of the variant female response to stroke with age.

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Dopamine receptor D5 expressed on dendritic cells promotes CD4+ T-cell-mediated autoimmunity

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Objective: Dendritic cells (DCs) are responsible for priming T-cells and for promoting their differentiation from naïve T-cells into appropriate effector cells. Emerging evidence points toward neurotransmitters not only mediating interactions into the nervous system, but also contributing to the modulation of immunity. Accordingly, we have analyzed the role of dopamine in the regulation of DCs function in the development of an autoimmune disease.
Methods: Bone marrow-derived DCs were obtained from wild-type or D5RKO C57BL/6 mice. Dopamine receptors (DARs) expression was analyzed by FACS. DCs were co-cultured with purified $CD4^+$ T-cells to evaluate activation and proliferation. Experimental Autoimmune Encephalomyelitis (EAE) was used as autoimmunity model. Clinical score was measure daily and phenotype of $CD4^+$ T-cell that infiltrate CNS was evaluated by flow cytometry. Animal work was performed according to institutional guidelines. Results shown are from three independent experiments and statistical significance was determined by unpaired Student's *t*-test or Mann–Whitney *U*-test.
Results: Our results show that DCs express the machinery necessary to synthesize and to store dopamine. They also express dopamine receptors D1 (D1R), D2R, D3R and D5R, but only D5R is significantly down-regulated after LPS-induced maturation. In vitro experiments indicate that lack of D5R in DCs impairs LPS-induced IL-12 secretion and consequently attenuates activation and proliferation of antigen-specific $CD4^+$ T-cells in co-culture experiments. To determine the relevance of D5R expressed on DCs in vivo, we studied the role of this receptor in the modulation of a $CD4^+$ T-cell-driven autoimmunity. Importantly, D5R-deficient DCs prophylactically transferred to a WT recipient were able to reduce the severity of Experimental Autoimmune Encephalomyelitis (EAE). Furthermore, we examined the phenotype of the $CD4^+$ T-cells infiltrated into the central nervous system during the peak of the disease. Mice transferred with D5R-deficient DCs showed a significant reduction in the percentage of infiltrating Th17 cells without differences in the percentage of Th1 cells when compared to animals transferred with wild-type DCs.
Conclusions: Our findings demonstrate that D5R expressed on DCs, by contributing to $CD4^+$ T-cell activation and differentiation to Th17 phenotype, is able to modulate the development of an autoimmune response in vivo.

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Preconditioning of microglia with Parkinson-linked alpha-synuclein affects the response induced by Toll-like receptor (TLR) stimulation

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Objective: Parkinson's Disease (PD) is characterized by the intraneuronal accumulation of aggregated α-synuclein in the Lewy Bodies. However, aberrant extracellular α-synuclein species have also been found in PD brains. In the last few years, the ability of mutant α-synuclein (found in familiar PD) to trigger microglia-mediated neuroinflammation has been recognized and linked to the robust inflammation observed in affected areas of the brain of PD patients. However, the molecular mechanisms that initiate and drive the development of sporadic PD remain to be elucidated. Intriguingly, several epidemiological and animal model studies have revealed an association between certain microbial infection events and the triggering or progression of sporadic PD. In this work, we sought to study the effect on the innate immunity of TLR stimulation on wild-type (Wt) α-synuclein-preconditioned microglia, modelling an underlying non-familiar PD-prone scenario.
Methods: Primary murine microglial cultures that had been preconditioned with extracellular Wt α-synuclein, were stimulated with a variety of TLR agonists. After incubation, several key interleukin and chemokine protein secretion levels were assayed. In addition, the change in the TLR expression levels was assayed by qRT-PCR and the impact of the different treatments on the phagocytic capacity of treated microglial cells, were performed. Finally, apoptosis/necrosis signalling pathway markers were measured by ELISA and analysed by immunofluorescence (IF).
Results: We herein report that certain stimuli can largely affect the TLR-mediated responses in the case of α-synuclein-preconditioning of microglia, either by altering certain chemokine secretion, or by substantially increasing or reducing pro-inflammatory or anti-inflammatory interleukins secretion levels, respectively. Furthermore, we show that a key apoptosis/necrosis signalling pathway protein is significantly augmented in α-synuclein-preconditioned microglia upon stimulation with particular ligands.
Conclusions: We conclude that certain TLR stimulation events can largely and differentially affect the innate immune response, as well as downstream cellular events, in microglia that had been previously exposed to Wt α-synuclein. Overall, we pose that our results might shed light onto the elusive link between bacterial infections and the initiation of sporadic PD and possibly of other related neurodegenerative disorders.

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Novel dominant and subdominant T cell epitopes of the CNS autoantigen, myelin oligodendrocyte glycoprotein, in mice and MS patients

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Objective: Amino acid (aa) residues 35–55 within the extracellular domain 1–125 of the autoantigen myelin oligodendrocyte glycoprotein (MOG) contain its only known encephalitogenic T cell determinant in C57BL/6 ($H\text{-}2^b$) mice. MOG 35–55 is also recognized by T cells in multiple sclerosis (MS) patients. In this study we examined T cell recognition of full length MOG 1–218, which includes its transmembrane and cytoplasmic domains.
Methods: Overlapping peptides encompassing the entire sequence of full-length MOG were tested for their capability to elicit proliferative

T cell responses and induce EAE in C57BL/6 (H-2^b) mice. Peripheral blood mononuclear cells from MS patients and healthy control (HC) were examined for the recognition of MOG peptides.
Results: Three novel T cell epitopes were identified in mice, an encephalitogenic determinant within transmembrane domain residues 119–132, and two nonencephalitogenic determinants, aa's 181–195 and 186–200, within the cytoplasmic domain. Clinical EAE, CNS inflammation and demyelination induced by p119–132 were as potent in severity as with p35–55. MOG 119–132-specific T cells were restricted by MHC II (I-A^b) molecules, produced Th1 and Th17 cytokines, and transferred EAE to naïve recipient mice. MOG p119–132 induced EAE in other H-2^b mouse strains, but not in mice of different haplotypes. Novel determinants, MOG 181–195 and 186–200, did not induce EAE by immunization or T cell adoptive transfer. Frequency of T cells that responded to p119-132 after immunization with full-length MOG was greater than to p35–55 or the non-encephalitogenic determinants, demonstrating that p119–132, not p35–55, is the immunodominant determinant of MOG. T cells from MS patients exhibited robust responses to 119–130 and 181–195, demonstrating that MS patients recognize epitopes in the transmembrane and cytoplasmic domains.
Conclusions: (1) Native MOG is a multideterminant T cell autoantigen in C57BL/6 mice that contains two nonencephalitogenic and two encephalitogenic determinants. (2) MOG 119–132, but not 35–55, is the immunodominant encephalitogenic T cell epitope. (3) Like mice, MS patients recognize epitopes in the transmembrane and cytoplasmic domains of MOG, which may be relevant to MS pathogenesis and development of myelin-specific therapeutics.

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Microglia/macrophage polarization to M2 phenotype is influenced by CD200R expression in the developing C57BL/6 mice brain following hypoxia/ischemia

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Recent works demonstrate that microglia are kept in a quiescent state in the intact central nervous system (CNS) by local interactions between the microglia receptor CD200 and its ligand, which is expressed on neurons. Injury to neurons stimulates nearby microglia whose activation is the first step in the protection and repair of damaged CNS tissues. However, the developing brain exhibits distinct morphological as well as physiological characteristics determining a peculiar response to injury showing an aggravated susceptibility to excitotoxicity and pro-inflammatory cytokines, along with an exacerbated inflammatory response. Hence, the aim of this study was to characterize the expression pattern of CD200-CD200R in the developing mice brain following Hypoxic/Ischemic (H/I) injury by immunofluorescence. Wild-type C57/BL6 mice postnatal day 7 was used for H/I injury induced by unilateral occlusion and hypoxia (55 min, 8% O_2) and samples were collected 3 h, 12 h, 24 h, 48 h, 72 h and 7 days after hypoxia. After H/I, CD200 immunolabeling was increased in the hippocampal fissure until 7 days post-lesion. CD200R+ cells were a subpopulation of Iba1+ reactive microglia/macrophages, showing ameboid/pseudopodic morphologies, and located in the damaged hippocampus and white matter tracts, persisting until 7 days post-lesion. Characterization of CD200R+ cells by triple immunofluorescence showed that most CD200R+ microglia expressed the mannose receptor CD206, characteristic of alternatively activated phenotype M2. In addition, some CD200R+ microglial cells showed markers of antigen presentation such as MHCII and CD86, and in specific survival times also expression of scavenger receptor 1 (CD204). We conclude that CD200R expression influences the microglia/macrophage polarization towards the alternatively activated phenotype in neonatal mice brain after H/I.
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IRF8 regulates the microglial response to neuronal injury

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Objective: The transcription factor interferon regulatory factor (IRF) 8 has a key role in the cellular response to IFN-γ and is involved in myeloid cell differentiation. We have identified IRF8 to be a constitutive and IFN-γ-stimulated nuclear factor in microglia — the brain-resident myeloid cells of the CNS. The objective of this study was to determine the role of IRF8 in the microglial response to sterile neuronal injury induced by axotomy.
Methods: Facial nerve axotomy (FNA) was performed in wild type (WT) and IRF8 KO mice and the brain removed at different times post-lesion. A subset of mice was injected with bromodeoxyuridine (BrdU) every 2 h during the 24 h prior to retrieval of the brain. Changes in the facial nucleus were examined by immunohistochemistry and histochemistry.
Results: In brains from IRF8 KO mice, NDPase histochemistry revealed gross alterations in the morphology of microglia, which were stunted and hypertrophied. Microglia from IRF8 KO mice also exhibited significant alterations in a number of key myeloid and immune markers including reduced levels of Iba1 and the chemokine receptors CCR2 and CCR5 but increased levels of F4/80, CD45 and tomato lectin (TL) binding. After FNA in WT mice, a progressive increase in microglial activation was observed in the lesioned facial nucleus that peaked at day 7 and was accompanied by dense staining for Iba-1, TL, CD11b, CD16/32 and NDPase. By contrast, in IRF8 KO mice, the microglial response was markedly attenuated and delayed with little staining for Iba-1 and CD16/32, while the density of staining for TL, CD11b and NDPase was significantly reduced. The attenuated microglial response to FNA in IRF8 KO mice was paralleled by a significant decrease (at day 3 post-FNA) in proliferation as monitored by BrdU and phosphohistone 3. The wrapping of individual motor neuron cell bodies by microglia involved in synaptic stripping and phagocytosis was incomplete in the absence of IRF8. Quantitative analysis showed that in IRF8 KO mice, the eneurodegeneration of axotomized motor neurons was slightly increased.
Conclusions: These studies suggest that IRF8: (1) is a key homeostatic transcriptional regulator of microglial cell function in the healthy brain, and (2) has a crucial role in the functional response of microglia to neuronal injury. Supported by NHMRC grant APP1007757.

Neurodegeneration

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CCL17 deficiency promotes beneficial neuroinflammatory responses and protects from cognitive decline in a mouse model of Alzheimer's disease

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Chemokines control microglia function and mononuclear phagocyte infiltration in inflammatory CNS conditions and therefore have been implicated in Alzheimer's disease (AD). The CC chemokine CCL17 is a key regulator of leukocyte trafficking during inflammation, but its role in the pathogenesis of AD is yet unresolved. This study demonstrates that CCL17 controls amyloid deposition, neuroinflammation and cognitive decline in an AD mouse model. CCL17 deficient mice ($CCL17^{E/E}$) crossed with APPswe/PS1dE9 (APP/PS1-$CCL17^{E/E}$) mice were fully protected against cognitive deficits associated with a reduction in oligomeric Amyloid β (Aβ) peptides, Aβ42 levels and a diminished neuronal loss in the brain. Neuroinflammatory responses were even enhanced in these animals with increased microgliosis and higher numbers of brain immigrating $Ly6C^{+}$ $CCR2^{+}$ macrophages/monocytes which expressed the mannose receptor. The findings that the anti-inflammatory cytokine IL-10 was higher expressed in the APP/PS1x $CCL17^{E/E}$ brain further suggested beneficial neuroinflammatory responses in the absence of CCL17. In addition we found that CCL17 deficient microglia released enhanced levels of IL-10 under inflammatory conditions and exhibited a superior capacity to phagocytose Aβ. While expression of the receptor for advanced glycation end products (RAGE) was reduced in the APP/PS1x $CCL17^{E/E}$ brain, the major Aβ degrading enzyme neprilysin was upregulated indicating that CCL17 modulated Aβ uptake as well as its degradation. Our study identified a unique role for CCL17 in regulating memory loss and microglia/macrophage function during neurodegeneration harboring therapeutic potential for the treatment of AD.

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Characteristic features of inflammatory demyelination and neurodegeneration in neuromyelitis optica spectrum disorder

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Background: Neuromyelitis optica spectrum disorder (NMOsd) is an inflammatory and demyelinating syndrome characterized by optic neuritis and myelitis. Several recent magnetization transfer MRI studies have revealed abnormalities in normal-appearing gray matter, and a French cohort study demonstrated the possibility of cognitive impairment in NMO. However, the characteristic features and pathogenesis of neurodegeneration in NMO brain remain elusive.
Objective: To confirm characteristic features of inflammatory demyelination and neurodegeneration in NMO.
Methods: The study was performed on brain materials from six patients with NMOsd and four controls with non-CNS inflammatory neurological diseases (one each with Lambert-Eaton syndrome, congenital myopathy, rhabdomyolysis, and abdominal hemorrhage) at autopsy.
Results: All cases with NMOsd had typical pathological findings of NMO in the spinal cord or optic nerves: pattern-specific loss of aquaporin-4 (AQP4) immunoreactivity and immunoglobulin deposits colocalizing with products of complement activation in a vasculocentric pattern around thickened hyalinized vessels in inflammatory demyelinating lesions. Neuropathological assessments in brains demonstrated that neuronal loss in cortical layers II, III, and IV was significantly prominent in NMO brains. All NMO cases showed preservation of myelin basic protein immunoreactivity within the cerebral cortex and no evidence of cortical demyelination and complement activation in a vasculocentric pattern. Meningeal inflammation was abundant in NMO brain, but B-cell follicle-like structures, which are a prominent feature in MS brains, were not detected in NMO meninges.
Conclusions: We demonstrate the inflammatory demyelination in spinal cord and optic nerves and the cortical neuronal loss in brain of NMOsd. These data indicate a pathological process consisting not only of inflammatory demyelinating events characterized by pattern-specific loss of AQP4 immunoreactivity but also cortical neurodegeneration in NMO brain.

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The morphology of microglia is altered in aging and in a mouse model of Alzheimer's disease

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Objective: Microglia derived from monocyte and macrophage lineages residing within the central nervous system (CNS) already during development once the vasculature is formed. The cells integrate within the neural tissue exhibiting a unique ramified morphology which allows them to constantly scan their territory and contribute to neuronal network function and repair. Since microglia cells are long-lived they are subjected to senescence processes. Although studies have shown that microglia senescence can be reflected by morphological changes, detailed quantification of such changes are not sufficiently established.
Methods: We developed a digitized tool that allowed us to quantitatively characterize the fine microglia structure in the brain's cortex of adult mice and the morphological changes the cells undergo with aging and the progression of Alzheimer's-like disease. In addition we established quantitative methods to evaluate the coverage of the neural tissue by resident microglia.
Results: We found that compared with microglia in young mice, microglia in mice aged 21 months labeled either by GFP expression under the CX3CR1 promoter or immunostained with anti-Iba-1, are less ramified, have decreased amount of branches and lower amount of fine processes. Notably, the morphological aberrations of microglia appeared in a mouse model of AD already at 9 months of age associated with the deposition of amyloid plaques. Overall, we demonstrate a significant reduction of the volume coverage by individual microglia with age, a process that is expedited in a mouse model of AD.
Conclusions: Our results indicate that microglia are subjected to age- and disease-related changes that may indicate their state of activation along with a compromised ability to support neuronal function and repair.

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Immune priming of microglia in a DNA repair deficient model of accelerated aging

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Ageing of brain tissue has been associated with enhanced activity and immune priming of microglia in mice, rats and primates. It is, however, not clear yet whether this age-related microglia activation is due to the intrinsic process of microglia aging or is an adapted response of microglia to the aging neural environment. Since age-related microglia immune priming forms a likely background for the development of neurodegenerative diseases, we decided to address its mechanism. We have investigated age-related microglia priming in *Ercc1*$^{\Delta/-}$ mice, a progeroid, DNA repair deficient mouse model that has been associated with accelerated senescence and neurodegeneration.

Upon aging, ERCC1 mice showed increased microglial proliferation and immune activation, suggesting a healthy and vigilant microglia phenotype. Furthermore, in mice where the ERCC1-related DNA repair deficit was targeted to forebrain neurons, immune-primed microglia were found specifically in forebrain areas. We suggest that the immune primed microglia phenotype in DNA-repair deficient, senescence accelerated mice represents an adequate response to the aging neural environment.

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Astrocytes trigger neurodegeneration through nitric oxide release after TrkB engagement: Implications for multiple sclerosis

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Objective: The neurotrophins are growth factors produced by neurons and glial cells that regulate neuronal survival, growth and differentiation. Astrocytes, the most abundant glial cells of the central nervous system, are essential for neuronal homeostasis and function, and till now the outcome of neurotrophin signalling in this cell type is unknown. Here we investigated the role of the neurotrophin receptor TrkB on astrocytes in neuroinflammation and neurodegeneration.
Methods: We checked the expression of TrkB in human normal brain, in multiple sclerosis (MS) lesions and in its animal model, the experimental autoimmune encephalomyelitis (EAE); moreover we performed EAE experiments in transgenic mice deficient for TrkB on astrocytes. Quantitative PCR, immunohistochemistry and immunofluorescence were used. In vitro study on human astrocytes and rat neuronal cultures were set up to analyze the effects of astrocytic activation on neuronal survival.
Results: We found that TrkB was strongly upregulated on astrocytes in MS and EAE lesions; furthermore the most abundant TrkB isoform in white matter lesions was the truncated isoforms TrkB-T1. EAE experiments performed in transgenic mice showed that these animals were protected from neurodegeneration and immune cells infiltration, indicating a fundamental role for astrocyte-TrkB in supporting experimental neuroinflammation. *In vitro* examination of the effects of astrocytic activation via the TrkB ligands BDNF, NT3 and NT4 on neuronal morphology and survival showed that this neurotrophin triggered a neurodegenerative process amplified by astrocytes. These detrimental effects were mediated by nitric oxide (NO) synthesized and released by astrocytes, and sustained by NO produced by neurons. NO synthesis during EAE was dependent on astrocyte TrkB, as transgenic mice showed decreased inducible NO synthase and nitrotyrosine immunoreactivity.
Conclusions: We identified a novel NO-mediated neurodegenerative pathway triggered by astrocyte TrkB Targeting this pathway represents a goal for novel neuroprotective therapies for MS.

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Regulatory T cells modulate disease progression in a murine model of Alzheimer's disease

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Objective: Alzheimer's disease (AD) is a neurodegenerative disorder characterized by progressive loss of memory and cognitive functions. Accumulation of Abeta peptide is considered the initiating cause of pathogenic lesions. Vaccination against Abeta provided encouraging results in experimental mouse models and, to a lesser extent, in a subsequent clinical trial (AN1792). Although the AN1792 trial had to be interrupted, due to meningoencephalitis attributed to pro-inflammatory T cell responses in 6% of the patients, preclinical murine models did not show evidence of T cell-related side effects. In addition, several reports suggest that Abeta-specific CD4$^+$ T cells may be implicated in the natural course of AD and could have a strong therapeutic potential as well. Our previous data indicate that regulatory T cells (Tregs) critically control the magnitude of Abeta-specific CD4$^+$ T cell responses in both physiological and pathological settings in response to Abeta vaccination. However, the actual role of Tregs in the pathophysiology of AD remains unknown. We analyzed the impact of Tregs on disease progression in a murine model of AD.
Methods: APPPS1 mice were depleted of Treg cells by using anti-CD25 antibodies (clone PC61), and the impact of Treg depletion on neuropathological features of the disease and cognitive proficiency of mice was evaluated.
Results: Depletion of Treg cells accelerated the onset of cognitive deficits in APPPS1 mice. Alteration in spatial memory was detectable as soon as 7 months in PC61-treated animals, while PBS-treated APPPS1 mice were not yet cognitively impaired as compared to wt animals. Early cognitive impairment in Treg-depleted APPPS1 mice was associated with alterations in neuropathological features of the disease.
Conclusions: Treg cells play a beneficial role in the pathophysiology of AD and delay disease progression in a murine model of the disease. These data open new perspectives in the development of Treg-based innovative immunotherapy approaches for the treatment of AD.

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Beneficial effects of fumarates in axon growth and regeneration: Crosstalk of immune and degenerative mechanisms

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Objective: The fumaric acid ester dimethylfumarate (DMF) is a new disease modifying therapy. Several studies have shown convincing data after DMF therapy in patients not only in autoimmune inflammatory diseases like relapsing–remitting multiple sclerosis but also in neurodegenerative disorders like Huntington's disease (HD). DMF potentially exerts neuroprotective effects via induction of the transcription factor "nuclear factor E2-related factor 2" (Nrf2) and detoxification pathways.

Although the exact mechanisms that lead to neurodegeneration are not fully understood some line of evidence speaks for the contribution of oxidative stress to neuronal tissue damage inducing neurodegeneration.

Methods: First, we thus investigated the therapeutic efficacy of DMF in R6/2 and YAC128 HD transgenic mice, which mimic many aspects of HD and are characterized by an enhanced generation of free radicals in neurons. Treatment with DMF significantly prevented weight loss, attenuated motor impairment and resulted in a significant preservation of morphologically intact neurons in histological analysis. DMF treatment resulted in an increased Nrf2 immunoreactivity in neuronal subpopulations, but not in astrocytes.

Second, analysing the underlying mechanisms, we use in vitro techniques for studying effects of DMF on axon growth and regeneration. Using dorsal root ganglia as tools for outgrowth assay and in vitro regeneration, we look at the effects of a sublethal dose of reactive oxygen species on regeneration and at the protective role for DMF. Furthermore we study the effect of DMF on ingrowing axons.

Third, getting an idea of downstream mechanisms of DMF, we quantified expression of affected genes by qPCR under different concentrations of DMF and with various durations of DMF-incubation.

Results: DMF exerts beneficial effects in axon growth and regeneration being effective not only in inflammatory but also in neurodegenerative diseases.

Conclusions: Given its excellent side effect profile, further studies with DMF and its downstream pathways are ongoing that hopefully lead to treatment of patients suffering from multiple sclerosis and neurodegenerative diseases like Huntington's and perhaps also Parkinson's disease.

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TNFR2-induced secretion of LIF in astrocytes promotes oligodendrocyte differentiation

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Objective: Despite promising results in mouse models of multiple sclerosis (MS), clinical trials with TNF neutralizing reagents in MS patients failed to ameliorate the disease and in some cases even led to disease exacerbation. One possible explanation for this failure is the dual role of TNF in the CNS. Whereas TNFR1 signaling mediates nerve demyelination, TNFR2 appears to be responsible for remyelination. However, the molecular mechanism or the cells involved in the regenerative properties of TNFR2 are not known yet.

Methods: To investigate the role of TNFR2 responses by astrocytes during myelination, we established an astrocyte–oligodendrocyte co-culture model, composed of primary astrocytes from huTNFR2-transgenic mice (TgE1335) and oligodendrocyte progenitor cells isolated from wildtype mice, which are capable of differentiating into mature myelinating oligodendrocytes. Using a TNF mutein, which is selective for the human TNFR2 (huTNFR2), it is possible to specifically activate huTNFR2 on astrocytes, without influencing mouse TNFR2 on oligodendrocytes.

Results: Activation of TNFR2 in primary astrocytes from wildtype or TgE1335 mice lead to upregulation of neurotrophic factors, including leukemia inhibitory factor (LIF). Using our astrocyte-oligodendrocyte co-culture model, we were able to show that specific activation of TNFR2 on astrocytes leads to an increased number of differentiated MBP-positive oligodendrocytes. After targeting LIF by adding a neutralizing antibody to the medium, the increased oligodendrocyte differentiation was abrogated, pointing towards an important role of astrocyte-secreted LIF during oligodendrocyte maturation.

Conclusions: Activation of TNFR2 in primary astrocytes evokes production of neurotrophic factors, including LIF, which are able to stimulate differentiation of neighboring oligodendrocyte progenitor cells. Our data underline the important role of TNFR2 during oligodendrocyte differentiation, identifying astrocytes as an important cell type for TNFR2-dependent myelination and show a possible mechanism of TNFR2 mediated remyelination.

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High levels of interleukin-17 in the cerebrospinal fluid of patients with Creutzfeldt-Jakob disease

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Objective: To investigate immune responses in the cerebrospinal fluid (CSF) in patients with Creutzfeldt-Jakob disease (CJD).

Methods: We conducted a case–control study. We enrolled 15 cases with CJD and 14 age-matched controls with non-inflammatory neurological disorders. The diagnoses of controls were normal pressure hydrocephalus (8), parkinsonian syndrome (3), myalgia/neuralgia (2), and headache (1). The concentrations of 27 CSF cytokines and chemokines were evaluated using the Bio-Plex suspention array system (Bio-Rad, San Francisco, CA). The concentrations were compared between the two groups using Mann–Whitney test. P values <0.05 were considered statistically significant.

Results: The values of following cytokines and chemokines were significantly elevated in the CJD group: interleukin (IL)-1 receptor antagonist (mean, 19.39 vs 0 pg/ml; $p<0.0001$), IL-12 (p70) (1.32 vs 0.42; $p=0.0353$), IL-17 (34.31 vs 3.45; $p=0.0001$), and platelet-derived growth factor BB (3.40 vs 1.20; $p=0.0072$). On the other hand, the values of IL-13 (1.65 vs 3.20; $p=0.0289$), granulocyte macrophage colony-stimulating factor (139.3 vs 339.6; $p<0.0001$), macrophage inflammatory protein-1α(MIP-1α)/CCL3 (0.09 vs 0.76; $p<0.0001$), and vascular endothelial growth factor (7.09 vs 12.88; $p<0.0001$) were significantly lower in the CJD group.

Conclusions: The high values of IL-17 indicated T cell-associated immune responses in patients with CJD. Furthermore, the production

of IL-1 receptor antagonist and IL-12 suggested coexistent anti-inflammatory reaction in CJD.

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Study of the effect of neuronal IKKβ upon the expression of inflammatory, myelin and neuronal genes during cuprizone-induced CNS demyelination

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The transcription factor NF-κB mediates the initiation of immune and inflammatory responses and protection of neurons against excitotoxic and ischemic injuries. In experimental autoimmune encephalomyelitis (EAE), an animal model for multiple sclerosis (MS), we have shown that the neuroprotective properties of NF-κB are sufficient for ameliorating clinical disease. Thus, mice conditionally deficient in IKKβ in $Ca^{(2+)}$/calmodulin-dependent kinase II alpha-expressing neurons (nIKKβKO)[1,2] which are deficient in NF-kB activation, showed more severe neurological deficits, enhanced axon damage, as well as increased inflammation and Th1 lymphocyte infiltration in the spinal cord compared to controls[3]. Here, we further investigated the effects of neuronal NF-κB in a model of compartmentalized CNS inflammation, representative of pattern III of MS, which is induced by the neurotoxin cuprizone and develops independently of T and B cells. nIKKβKO and $IKK\beta^{F/F}$ control mice were fed *ad libitum* with cuprizone-supplemented diet and sacrificed at the onset and peak of demyelination and at the onset of remyelination after cuprizone withdrawal[4]. The expression of selected gene markers, relative to that of the Gusb gene, was measured in total brain mRNA by quantitative RT-PCR. Similar to EAE, the expression of inflammatory genes (Cxcl16 and Tlr2) was increased while that of myelin (MBP, Olig2) and neuronal (Nrg1) genes was reduced in nIKKβKO brain during the demyelination phase. Neuropathological analysis of brains taken at the same disease time-points confirmed an increase in the number of activated astrocytes and microglia, a decrease in the amount of myelin and extensive neurodegeneration in the nIKKβKO brains. These results show that neurons contribute to CNS pathology during cuprizone-induced demyelination and that neuronal NF-κB is critical for suppressing inflammation and axonal damage. Interestingly, expression of the synaptic marker Snap25, was higher in nIKKβKO during remyelination suggesting there might be improved neurite outgrowth in these animals during the repair phase. Our results provide further evidence for an immunosuppressive role of neurons and neuronal NF-κB and further stress the importance of neuroprotective therapies for the treatment of CNS inflammatory diseases.

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Immune responses in rapid dementia — A comparative study on neuroinflammatory markers in CJD, AD, rpAD and MS patients

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Objective: Immunological responses in neurodegenerative disease pathogenesis such as in Alzheimer's disease (AD) and prion disease have become of increasing scientific interest in the past years. A main neuropathological hallmark of both neurodegenerative diseases constitutes the extracellular deposition of amyloid fibrils of A-beta (Aβ) for AD and PrP^{Sc} for Creutzfeldt-Jakob disease (CJD), leading to subsequent neurodegeneration. In these aggregational or conformational disorders it was shown that amyloid depositions co-localize with a broad variety of inflammation-related proteins (complement factors, acute phase proteins, proinflammatory cytokines) and activated microglia, suggesting a major influence of the brain's innate immune system and a chronic inflammatory process in disease pathogenesis.
Methods: Using a cytokine multiplex array we studied 17 pro- and anti-inflammatory cytokines with candidate cytokines of a TH 1, TH2 and TH17 immune answer as well as candidate chemokines for the innate immune answer in cerebrospinal fluid (CSF) and serum samples in patients with Creutzfeldt-Jakob disease (CJD, n = 10) and Alzheimer's disease (AD, n = 10) including a subgroup of rapid progressive AD (rpAD n = 6). As controls we included patients with multiple sclerosis (MS, n = 10) as the most frequent chronic inflammatory disease of the central nervous system causing neurodegeneration and controls (n = 10). CSF and serum samples were collected in our prospective Alzheimer's disease surveillance study as well as in the framework of the German CJD Surveillance study and from the Neurology clinics.
Results: As major findings we found significantly elevated concentrations of proinflammatory cytokines (IL-6, IL-7, IL-13, TNF-α and G-CSF) in serum of patients with rpAD when compared to AD, CJD, MS and controls. G-CSF was significantly elevated in serum both of rpAD und AD patients. The immune response profile was reflected predominantly by TH2-associated cytokines (IL-6, IL-13 and G-CSF). In CSF we revealed significantly elevated levels of IL-8 und MCP in CJD (IL-8, MCP-1) und AD (MCP-1) patients. Both cytokines are potent proinflammatory chemokines related to an innate immune response.
Conclusions: Elevated levels of proinflammatory cytokines in serum of rpAD patients represent a novel finding. In the context of rapid dementia, rpAD was recently identified representing an aggressive subtype of AD. Our findings might reflect a systemic immune response in this group that was not presented in the same way in MS, AD or CJD as disease controls. Elevation of the chemokines IL-8 and MCP-1 in CSF in CJD and AD were partially reported before. As these markers are mostly involved in mechanism of the innate immune answer they might represent an atypical neuroinflammatory response as reflected by microglia activation and astrogliosis in the neurodegenerative process. Our findings show in two different ways an activation of the immune system in rapid dementia that was distinguishable from MS and controls. An understanding of the neuroinflammatory process in neurodegenerative dementias becomes more and more important as it might reveal new insights in the pathogenesis especially in rapid progressive forms (rpAD, CJD).

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Telomere dysfunction leads to changes in microglial numbers and morphology in a mouse model of premature aging

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Objective: The susceptibility of the aging brain to neurodegenerative diseases may in part be attributed to the intrinsic senescence of the

microglia. Cellular aging or senescence is linked to telomere shortening/dysfunction. Evidence is accumulating that aging microglia show morphological changes, referred to as microglial dystrophy characterized by accumulation of iron-binding protein, ferritin. Furthermore, microglia from Alzheimer's brains have been reported to have shorter telomeres. Here, we investigated the effect of telomere shortening on microglial morphology, numbers, and activation state in telomerase deficient ($Terc^{-/-}$) mice, a model of premature aging due to shortened telomeres.
Methods: Male $Terc^{+/+}$ mice and 3rd generation $Terc^{-/-}$ mice, which show a clear aging phenotype, were perfusion-fixed with 4% paraformaldehyde. Brains were processed into 60 μm thick horizontal sections. Every fourth sections was used for stereological estimation of Mac-1^{+} microglia in the molecular layer of the dentate gyrus by using the optical fractionator method. Sections were also stained for the microglial markers Iba-1 to monitor microglial morphology and CD45 to study microglial activation. Further sections were stained for the apoptosis marker Poly (ADP-ribose) polymerase (PARP).
Results: Unlike resting microglia in littermate wild type (WT) $Terc^{+/+}$ mice, which display thin, finely branched processes, microglia in $Terc^{-/-}$ mice exhibited partial retraction and hypertrophy of processes. Microglial CD45 immunoreactivity was similar in $Terc^{+/+}$ and $Terc^{-/-}$ mice. Absolute numbers of Mac-1^{+} microglia was significantly reduced in young and old $Terc^{-/-}$ versus $Terc^{+/+}$ mice. An increased microglial density in old $Terc^{-/-}$ versus $Terc^{+/+}$ mice could be attributed to a volume reduction of the dentate gyrus. We also observed an increased number of $PARP^{+}$ cells in old $Terc^{-/-}$ versus $Terc^{+/+}$ mice. This increase was most prominent in the sub-granular zone, an active site of neurogenesis, but also occurred in the molecular layer of the dentate gyrus and might reflect microglial apoptosis.
Conclusions: Our findings suggest that telomere shortening impairs microglial capacity for self-renewal. Surprisingly the change in the microglial morphology in the $Terc^{-/-}$ mice was observed with no upregulation in CD45. Ongoing studies will show if microglia in the $Terc^{-/-}$ mice show additional signs of cellular senescence and dystrophy, including accumulation of ferritin.

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Overcoming chondroitin sulfate proteoglycan inhibition of oligodendrocyte-lineage cells and remyelination

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Objective: The extracellular matrix (ECM) has been shown to significantly influence neural cells. Chondroitin sulfate proteoglycans (CSPGs) are a family of ECM molecules that impede regenerating axons following traumatic CNS injury. Recently, we determined that CSPGs are highly upregulated in a mouse model of demyelination and that reduction of CSPG biosynthesis improved remyelination (Lau et al., Annals Neurol, in press). These findings led us to postulate that enzymatic degradation of CSPGs or targeting downstream signaling pathways would promote OPC and oligodendrocyte maturation and remyelination following a demyelinating insult.
Methods: We isolated oligodendrocyte precursor cells (OPCs) from adult human or neonatal mouse brains. We examined the impact of enzymatic degradation of CSPGs using ADAMTS-4 and chondroitinase ABC (ChABC) on OPCs and oligodendrocytes. Downstream signaling inhibitors of several pathways including ROCK, EGFR, and MAPK were also tested. Lastly, the impact of *in vivo* infusion of ADAMTS-4 was tested on adult female mice subjected to lysolecithin demyelination.
Results: To investigate the selective effect of CSPGs, human oligodendrocytes were plated onto molds that alternated between albumin and CSPG stripes. Few oligodendrocytes and processes were found on CSPG compared to albumin stripes. The inhibitory effect of CSPGs on human OPC adhesion was overcome with the protease ADAMTS-4, which degrades the core protein of CSPGs, but not by ChABC, which removes only the glycosaminoglycan side chains. Both enzymes relieved CSPG inhibition of oligodendrocyte process outgrowth. These findings implicate the protein core of CSPGs as the inhibitory components for oligodendrocyte adhesion, and the glycosaminoglycan moieties as impediments of morphological maturation. The results of application of various signaling inhibitors in culture along with the effect of ADAMTS-4 on remyelination *in vivo* on the demyelinated spinal cords of mice are being tabulated.
Conclusions: Our results identify CSPGs as novel impediments to human oligodendrocytes and suggest that future remyelinating therapies may include reducing levels of CSPGs or targeting their signaling mechanisms.

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The neuroprotective role of microglia treated with milk fat globule-EGF factor 8

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Objective: Milk fat globule-EGF factor 8 (MFG-E8) expressed on microglia is a phosphatidylserine receptor that mediates the phagocytosis of apoptotic cells. The mechanisms by which microglia produce MFG-E8 and the precise functions of MFG-E8 are unknown. Soluble oligomeric amyloid β (oAβ) contributes to neurotoxicity such as synaptic dysfunction in Alzheimer's disease (AD). Microglia accumulate at senile plaques in AD, and have both neurotoxic and neuroprotective properties. We examined the role of microglia treated with MFG-E8 against oAβ toxicity.
Methods: The release of MFG-E8 from microglia treated with conditioned medium from neurons exposed to oAβ was measured by ELISA. The neuroprotective effects of MFG-E8 and MFG-E8 — induced microglial phagocytosis of oAβ were assessed by immunocytochemistry. The effects of MFG-E8 on the production of the anti-oxidative enzyme hemeoxygenase-1 (HO-1) and the inhibition of inflammatory molecules were determined by ELISA and immunocytochemisty.
Results: MFG-E8 was induced in microglia treated with conditioned medium from neurons that had been exposed to oAβ. MFG-E8 significantly attenuated oAβ-induced neuronal cell death in a primary neuron — microglia coculture system. Microglial phagocytosis of oAβ was accelerated by MFG-E8 treatment due to increased CD47 expression. MFG-E8 induced anti-oxidant enzyme HO-1 in microglia. Moreover, MFG-E8 suppressed the production of TNF-α, IL-1β, IL-6, glutamate, nitric oxide and reactive oxygen species by microglia activated with LPS.
Conclusions: MFG-E8 may have novel roles as a neuroprotectant that is produced from microglia in AD.

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Dimethyl fumarate protects the central nervous system against excitotoxicity

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Objective: Dimethyl fumarate (DMF), a novel oral treatment for Multiple Sclerosis (MS), has been shown to significantly reduce gadolinium-enhancing lesions and disease progression in relapsing-remitting MS (RRMS) in two Phase III clinical trials. However, the possible neuroprotective function of DMF has not been fully investigated. Our preliminary data suggest that DMF upregulates heme oxygenase-1 (HO-1), an anti-oxidative and anti-inflammatory enzyme in the central nervous system (CNS). Interestingly, previous studies showed that overexpression of HO-1 in neurons protects neurons against toxicity caused by H_2O_2 and MPP. Based on these findings, our hypothesis is that DMF exerts its neuroprotective effects in MS through HO-1 induction.
Methods: In the current study, an excitotoxic animal model was induced by AMPA microinjection into the spinal cord at lumbar section. To evaluate DMF's neuroprotective effect, DMF was fed to naïve mice for 3 days followed by AMPA microinjection. Neuronal loss and behavior performance were evaluated 24 h after injection. To determine if HO-1 expression is altered, DMF was fed to naïve mice for 3 days, and then HO-1 mRNA and protein expression in the brain and spinal cord were respectively determined by PCR and ELISA.
Results: Our results demonstrate that DMF pre-treatment reduces neuronal damage by 30% and improves behavioral outcomes as compared to vehicle-treated mice, indicating the neuroprotective effects of DMF. Additionally, both HO-1mRNA and protein expression were significantly upregulated in the spinal cord in the DMF feeding mice compared to vehicle-treated mice, suggesting HO-1 as a potential target of DMF.
Conclusions: Our data demonstrate that DMF protects the spinal cord against excitotoxicity possibly through upregulation of HO-1in the CNS.

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Neuroprotective effect of silymarin in a mouse model of Parkinson's disease

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Objective: Parkinson's disease (PD) is a neurodegenerative disease secondary to the loss of dopaminergic neurons in the substantia nigra. 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) produces in mice and primates histopathological changes similar to PD in humans. A common feature of PD and MPTP models is neuronal death and dopamine depletion. At present the treatment of PD is principally based on the replacement of dopamine levels by administration of levodopa, however, modulation of neuronal death is important in order to modify disease progression. Silymarin is a polyphenolic flavonoid derived from the seeds and fruits of the plant *Silybum marianum* and has mainly antioxidant, anti-inflammatory, cytoprotective and neuroprotective effects.

In this work we evaluated the neuroprotective effect of silymarin in a mouse model of PD. and determined the concentration of dopamine in the striatum of MPTP-intoxicated mice and MPTP-intoxicated mice treated with silymarin.
Methods: In order to explore whether silymarin has a neuroprotective effects in PD we determined the concentration of striatal dopamine by high resolution liquid chromatography with electrochemical detection, the number of apoptotic cells with in situ detection of fragmented DNA (TUNEL assay) and the number of tyrosine hydroxylase positive neurons (TH+) in substantia nigra by immunohistochemistry of vehicle-treated, silymarin-treated, MPTP-intoxicated and MPTP-silymarin treated mice.
Results: Silymarin treatment partially recovered dopamine depletion by MPTP treatment (48%, 62%, 69%, 55%, 43%, 33% and 29% compared to control group at 25, 50, 100, 200, 250, 300 and 400 mg/kg, respectively), the most effective dose was 100 mg/kg. In addition, silymarin treatment significantly reduced the number of apoptotic cells induced by MPTP intoxication. These results are directly related to the number of TH+neurons observed in the sustantia nigra of mice from the different experimental groups. MPTP treatment reduced significantly the TH+neurons, while silymarin treatment in MPTP-intoxicated mice preserved significantly the TH+neurons.
Conclusions: The current study shows evidence of the protective properties of silymarin in a MPTP-induced PD model. Silymarin preserved striatal dopamine levels by diminishing apoptosis in the substantia nigra and preserving the TH+neurons. These results may be of interest in the treatment of PD.

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Phosphorylation of PPAR-gamma in ALS

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Objective: Oxidative stress, mitochondrial dysfunction and neuroinflammation are pathological processes involved in amyotrophic lateral sclerosis (ALS), a neurodegenerative disorder characterized by a loss of upper and lower motor neurons. In animal models of neurodegeneration several experiments using Thiazolidinediones (TZD), synthetic PPARγ agonists, suggest a regulatory role of PPARγ in inflammatory pathways. However, clinical trials in ALS patients treated with agonists for PPARγ have failed. These findings, together with the regulation of PPARγ activity mediated by post-translational modifications, brought us to study the specific inactivating phosphorylation site (Ser112/Ser82) of PPARγ in human ALS tissue and in G93A SOD1 mice, an animal model of ALS.
Methods: G93A SOD1 mice from Jackson Laboratory were sacrificed at 30, 60, 100 and 120 days of age. Cortex and spinal cord were analyzed in a total of 6 females and 3–6 males per age group. ALS and control human spinal cord tissue was supplied by the Neurological Tissue Bank-University of Barcelona-Hospital Clínic-IDIBAPS.

The phospho-PPARγ protein content was studied by western blot, quantified by Quantity One Analysis software (BioRad) and analyzed by GraphPad Prism[R].
Results: We found an age-dependent effect on phosphorylation of PPARγ in the spinal cord of mice, statistically significant in 100- and 120-day-old G93A SOD1 mice.

In human samples an increasing tendency of phosphorylated PPARγ protein was observed between ALS and control cases. That tendency was clearer when tissue sample were sorted in those from pure ALS and ALS with some other pathology (fronto-temporal lobar degeneration or Alzheimer's disease related pathology).
Conclusions: The increment of inactive phosphorylated PPARγ levels in the spinal cord of the clinically affected mice (100- and 120-day-old G93A SOD1), prompt a possible explanation for the inflammatory processes in the brain, one of the hallmarks of ALS and other neurodegenerative disorders.

The failure of clinical trials with PPARγ agonist in ALS patients could be explained by the increased levels of phosphorylated PPARγ found in the human samples.

The levels of active/inactive PPARγ seem to be important not only with regard to the clinical presentation of the disease, but also regarding to treatment efficiency. Understanding of the PPARγ activation/inactivation mechanisms will help in the development of new treatment regimens for ALS and other neurodegenerative diseases.

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a-Synuclein vaccination prevents pathological inclusions accumulation in striatum via Treg recruitment

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Objective: It is becoming evident that the peripheral immune system takes an active role in Parkinson's Disease (PD) progression, as supported/suggested by the induction of the antigen presenting protein HLA-DR (human homolog to MHC II) in diseased brain and the infiltration of lymphocytes; as well as the presence in serum of alpha-synuclein specific antibodies. We designed a vaccination strategy to attempt to harness these processes and mediate protection against disease progression in a rat model of PD.
Methods: Using recombinant adeno-associated viral vector (rAAV) we unilaterally over-expressed human alpha-synuclein in the rat substantia nigra (SN) to induce progressive neuropathology. Prior to stereotactical delivery of rAAV-alpha-synuclein, animals were vaccinated in order to induce a memory T cell population against the protein.
Results: This therapeutic approach resulted in the accumulation of CD4+ MHC II+ ramified microglia in the SN, long lasting infiltration of CD4+Foxp3+ cells throughout the nigrostrial system, and 66% fewer pathological aggregates in the striatum compared to control animals that received a mock vaccine. These events were correlated with the long-term increase in GDNF levels in the striatum (8 weeks post-virus injection).
Conclusions: Together, these results show that a protective vaccination strategy requires the induction of regulatory T cells and distinctly activated microglia (CD4+ MHC II+) that induce immune tolerance against alpha-synuclein.

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Brain markers of neurodegeneration in sepsis survivor rats

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Objective: Survivors from sepsis presented cognitive deficits associated with decreased quality of life and increased long-term morbidity. Some of these alterations resembled the pathophysiological mechanisms of neurodegenerative diseases. The receptor for advanced glycation (RAGE) has been increasingly implicated in the progression of neuronal death in many neurodegenerative diseases, such as Alzheimer's disease (AD), and is also an important pro-inflammatory mediator. For this reason, we analyzed biochemical parameters related to neurodegeneration in rats that survived sepsis, and their relation to cognitive dysfunction.
Methods: Rats were subjected to sepsis by cecal ligation and puncture (CLP), and thirty days after surgery the hippocampus and pre-frontal cortex have been isolated just after cognitive evaluation by the inhibitory avoidance test. The immunocontent of β-amyloid peptide (Aβ), RAGE and synaptophysin were analyzed by western blot.
Results: Aβ was increased in septic animals the hippocampus, but not in the pre-frontal cortex. RAGE was upregulated in both structures after sepsis, and the immunocontent of synaptophysin was decreased only in the pre-cortex, and inversely correlated to Aβ levels. Pre-frontal levels of synaptophysin correlated with performance in the inhibitory avoidance, as well as hippocampus levels of Aβ.
Conclusions: In conclusion, brain from sepsis survivor animals presented several markers of neurodegeneration and RAGE and this was related to cognitive performance suggesting that this alterations could be responsible to long-term cognitive deficits in sepsis survivors.

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The PARK18 SNP is associated with altered expression and regulation of major histocompatibility complex II genes in Parkinson's disease patients

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Objective: Microglia are the myeloid-derived antigen presenting cells (APCs) of the brain involved in immune surveillance. Yet chronic activation of microglia can lead to overproduction of pro-inflammatory cytokines and chemokines and recruitment of peripheral immune cells to the CNS all of which can hasten degeneration of vulnerable neurons. A single nucleotide polymorphism (SNP) in the first intron of the HLA-DRA gene termed *PARK18* was recently shown to be associated with a higher risk of Parkinson's disease (PD) in a genome-wide association study (GWAS). Individuals homozygous for the rs3120882 G SNP (PARK18GG) have a 1.7 fold higher risk ($p = 5 \times 10^{-8}$) for PD than individuals homozygous for the A allele (PARK18AA). HLA-DRA is a located in the major histocompatibility complex II (MHCII) locus on chromosome 6 and encodes proteins that present antigens on the surface of APCs to activate the adaptive immune system. Implication of this genetic locus in PD provides a direct link to explain how adaptive immunity can exacerbate neuroinflammation in the context of this neurodegenerative disease.
Methods: We used flow cytometry and real-time PCR to investigate whether peripheral monocytes from PD patients with the PARK18 GG allele display differences in HLA-DR and HLA-DQ expression compared to control subjects with the PARK18 AA allele under resting or stimulated conditions. We also used luciferase reporter assays to investigate whether the GG allele is associated with regulatory activity at the PARK18 locus.
Results: Our examination of MHCII gene and protein expression in APCs, specifically B cells and monocytes, from the peripheral blood of PARK18GG PD patients compared to PARK18AA healthy age-matched controls revealed interesting differences in the levels of HLA-DR and HLA-DQ expression. Monocytes from these patients also responded differently to interferon-γ stimulation with respect to MHCII expression. Preliminary results using human monocyte cell lines suggested the existence of regulatory activity at the PARK18 locus.
Conclusions: Our initial findings indicate that PARK18GG is clearly linked to altered expression of MHC II genes in PD patients and provide evidence to implicate the role of adaptive immunity in the pathophysiology of this neurodegenerative disease.

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Neuronal specific T cells are pathogenic in Biozzi mice — Implications for MS

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Objective: Multiple sclerosis (MS) is a demyelinating and neurodegenerative disease of the central nervous system (CNS) characterized by inflammation, oligodendrocyte damage, axonal damage and astrogliosis. Despite a link between neuronal damage and clinical disability the mechanism of neurodegeneration is unknown. Similar to aberrant immune responses that damage myelin, immune responses to neuronal proteins may be pathogenic in MS. In MS, T cell responses to the neuronal cytoskeleton protein neurofilament light (NF-L) are observed while antibody levels to NF-L are associated with disease progression.

Our objective is to examine how axonal-reactive T cells arise and the mechanisms involved in immune mediated neuronal damage.
Methods: We have developed a model of neurodegeneration and spasticity following immunization of ABH mice with the NF-L protein. Previous studies show that the pathology is a direct effect of an immune attack on neurons/axons. Detailed analysis of the pathology was investigated by confocal microscopy. The pathogenicity and specificity of the NF-L reactive T cells was investigated by epitope mapping and adoptive transfer of NF-L reactive T cells in mice. The mechanisms of T cell activation in the CNS was examined using phagocytosis assays and by pathology studies of MS lesions.
Results: In mice T cell responses to NF-L peptides reveal pathogenic epitopes that induce spasticity following T cell transfer. In diseased mice CD4+ cells dominate the CNS lesions and colocalize with damaged axons. Neurons do not express MHC class I and CD8+ cells are rarely observed suggesting a role for cytotoxic CD4+ cells. In MS lesions, phagocytosis of damaged neurons is observed as evidenced by NF-L in HLA-DR+ cells. NF-L was observed in cerebrospinal fluid cells from MS patients, suggesting neuronal debris is drained by this route.
Conclusions: We show that NF-L reactive T cells induce neurological disease in mice. Since CD4+ T cell dominate the lesions in the absence of MHC class I we suggest that cytotoxic CD4+ T cells play the dominant role in disease. In MS neuroaxonal debris is engulfed, phagocytosed and degraded by HLA-DR+ cells. Although uptake is essential for clearing neuronal debris, phagocytic cells could also play a role in augmenting autoimmunity to neuronal antigens.

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AAV2/1-mediated hippocampal gene delivery of neuronal anti-inflammatory molecule CD200 enhances neurogenesis and amyloid clearance in Alzheimer's disease mouse model

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Objective: Alzheimer's disease (AD) is a neurodegenerative disease characterized by progressive decline of cognitive function and memory formation. It is distinguished by the build-up of β-amyloid protein (Aβ) into plaques and intracellular tau accumulation. There is currently no therapy that can halt or reverse its progression. It has been shown that, even in healthy brains, there is an age-dependent increase in neuroinflammation and in the AD brain specifically, there is significant up-regulation of pro-inflammatory cytokines and increased activation of microglial cells. This suggests that dysregulated inflammation in the aged brain may result in the neuronal damage seen in AD. Up-regulation of specific pro-inflammatory cytokines and microglial activation, as well as toxic Aβ, negatively affect the capability of neural stem cells to mature and survive. Patients with AD exhibit an increase in cellular proliferation in the hippocampus that is trounced by a reduction in neuronal maturation. We hypothesize that the chronic neuroinflammation seen in AD is a major contributor to overall suppression of neurogenesis in the subgranular zone of the hippocampus. CD200 is an anti-inflammatory glycoprotein expressed in many cell types, including neurons, and its receptor is expressed in cells of the myeloid lineage, such as microglia. It has already been shown in both human AD patients and animal models of AD that an age-related increase in microglial activation is accompanied by an age-related or Aβ-induced decrease in CD200 expression.
Methods: In this study, the AD transgenic mouse model expressing APP mutant (Tg2576) was intracranially injected with adeno-associated virus expressing CD200 into the hippocampus at the pre-symptomatic disease stage. Following treatment with CD200, neurogenesis was measured in the brain by using both proliferation (BrdU) and differentiation markers (Dcx). Mice were also assessed for Aβ loads. Neurogenesis was also examined *in vitro* by co-culture of neural stem cells with microglia stimulated by CD200 with or without Aβ.
Results: CD200 expression in transgenic mice enhanced both proliferation and differentiation of neural stem cells in the dentate gyrus of the hippocampus and significantly reduced Aβ load in the hippocampus and cortex.
Conclusions: These data indicate that CD200 may be able to delay the onset of AD-like pathology and subsequent decrease in neurogenesis, thus giving it high potential as a therapeutic against neurodegeneration.

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Reducing Alzheimer's disease β-amyloid pathology by manipulating IL-12/IL-23 signaling

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Objective: Alzheimer's disease (AD) pathology displays an inflammatory component characterized by the presence of pro-inflammatory cytokines particularly in response to β-amyloid. Cytokines such as IL-1β and TNFα, reactive oxygen species and other signs of inflammation have been described as the local cellular response to Aβ plaques. However, despite descriptive evidence, functional studies on proinflammatory mediators in AD are relatively scarce.
Methods: Using transgenic *APPPS1* mice serving as a model of AD we first assessed the expression of proinflammatory cytokines in microglia. We crossed APPPS1 mice with animals harboring targeted deletions of cytokine subunits and analyzed cerebral plaque load. Additionally, we studied bone-marrow chimeric mice to dissect the functional impact of cytokine deletion in the radiation resistant vs non-resistant cellular compartment. Moreover, we analyzed the impact of antibody-mediated neutralization of IL-12/23 signaling on plaque load and behavioral performance.

Results: We observed the production of the common interleukin (IL)-12 and IL-23 subunit p40 by microglia. Genetic ablation of p40 and two other IL-12/IL-23 subunits, namely p35 and p19, resulted in a drastic decrease in cerebral amyloid plaque load. Although deletion of IL-12/IL-23 signaling from the radiation-resistant glial compartment of the brain either by removing p40 or its respective receptors was most efficient in mitigating cerebral amyloidosis, peripheral administration of a neutralizing anti-p40 antibody likewise resulted in a significant reduction of cerebral β-amyloid in *APPPS1* mice. Furthermore, intracerebroventricular delivery of anti-p40 antibodies reversed cognitive deficits in aged *APPPS1* mice.
Conclusions: IL-12/IL-23 signaling seems to be crucially involved in controlling not only the amount of a central component of AD pathology, namely Aβ plaques, but also cognitive impairment. Our results suggest that blockade of IL-12/IL-23 signaling ameliorates cerebral amyloidosis and poses a novel potential pharmacological approach to combat AD.

Stem cells

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Can MSCs use the cholinergic system to carry out their immunomodulatory functions?

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Objective: Mesenchymal stem cells (MSC) are adult stromal progenitor cells displaying immunomodulatory activities including inhibition of proliferation of T cells, B cells and also dendritic cells (DC) maturation in vitro. In mice, MSCs intravenous injection leads to rapid entrapment in the lungs where they interact with resident macrophages. The lungs, like other viscera, are innervated by vagal endings. Vagal signaling has been shown to downregulate inflammation, and the mechanism is thought to involve acetylcholine binding to the alpha-7 subunit of the nicotinic acetylcholine receptor on macrophages and dendritic cells. In a recent study we demonstrated how intravenous administration of MSC blocks, almost instantaneously, the migration of subcutaneously administered ovalbumin-pulsed DC to the draining lymph nodes. Since the effect of MSC on migration of DC is extremely rapid we hypothesize that this effect can be mediated, at least in part, by the vagus nerve.
Methods: To determine whether vagus is activated in response to MSC administration, we investigated heart rate variability (HRV) by ECG in previously anesthetized mice. Moreover, administration of MSC inhibited the increase in serum TNFa concentration and resulted in down-regulation of pro-inflammatory genes in lungs and spleen. We used Mecamylamine (1 mg/Kg i.p), an antagonist of the nicotinic acetylcholine receptor to inhibit the functional of vagus nerve.
Results: In MSC treated mice we observed evident bradycardia, early arrhythmias (ventricular extrasystoles), episodes of gasp and, within 1–2 min, atrio-ventricular (AV) block of second grade. In mice treated with fibroblasts, as controls, we noticed a trend to tachycardia, few sporadic extrasystoles (<5) and a first grade block. We repeated the same experiments described above with MSC pretreated with nicotine, a nicotinic agonist, and we observed no effect of these cells on HRV, suggesting that MSCs are able to influence the vagus nerve only if their acethylcholine receptor is available. The production of TNFa in $CD4^+$T cells and the proliferation assay from mecamylamine treated mice is more severe compare to controls mice with or without stimulation with anti CD3 antibody. Interestingly, when we intravenously injected MSC, in these mice, we could not observe inhibition of the increase in serum TNFa concentration and down-regulation of pro-inflammatory genes in lungs and spleen.
Conclusions: These finding suggest that MSC requires a functional cholinergic innervation to carry out their immunomodulatory function.

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Herpes simplex virus type 1 alters neuronal precursor differentiation from neural stem cells

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Objective: Herpes simplex virus type 1 (HSV-1) is one of the most widespread human infections that accounts for more than 90% of the cases of herpes simplex encephalitis (HSE), causing severe and permanent neurological sequelae among the treated cases, such as anterograde memory loss, anosmia (loss of smell), and dysphasia (loss of language). While our previous results in an animal model of HSV-1 encephalitis revealed HSV-1 infects cells in the subventricular zone (SVZ) where neural stem cells (NSCs) reside, the link between HSV-1 infection and lifelong severely impaired neurological functions experienced by HSE survivors is presently not understood. We hypothesize the neurological sequelae observed in encephalitic patients following HSV-1 infection are the consequence of HSV-1 infection of NSCs.
Methods: A GFP-expressing mouse NSC line was used in this study. NSCs were seeded in plates containing growth medium and infected them with different concentrations of HSV-1 (McKrae strain). Supernatants were collected at different time points and assayed for viral content (by plaque assay) and expression of select chemokines including CXCL1, CXCL5, CXCL9, and CXCL10. Viral antigen expression by NSCs was also conducted using immunocytochemistry (ICC). In other experiments, NSCs were added onto Matrigel matrix covered plates and evaluated for differentiation potential using markers selective for neurons (β-III tubulin and doublecortin), astrocytes (GFAP), and oligodendrocytes (GALC and Ng2) using ICC labeling for confocal microscopy and western blot techniques in infected and uninfected NSC cultures.
Results: NSCs are susceptible to HSV-1 evident by the recovery of copious amounts of virus in the supernatant and HSV-1 antigen expression by the cells following infection. Of the chemokines evaluated, only CXCL10 levels were altered (reduced) in NSC cultures infected with HSV-1. Analysis of differentiation patterns in NSC cultures revealed a dramatic and specific loss of the neuronal phenotype with no significant changes in the percentage of astrocytes or oligodendrocytes following viral infection.
Conclusions: NSCs are highly susceptible to HSV-1 which impacts on the differentiation process with a specific loss of neuronal precursors.

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Intrathecal transplantation of adult neural stem/precursor cells dampens CNS-restricted inflammation of experimental autoimmune encephalomyelitis

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Objective: Transplantation of neural stem/progenitor cells (NPCs) in rodent and non-human primate models of multiple sclerosis (MS) have consistently shown that NPCs promote neuroprotection through pleiotropic mechanisms, including immunomodulation, neurotrophic support and stimulation of endogenous repair processes. To foster the efficacy of NPC transplantation avoiding unwanted side effects caused by their multifaceted way of action, it is still mandatory to dissect the cellular and molecular mechanisms underlying their therapeutic effect.

Our work investigated the cellular and molecular interactions underlying the therapeutic efficacy of NPCs on CNS-confined inflammatory processes of the experimental autoimmune encephalomyelitis (EAE), the animal model of MS.
Methods: EAE was induced in C57 Bl/6 mice by immunization with the MOG_{35-55} peptide. At the peak of inflammation (20 days post-immunization, dpi) EAE mice were intrathecally transplanted with GFP-labeled syngenic adult SVZ-derived NPCs or vehicle. Neuropathology was assessed at 40 and 80 dpi, whereas *ex vivo* flow cytometry analysis of CNS inflammatory infiltrate was performed at 30 dpi. The direct interference of NPCs with inflammatory cells was investigated *in vitro* by co-culture of NPCs with dendritic cells (DCs) and helper T (Th) cells, followed by flow cytometry, protein and gene expression analyses.
Results: Intrathecal transplantation of NPCs significantly reduced clinical disability, demyelination and axonal loss of EAE when compared to sham treatment. At 40 dpi, transplanted NSCs persisted undifferentiated, localizing preferentially in subarachnoid and perivascular spaces close to the inflammatory infiltrate. Analysis of CNS inflammatory infiltrate revealed that NPCs significantly reduced blood-born myeloid DCs, macrophages as well as of encephalitogenic Th17 and Th1 cells. In vitro experiments demonstrated that NPCs – through secreted mediators – restrain antigen-presenting properties of DCs impairing maturation and re-activation of myelin antigen-specific T cells.
Conclusions: This work confirms the efficacy of the intrathecally transplanted NPCs in ameliorating disease severity of experimental MS and suggested that NPCs drive neuroprotection by interfering in the subarachnoid and perivascular spaces with antigen re-call and maturation of encephalitogenic T cells. By dampening this process, NPCs prevent CNS accumulation of blood-born inflammatory cells responsible for disease progression in MS.

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Three chemokines, SDF1α, MCP-1 and hepatocyte growth factor attract transplanted neural stem cells during neuroinflammation

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Objective: Multiple sclerosis (MS) is a multifocal disease, and precursor cells need to migrate into the multiple lesions in order to exert their therapeutic effects. Therefore, cell migration is a crucial element in regenerative processes in MS, dictating the route of delivery, when cell transplantation is considered. We have previously shown that inflammation triggers migration of multipotential neural precursor cells (NPCs) into white matter of experimental autoimmune encephalomyelitis (EAE) rodents, a widely used model of MS. Here we investigated the molecular basis of this attraction.
Methods: Neurospheres were grown from E13 embryonic brains of wild type, GFP transgenic or CCR2 knock-out mice. Neurospheres were studied in-vitro for cell motility, receptor expression and cell differentiation, using inverted and fluorescent microscopy and PCR. To investigate the role of chemokine signaling in cell migration in EAE, chemokine receptors were blocked on neurosphere cells. BrdU-labelled treated spheres and non-treated GFP spheres (as controls) were transplanted to the lateral cerebral ventricles of PLP-EAE SJL mice or of MOG-EAE C57Bl/6 mice. Fluorescent microscopy was used to compare migration of treated versus non-treated cells.
Results: Inflammation-triggered NPC migration into white matter tracts was dependent on a motile NPC phenotype and tissue-derived chemokines. Specifically, growing NPCs with epidermal growth factor (EGF) induced a motile phenotype, enabling their response to inflammation. Stromal Cell-Derived Factor-1α, Monocyte Chemoattractant Protein-1 and Hepatocyte Growth Factor were expressed in the EAE brain and specifically in microglia and astrocytes. Their cognate receptors, CXCR4, CCR2 or c-Met were constitutively expressed on NPCs. Selective blockage of CXCR4, CCR2 or c-Met partially inhibited NPC migration in EAE brains. Blocking all 3 receptors resulted in profound inhibition of NPC migration, as compared to extensive migration of control NPCs.
Conclusions: NPC migration depends on a motile phenotype induced by EGF. Signaling via three chemokine systems accounts for most of the inflammation-induced, tissue-derived attraction of transplanted NPCs into white matter tracts during EAE.

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Time associated decline in neurotrophic properties of neural stem cell grafts render them dependent on brain region-specific environmental support

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Objective: NPCs therapeutic value in neurodegenerative diseases is critically dependent on their long term survival independent of any exogenous growth factor and enduring functional neurotrophic properties. Here, we examined whether NPCs survive and maintain their properties for extended periods of time, or become dependent on environmental support.
Methods: Fetal GFP(+) NPC spheres were grafted into the naïve/irradiated hippocampus, ventricles and naïve/6-OH DA lesioned striatum. Their survival and fate were determined by computerized image analysis, including immunofluorescent stainings. We characterized tissue elements that support graft survival and blocked their signaling by

continuous infusion of blocking antibodies. Time associated changes in NPC spheres *in-vitro* were examined using immuno-fluorescent stainings and two functional assays.
Results: Two months following transplantation to naïve brains, large grafts were detected in the ventricles and hippocampus (70 ± 16%), but only little survival was evident in the striatum (17 ± 3.7%). To point at possible regional characteristics which underlie the differential survival of NPC grafts we performed several manipulations of the brain environment. Brain irradiation, which totally eliminated hippocampal neurogenesis resulted in 90% reduction of hippocampal graft survival. Acute neurotoxic injury with 6-hydroxydopamine induced a 3-fold increase (52.4 ± 6%) in striatal graft survival, associated with induction of nestin, CD31, β1-integrin, GFAP and cycling cells, all established components of the stem cell niche. Disruption of the extracellular matrix structure of this reactive niche by continuous blockage of host striatum β1-integrin caused 73% reduction in graft survival. In correlation with *in vivo* findings, long term cultured neural precursors exhibited an increase in apoptotic cells and dramatic decline in neurotrophic effects, as indicated by their decreased effect on neurite outgrowth in PC12 cells and reduced induction of OPCs proliferation, two *in vitro* functional assays.
Conclusions: We conclude that long-term changes in transplanted NPC properties render them dependent on region specific environmental support. We suggest that proximity to functional neurogenic/stem cell niches enables improved NPC graft survival. Furthermore, the neurotrophic effects of NPCs transplantation are limited in time and represent an important shortcoming for clinical translation.

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Time limited immunomodulatory functions of transplanted neural precursor cells

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Objective: A major issue in developing neural stem/precursor cell (NPC) therapy for chronic neurological disorders is whether cells maintain their immune-regulatory properties for a prolonged period of time. This is crucial to allow allogeneic NPC grafts to evade rejection from the host brain and to continue down-regulating neuroinflammation. Fetal NPCs possess powerful immunomodulatory properties which enable them to protect the brain from immune-mediated injury. We examined here whether intracerebrally-transplanted NPCs are able to inhibit early versus delayed induction of autoimmune brain inflammation and to inhibit an allogeneic rejection reaction against the graft.
Methods: Fetal GFP(+) NPC spheres were grafted into the 6-OH DA lesioned striatum or the ventricles of experimental autoimmune encephalomyelitis (EAE) mice. Their fate was determined by computerized image analysis of immunofluorescent stained sections. Fetal and long term spheres effects on Concanavalin A -stimulated lymph node cells were examined *in-vitro* by RT-PCR and 3H-thymidine incorporation.
Results: Allogeneic fetal NPC grafts elicited a strong immune reaction of T cell and microglial infiltration and were rejected from the host brain. Singeneic intraventricular fetal NPC grafts survived in the ventricles and entered a quiescent, non-proliferative and non-differentiated state. These transplants attenuated clinically and pathologically brain inflammation during early EAE relapse but failed to inhibit delayed relapse. In correlation, long term cultured neural precursors that survived in a non-proliferative state lost their capacity to inhibit immune cell proliferation *in vitro*.
Conclusions: Long-term functional changes in transplanted neural precursor cells lead to loss of their therapeutic immune-regulatory properties, resulting in lack of EAE attenuation. Moreover, these changes render allogeneic grafts vulnerable to immunologic rejection. Thus, the immunomodulatory effects of neural precursor cell transplantation are limited in time.

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The inflammatory fingerprint of Neural Stem Cells

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Objective: Neural stem progenitor cells (NPCs) reside in specialized areas of the adult mammalian brain where they undergo self-renewal and differentiation. This concept supports the idea that the adult CNS has the intrinsic potential to undergo repair/regeneration upon injuries. In contrast with this, others and we have shown that inflammation halts the proliferation and differentiation capacities of the endogenous brain stem cell compartment, thus restraining its intrinsic potential to contribute to tissue repair and/or regeneration. The mechanisms that characterize this response of brain stem cells to inflammation are still far to be identified. In this work we want to obtain a comprehensive, molecular profile of the changes induced in vitro by inflammation on the biology of neural stem cells.
Methods: We have developed an integrated 'omic' approach that draws a comprehensive picture of the NPCs' molecular response to inflammation. Mouse sub ventricular zone (SVZ)-derived NPCs were cultured in vitro with serum-free media in the presence or not of either Th1 pro-inflammatory or Th2 anti-inflammatory cytokines mixes that mimic a putative inflammatory microenvironment and characterized their transcriptome using small RNA-Seq and long RNA-Seq.
Results: Only Th1 cytokines induced a strong up-regulation of the Jak/Stat signalling pathway and interferon responsive genes, together with regulating cell cycle genes towards a block in G0/G1. Exposure to Th1 cytokines also induces several genes involved in antigen presentation, such as MHC-I and MHC-II subunits and immunoproteasome. In addition, we found 5 miRNAs that were differentially expressed after Th1 cytokines and using an integrative approach combining the long RNA-Seq with the miRNAs expression data we identified miR-155 as a candidate master regulator of the NPCs' inflammatory response. Th2 cytokines did not evoke any detectable effect on NPCs.
Conclusions: Identifying the inflammatory fingerprint of NPCs, and understanding its impact on neural stem cell biology, will help to understand the dysfunction of the endogenous brain stem cell compartment in experimental inflammatory disorders of the CNS. We envisage that such an integrated approach will have realistic chances to help us towards the development of novel reparative therapeutic approaches aimed at restoring the function of the endogenous brain stem cell compartment.

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Comparative study on human mesenchymal cells treatment in a murine model of multiple sclerosis

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Multiple sclerosis (MS) is a chronic neurological disorder characterized by focal lesions with inflammation, demyelination and neurodegeneration of the central nervous system (CNS). The current treatments for MS are only partially effective and this results in chronic disability and irreversible axonal and neuronal damage. A primordial interest in the MS treatments would be to provide mechanisms for remyelination and regeneration.

Human mesenchymal stem cells (hMSCs) are an attractive approach for future treatments of MS due to their immunomodulatory and neuroprotective properties. Several studies have demonstrated the usefulness of these cells (syngeneic and xenogeneic MSCs) in experimental autoimmune encephalomyelitis (EAE). Therapeutic protocols considerably differ and comparative studies about these protocols are limited.

The use of hMSCs may offer multiple theoretical advantages, however, several experimental questions might be considered related to cell size, number of cells, day and route of administration.

In this study, we used hMSCs to treat myelin oligodendrocyte glycoprotein MOG_{35-55} induced EAE in C57BL/6 mice. We compared three routes of administration (intravenous, intraperitoneal and intracardiac), different numbers of cells (from 0, 5 to 2^6 cells) and transfer in different days of the disease course: 2, 7 and 14 days after EAE induction. Bone marrow was used as a source of hMSCs, and cells were cultured and expanded up to passage 5 and 6.All mice were evaluated daily for EAE signs and weight loss.

All untreated EAE mice developed full blown EAE with severity scores ranging from 2 to 4, in a scale of 0–6. The clinical course of EAE mice treated with 0.5-1.0^6 cells was similar to the one of untreated EAE animals, independently of the route used. However, intravenous delivery of 2×10^6 hMSCs up to day 14 post induction achieved a marked reduction of the clinical severity in EAE mice, and compared to the other routes proved to be the safest and most reliable route for cell administration.

hMSCs should be expanded and criopreserved due to the large numbers of cells used in the treatment of this EAE model. These cells have previously been reported to be susceptible to cryopreservation and they can undergo a limited number of passages before their potential is compromised. In this study, we have repeatedly observed the differences using “fresh” and freeze-thawed cells.

These results suggest the preferential use of the intravenous route with not less than 2x10^6 cells avoiding repeated freezing and thawing for maintenance of their anti-inflammatory and immunomodulatory characteristics when using hMSCs to this EAE model.

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β2-microglobulin regulates the age-related decline in adult neurogenesis and cognitive function

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Objective: The discovery of neural stem cells (NPCs) in the adult brain has incited possibilities for regenerative therapeutics aimed at restoring cellular and associated cognitive functions in the aging nervous system. However, advancements must first be made in understanding how normal environmental changes during aging alter the regenerative capacity of NPCs. Previously, using a proteomic approach to profile changes in aging blood, together with heterochronic parabiosis – in which the circulatory systems of young and old animals are connected – we identified β2-microglobulin (B2M) as a potential age-related regulator of neurogenesis. The goal of this study was therefore to investigate the role of B2M in mediating the decline in adult neurogenesis, and impairments in learning and memory, observed during aging.
Methods: Here we use primary NPC models to investigate whether direct exposure to recombinant B2M is sufficient to inhibit NPC proliferation and neural differentiation *in vitro*. We combine a pharmacological approach with stereotaxic injections into the dentate gyrus of the hippocampus to examine whether direct exposure to B2M inhibits adult neurogenesis *in vivo*. Subsequently, using the radial arm water maze and fear conditioning paradigms we examine the effect direct exposure to B2M has on hippocampal-dependent learning and memory. Finally, we make use of a genetic mouse model lacking expression of B2M to investigate whether the absence of B2M can mitigate the age-dependent decrease in adult neurogenesis and improve associated cognitive functions.
Results: We observed that exposure to recombinant B2M in vitro is sufficient to inhibit NPC proliferation and neural differentiation in an MHC I dependent manner. Additionally, we detected a decrease in neurogenesis in the adult hippocampus after local administration of exogenous B2M, which could be mitigated in the absence of MHC I expression *in vivo*. Furthermore, local exposure to B2M resulted in impairments in hippocampal dependent learning and memory. Lastly, we observed an age-dependent enhancement in hippocampal neurogenesis, and associated cognitive processes, in old mice lack B2M expression.
Conclusions: Cumulatively, our data indicate that B2M negatively regulates adult hippocampal neurogenesis and associated cognitive process during aging.

T cells

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Characterization and behavior of T regulatory cells in HTLV-1 associated myelopathy/tropical spastic paraparesis

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Objective: HTLV-1 primarily infects primarily CD4+CD25+ T cells leading to proliferation and activation. A subset of these cells are regulatory (typically CD4+CD25+hi -Tregs), and have been shown to have decreased suppressive capacity in HAM/TSP patients as compared to normal donors (NDs). Due to the inflammatory component of HAM/TSP, markers normally used to characterize Tregs, such as CD25, CTLA4 and GITR are limiting in identifying T regulatory cells. To more precisely characterize Treg cells, we used a novel method that analyzes the methylation status of specific CpGs in the FoxP3 locus in CD4+CD25+ T cells that stably express FoxP3. Tregs stably express FoxP3 which is associated with a completely demethylated locus.
Methods: CD4+CD25+ cells were enriched as the most likely subset to contain these cells and then the DNA of these cells were analyzed by qPCR with primers directed against the Treg cells specific demethylation region (TSDR).

Results: We show that there is decreased demethylation in this population from HAM/TSP patients as compared to NDs, despite the increased CD4+CD25+ population size in HAM/TSP. Further we show that this correlates with an overall expanded population of CD4+CD25lo cells with no change in the CD4+CD25hi group between NDs and HAM/TSP patients.
Conclusions: Despite having a similar percentage of Tregs (as defined by CD4+CD25hi) compared to NDs, HAM/TSP patients have a lower number of functional Tregs, as noted by a lower %unmethylated in the FoxP3 locus. It may be that in HAM/TSP patients, Tregs are unable to suppress the overwhelming number of inflammatory cells present in the disease.

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Defining the T-cell effect of the MS associated SNP, rs6897932, on IL7/IL7Rα signaling

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Objective: Multiple sclerosis (MS) is an autoimmune disease that is caused by myelin loss in the central nervous system and results in severe disability. Recent genetics studies have confirmed an association between a non-synonymous SNP in the interleukin 7 receptor alpha chain (*IL7Rα*) gene, rs6897932 (C/T), and the risk of developing MS. *IL7Rα* encodes a transmembrane protein that is expressed in several immune cell subpopulations. Signaling via the IL7 pathway plays a crucial role in the maintenance of immune system homeostasis. The associated SNP occurs within exon 6 of *IL7Rα* which encodes the transmembrane domain of the receptor. The non-synonymous risk allele causes a T244I amino acid change that results in increased skipping of exon 6 and elevated levels of the soluble isoform of the protein. Based on the autoimmune characteristics of MS and the important role of *IL7Rα* in the immune system homeostasis, the aim of our study is to investigate the functional role of the associated SNP to MS pathogenesis in T cell subpopulations.
Methods: To verify the impact of rs6897932 on IL7/IL7Rα signaling we investigated the levels of phosphorylated STAT5 by flow cytometry in primary T cell cultures after IL7 stimulation. Naïve and memory CD4+ and CD8+ T cells were isolated from peripheral blood of relapsing-remitting MS patients and controls homozygous for the alternative rs6897932 alleles.
Results: Our preliminary analyses in naïve CD4+ T cells show that STAT5 phosphorylation is increased about 2-fold in subjects carrying the risk allele C. Focusing specifically on individuals with the same genotype, we have observed a 1.5 fold decrease of phosphorylated STAT5 in homozygous 'TT' patients compared to controls; whereas, no differences are seen among patients and controls homozygous 'CC'. A similar trend was also detected in naïve CD8+ and memory CD4+ T cells.
Conclusions: Based on our observations we speculate that the IL7Rα activity is affected by the two alternative alleles of rs6897932. Specifically, the presence of the T allele causes the change of the threonine (polar) with isoleucine (nonpolar) within the transmembrane domain. This modification might affect the protein folding resulting in a decrease of the receptor activity that can be explained as less accessibility of the intracellular domain to STAT5; and/or decrease the stability of the receptor on the cell membrane. However, further analyses are being carried out to verify and confirm our preliminary results.

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Interleukin-15 amplifies the pathogenic activity of CD4+CD28− T cells in multiple sclerosis patients

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Objective: Ageing of the immune system, or immunosenescence, contributes to the increased morbidity and mortality seen in the elderly. Premature immunosenescence is shown to occur in patients with autoimmune diseases including multiple sclerosis (MS). The main characteristic of immunosenescence is the expansion of $CD4^+CD28^-$ T cells in the peripheral blood. We demonstrated that these cells accumulate in brain lesions of MS patients. $CD4^+CD28^-$ T cells have a cytotoxic profile and share many properties with natural killer (NK) cells. These features include intracellular stores of the cytotoxic molecules perforin and granzyme B, and the expression of NKG2D, an activating NK cell receptor. For NK cells, it is known that NKG2D expression and cytotoxicity are increased in response to interleukin (IL)-15. Moreover, IL-15 is upregulated in the serum and cerebrospinal fluid of MS patients. The aim of our study is to investigate whether IL-15 enhances cytotoxicity of $CD4^+CD28^-$ T cells as seen for NK cells.
Methods: Flow cytometric analysis was performed to investigate proliferation, expression of cytotoxic molecules and degranulation of $CD4^+CD28^-$ T cells in response to IL-15. Coculture with the NKG2D ligand expressing cell line U251 assessed the contribution of NKG2D ligation to degranulation of $CD4^+CD28^-$ T cells. To identify IL-15 producing cells in the brain, immunohistochemistry was performed on MS lesion tissue and normal brain tissue of non-demented controls.
Results: Our in vitro results show that IL-15 preferentially induces proliferation of $CD4^+CD28^-$ T cells as compared to their $CD28^+$ counterparts. Phenotypically, IL-15 significantly increases expression of NKG2D, perforin and granzyme B by $CD4^+CD28^-$ T cells. Also, the production of interferon-gamma is increased, and this increase is significantly higher in MS patients. When IL-15 is presented to $CD4^+CD28^-$ T cells, the release of cytotoxic granules from the cells is significantly enhanced. This degranulation is not dependent on NKG2D ligation, but blocking of NKG2D diminished this process. In MS lesions, we found that microglia/macrophages are the main IL-15 producing cells, and that $CD4^+$ T cells are found in close proximity to them, suggesting in vivo stimulation.
Conclusions: In summary, our findings indicate that $CD4^+CD28^-$ T cells, which accumulate in brain lesions of MS patients, are functionally boosted by IL-15 producing cells. This process amplifies their cytotoxicity, contributing to local damage.

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CD8+ effector and memory T cell subsets in relapsing-onset Multiple Sclerosis

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Objective: In this study we evaluated the percentages of the different subsets of $CD8^+$ T effector and memory cells expressing T-bet and Eomesodermin (Eomes) during the course of relapsing-onset Multiple Sclerosis (MS). Then we correlated the percentages of these $CD8^+$ T cell subsets with age, gender, disease duration and disability score.

Methods: We analyzed by flow cytometry the percentages of circulating $CD8^+CCR7^-CD127^{low}$, (Short Lived Effector Cells,SLEC), $CD8^+CCR7^-CD127^{high}$ (Memory Precursor Effector Cells,MPEC) and $CD8^+CCR7^+CD127^+$ (Central Memory,CM) T cells in 71 patients affected by relapsing–remitting MS (RRMS) in different phases of disease, 25 patients with secondary progressive MS (SPMS) and in 54 age and sex-matched healthy subjects (HS). In a subgroup of RRMS and SPMS patients we evaluated the expression of T-bet and Eomes in the different subsets of $CD8^+$ T cells.
Results: We found higher percentages of circulating $CD8^+$ SLEC in MS patients with RRMS and SPMS compared to HS. $CD8^+$ CM were increased in MS patients compared to HS. The percentages of $CD8^+$ CM were higher in RRMS in both phases of disease compared to SPMS patients and HS. The percentages of $CD8^+$ MPEC and CM positively correlated with age in controls but not in relapsing–onset MS patients. The percentage of circulating $CD8^+$ CM correlated negatively with EDSS score in RRMS and SPMS patients in stable phase of the disease. Circulating T-bet^+ SLEC were increased in relapsing RRMS patients and SPMS patients. T-bet^+ MPEC were also increased in relapsing RRMS patients. The percentages of circulating $CD8^+$ MPEC and $Eomes^+$ CM were higher in RRMS in both phases of the disease compared to SPMS patients.
Conclusions: We found higher percentages of circulating $CD8^+$ SLEC in MS patients with RRMS and SPMS compared to HS. $CD8^+$ CM were increased in MS patients compared to HS. The percentages of $CD8^+$ CM were higher in RRMS in both phases of disease compared to SPMS patients and HS. The percentages of $CD8^+$ MPEC and CM positively correlated with age in controls but not in relapsing–onset MS patients. The percentage of circulating $CD8^+$ CM correlated negatively with EDSS score in RRMS and SPMS patients in stable phase of the disease. Circulating T-bet^+ SLEC were increased in relapsing RRMS patients and SPMS patients. T-bet^+ MPEC were also increased in relapsing RRMS patients. The percentages of circulating $CD8^+$ MPEC and $Eomes^+$ CM were higher in RRMS in both phases of the disease compared to SPMS patients.

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Termisartan inhibits human Th17 differentiation

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Objective: To determine whether telmisartan (TLM) inhibits human Th17 differentiation.
Background: T helper cells secreting interleukin (IL)-17 (Th17) play a crucial role in the pathogenesis of autoimmune diseases such as multiple sclerosis. In addition, Th17 is associated with secondary inflammation induced by cranial infarction. TLM can be pharmacologically categorised as an angiotensin II type 1 receptor blocker (ARB) as well as a peroxisome proliferator-activated receptor gamma (PPER gamma), commonly used as antihypertensive medication. Previous studies demonstrated that PPER gamma agonists (pioglitazone) and other ARBs inhibit Th17 differentiation. However, the effect of telmisartan on Th17 differentiation remains unknown.
Methods: Naïve T cells were isolated from five healthy volunteers using magnetic beads kit. For Th17 differentiation, the naïve T cells were stimulated by anti-CD3, anti-CD28, IL-1beta, IL-6, IL-21, IL-23, and tumour growth factor beta 1, with/without TLM.

The Th17 population was measured by flow cytometry using anti-IL-17 antibodies.

The activated form of phospho-nuclear factor (NF)-κB in the cell lysate was measured using enzyme immunoassay.

Total RNA was isolated from stimulated cells, and mRNA expression of IL-17 and RAR-related orphan receptor (ROR) gamma t was measured using a quantitative real-time polymerase chain reaction method.
Results: Following stimulation with cytokines with/without TLM for 1 week, IL-17+ cell populations were measured using flow cytometry. The number of IL-17+ cell populations was lower when TLM was used for stimulation compared with that when TLM was not used. To analyse the mechanism by which TLM inhibits Th17 differentiation, phospho-NF-κB level was measured after a 30-minute TLM simulation, and it was found to be significantly higher after TLM stimulation. Relative mRNA expression of IL-17 was significantly decreased by TLM stimulation, and this effect was blocked by BAY-11-7085 (NF-κB inhibitor).
Conclusions: These findings suggest that TLM inhibits Th17 differentiation via the NF-κB pathway, and it may regulate harmful secondary inflammation induced by cranial infarction in patients with ischemic stroke.

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The inhibitory effect of Tim-3 signaling in Th1 cells and activated CD8+ T cells in EAE

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Objective: Multiple sclerosis (MS) is an inflammatory, demyelinating disease of the CNS that is mediated by autoreactive, myelin-specific CD4+ and CD8+ T cells. Tim-3 is a cell-surface molecule expressed on Th1 cells, activated CD8+ T cells, microglia, and DCs. Tim-3 signaling has been shown to induce Th1 cell death and ameliorates Th1-mediated experimental autoimmune encephalomyelitis (EAE), an animal model for MS. However, the function of Tim-3 in other cell types is not known. We study the function of Tim-3 in myelin-specific CD8+ T cells, DCs, and microglia using both CD4+ and CD8+ T cell-mediated EAE models.
Methods: CD8+ T cell mediated EAE can be actively induced with vaccinia virus infection in MBP-specific transgenic mice. CD4+ T cell-mediated EAE can be actively induced with rMOG/CFA immunization and pertussis toxin. Two types of EAE can be induced with this method: classic EAE is characterized by spinal cord inflammation and Th17:Th1 cell ratio close to 1; atypical EAE is characterized by inflammation localized both in the brain and spinal cord with Th17:Th1 cell ratio higher than 1 in the brain.
Results: Tim-3 signaling induces cell death in activated MBP-specific TCR transgenic CD8+ (8.8) T cell. Upon CD8+ T cell-mediated EAE induction, Tim-3 KO 8.8 mice had an earlier disease onset compared to WT mice due to an increased accumulation of MBP-specific CD8+ T cell in the CNS. Furthermore, Tim-3 blocking antibody treatment in vivo exacerbates CD8+ T cell-mediated EAE. In CD4+ T cell-mediated EAE induction, Tim-3 KO mice exhibit a lower incidence of atypical disease compared to WT mice. An increased numbers of Th1 cells were found in the brains of Tim-3 KO mice compared to WT mice but the numbers of Th17 cells were not different. Our data suggest that the lack of Tim-3 signaling in Tim-3 KO mice allows Th1 cells to accumulate and lower the Th17 to Th1 cell ratio in the brains, resulting in a lower incidence of atypical EAE in Tim-3 KO mice compared to WT mice.
Conclusions: Tim-3 signaling negatively regulates autoreactive, myelin-specific CD8+ T cells in CNS autoimmunity. Furthermore, this is the first time Tim-3 signaling in CD4+ T cells has been shown to affect

the localization of inflammatory lesions in CNS autoimmunity. Our results in both CD4+ T cell- and CD8+ T cell-mediated EAE models have implications for therapeutic strategies targeting Tim-3 in MS.

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Immune regulatory functions of neuronal adhesion molecule Amigo2 and its role in immune diseases

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Objective: The nervous and immune systems are communicating with each other constantly and delicately to maintain their own homeostasis. So far, many molecules have been identified to be expressed and utilized by both systems. In this research, we aim to elucidate the roles played by neuronal adhesion molecules in neuroimmune cross-talk, in order to provide a novel and global understanding of the interaction between these two systems, especially how neurons control immunity. AMIGO2 belongs to the amphoterin-induced gene and ORF (AMIGO) protein family, which was originally found to be induced by the high mobility group box-1 in rat hippocampal neurons. AMIGO2 is expressed mostly by endothelial, neuronal and immune cells. It has been shown to inhibit apoptosis and promote neuronal survival and its *de novo* deletion may lead to mental retardation, while little is known about its immune regulatory function.
Methods: To study the functions of AMIGO2, we generated a conventional AMIGO2 knockout (KO) mouse line by replacing the Amigo2 gene with alkaline phosphatase reporter gene. To investigate AMIGO2's role in T cell development, we immunophenotyped the splenocytes and thymocytes from AMG2KO mice by flow cytometry. To address its role in T cell activation and proliferation, we stimulated the splenocytes with CD3 ligation or ConA and checked the percentage of activated or proliferated T cells. We also tested the survival rate of AMG2KO T cells *in vitro*. To further check whether AMIGO2 has any immune function *in vivo*, we used the acute experimental autoimmune encephalomyelitis (EAE) model by immunizing mice with MOG_{35-55} peptide emulsion.
Results: T cells were slightly increased in AMG2KO mice. AMG2KO T cells were significantly more activated and proliferative in response to CD3 ligation or ConA stimulation. In addition, AMG2KO T cells survived better under mild stimulation. Interestingly, AMG2KO mice had delayed onset of the autoimmune disease development. Currently, we are studying AMIGO2's role in regulating T cell differentiation and characterizing the molecular mechanisms of its immune regulatory functions, which may help us interpret the *in vitro* and *in vivo* results.
Conclusions: Our preliminary results suggest that the absence of Amigo2 leads to enhanced T cell survival, activation, and proliferation *in vitro*, and increased amount of T cells and more resistance to EAE autoimmune disease *in vivo*.

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Recovery of the T cell repertoire in chronic inflammatory neuropathies during treatment with intravenous immunoglobulins

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Objective: Chronic inflammatory demyelinating polyneuropathy (CIDP) is an autoimmune disorder of the peripheral nervous system (PNS) with pronounced heterogeneity of disease severity and treatment response. The pathogenic role of T cells remains controversial. Intravenous immunoglobulins (IVIg) are effective in CIDP but their effect on antigen specificity of PNS autoreactive T cells is unknown.
Methods: The T cell receptor (TCR) repertoire of $CD4^+$ and $CD8^+$ T cells in the peripheral blood was analyzed using CDR3 spectratyping. CIDP patients were included without and with IVIg treatment. To simplify the spectratyping analysis, we introduced a new classification system of alterations in the TCR length distribution and correlated this with the statistical values skewness and kurtosis.
Results: While the TCR length distribution of CIDP patients was only moderately altered for most of the Vbeta elements of $CD4^+$ T cells, the $CD8^+$ population displayed extensive oligoclonal expansions in all analyzed 24 Vbeta elements. Treatment with IVIg reduced the oligoclonal expansions within both the $CD4^+$ and $CD8^+$ population. The evaluation of the simplified analysis of spectratyping data correlated with the degree of alterations.
Conclusions: Our data demonstrate that cytotoxic $CD8^+$ T cells exhibit a much broader activation than $CD4^+$ T cells indicating a potentially crucial role of $CD8^+$ T cells in the immunopathogenesis of CIDP. The profound oligoclonal response in T cell activation suggests that multiple peptides may induce and propagate this autoimmune driven disease. The observed reduction of highly activated T cells may contribute to the therapeutic effects of IVIg. The validation of the classification system integrated in a reduction the of time consuming analysis of spectratyping data.

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Posttranslational modifications of myelin antigens and their role in MS pathology

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Objective: Our study aims at characterizing the adaptive immune response towards citrullinated myelin proteins in multiple sclerosis (MS). More precisely, specific T cell responses towards modified peptides were assessed for a pathophysiological role in disease progression. Autoimmune diseases, such as MS, might appear from breakdown of immunological self-tolerance, induced by recognition of self-proteins that have undergone posttranslational modifications (PTM) under inflammatory conditions. Citrullination is such a PTM, a process catalyzed by peptidylarginyl deiminase enzymes, whereby arginine is deiminated to citrulline. In MS, disease severity seems to follow the citrullination degree of myelin basic protein (MBP), T cell responses to citrullinated MBP are detected in MS patients, and citrullination of MBP epitopes elicit stronger Th1 responses in vitro. Hence, given the high level of citrullinated MBP protein in brain tissue of MS patients, a predominant response to *de novo* formed citrullinated MBP-peptides may be present and could promote disease.
Methods: A set of immunodominant peptides of MBP and myelin oligodendrocyte glycoprotein (MOG) where generated in a non-modified and citrullinated version. Cytokine production and T cell phenotype was subsequently assessed by cytometric bead array in peptide-stimulated peripheral blood mononuclear cells (PBMCs) of MS patients and healthy controls.
Results: Preliminary results revealed a set of peptide pairs that significantly modulated cytokine responses in MS patients compared to healthy controls. Patients stratified according to HLA types and

disease progression will be further screened with a newly developed technique based on flow cytometry, which allows for detection of activated T cells present at very low frequencies.
Conclusions: Taken together, using such modified myelin peptides as antigens, we are able to study MHC class II peptide interactions and T-cell immunity, and we are provided with leads to study fine specificity and regulation of T- and B-cell responses in different stages of disease development in humans.

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Autoreactive T cell maturation prevented in CCL2-expressing thymus

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Objective: CCL2 is a chemokine that induces the recruitment of immune cells to tissues during inflammation. CCL2 is implicated in experimental autoimmune encephalomyelitis (EAE), a mouse model of multiple sclerosis. Mice lacking CCL2 or its receptor CCR2 are resistant to EAE.

Dendritic cells (DC) are involved in autoreactive T cell deletion in the thymus. Subpopulations of DC express CCR2 and can migrate to thymus.

We studied transgenic mice that ectopically express CCL2 in thymus (MBP/CCL2). Our goal was to investigate whether thymic expression of CCL2 induced recruitment of DC and tolerized autoreactive T cells.
Methods: We immunized C57Bl/6 and transgenic mice with MOGp35-55 (myelin oligodendrocyte glycoprotein) to induce EAE and analyzed CD4 T cell response using flow cytometry and CFSE dilution. Thymic cell populations were separated by Percoll gradient centrifugation and transcripts were measured by quantitative PCR.
Results: Transgenic MBP/CCL2 mice were resistant to EAE. MBP/CCL2 crossed with 2D2 mice, whose T cells express a T cell receptor (TCR) specific for MOGp35-55, were also resistant to EAE. Lymph node cells from MOG-immunized mice that ectopically expressed CCL2 in thymus proliferated poorly in response to MOGp35-55 *in vitro*.

We showed that MOG-specific $CD4^+$ T cell development was severely impaired in 2D2xCCL2 mice. We also showed that CCL2 transcripts were expressed in thymic stromal cells that include thymic epithelial cells, DC, macrophages, fibroblasts. To confirm that CCL2 was specifically involved in regulation of autoreactive T cell development, we crossed MBP/CCL2 with OT2 mice, whose T cells express a TCR specific for the non-self-antigen ovalbumin (OVA). CCL2 transcripts were not expressed in thymus and OVA-specific $CD4^+$ T cells were relatively unaffected.
Conclusions: We conclude that in CCL2 transgenic mice, interaction between autoreactive thymocytes and thymic stromal cells induces ectopic expression of CCL2, which is involved in impairment of autoreactive T cell development and leads to protection against EAE. This identifies a novel role for CCL2. Our goal now is to investigate which thymic stromal cell(s) are involved, in order to determine how autoreactive T cell development is impaired, which could lead towards new therapeutic targets for multiple sclerosis.

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MAIT cells display proinflammatory characteristics in multiple sclerosis

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Objective: Multiple sclerosis (MS) is an autoimmune disease that affects the central nervous system. We have previously shown that in MS patients a specific subset of $CD8^+$ T cells expressing high levels of CD161 is significantly more frequent than in healthy donors. These consists of a population of T cells, mostly present in the gut, that display a semi-invariant T cell receptor (Vα7.2), named Mucosal-associated invariant T (MAIT) cells, that recognize bacterial products loaded on the evolutionarily conserved MHC-related molecule 1 (MR1).
Methods: In this study we show that MAIT cells in MS patients are stronger producers of proinflammatory cytokines compared to healthy donors. For this purpose, we studied this cell subset from peripheral blood of MS patients and healthy donors. We observed different patterns of cytokine production from these cells, *ex-vivo* and/or *in-vitro*, through flow cytometric analysis of samples obtained from homozygotic twins discordant for MS.
Results: In homozygotic twins discordant for MS, the affected twin consistently shows a higher frequency of proinflammatory MAIT cells in the peripheral blood. This expansion could be due to stimulation of this cell subset by bacterial strains with prominent pro-inflammatory inducing abilities. The "hygiene hypothesis" states that the modification in the lifestyle of western countries, with improved sanitation, may have altered the colonization of intestinal microorganisms, leading to an imbalance between tolerogenic and inflammatory members of the microbiota. Thus, it is possible to hypothesize that in MS patients such an imbalance favours the generation and amplification of proinflammatory effector cells whose effects reach way beyond the intestinal mucosa. The result of such stimulation may thus be represented by the expanded population of IL-17 + MAIT cells, which are a unique "handle" to haul research efforts in this direction. The functional studies performed ex vivo also suggest that in the affected twin MAIT cells produce higher levels of proinflammatory cytokines, further adding strength and vigour to the immune response.
Conclusions: These results are in agreement with the hypothesis that dysbiosis of the gut microbiota may determine a dysfunction of mucosal responses and consequently may favour the development of systemic inflammatory and autoimmune diseases.

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Th17-mediated autoimmunity involves separate phases of inflammation driven by distinct populations of IL-17-producing T cells

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Objective: We have previously shown that NR4A2 (Nurr1) is among the most highly upregulated genes in peripheral blood T cells from patients with multiple sclerosis (MS). In the murine model of MS, experimental autoimmune encephalomyelitis (EAE), pathogenic T cells infiltrating the central nervous system (CNS) express NR4A2. In this study, we examined the role of NR4A2 in controlling pathogenic T cell differentiation that causes autoimmune inflammation of the CNS.

Methods: We generated C57BL/6 mice that have a CD4-specific deletion of NR4A2. We immunised these mice with MOG peptide to induce EAE then monitored clinical disease and examined the responses made by T cells infiltrating the CNS. Using *in vitro* cell culture, we tested a requirement for NR4A2 in different types of T cell differentiation, to produce IL-17-secreting T cells (Th17 cells).
Results: When EAE was induced in CD4-specific NR4A2 conditional knock-out mice, large numbers of T cells infiltrated into the CNS, but such cells produced reduced levels of IL-17. Critically, despite such infiltration, these NR4A2$^{-/-}$ mice were protected from acute clinical EAE. However, such mice instead developed a late onset chronic EAE, with clinical symptoms commencing several weeks after the conventional peak EAE and establishment of IL-17 production by CNS-infiltrating T cells. Additionally, NR4A2 appears to be required for conventional Th17 differentiation that results from activation of naïve T cells in the presence of IL-6 and TGF-β. However, alternative Th17 differentiation, where IL-1β, IL-6, and IL-23 is added to T cell cultures, is unaffected by NR4A2 deficiency.
Conclusions: We have demonstrated that NR4A2 is required for both early/acute autoimmune inflammation and conventional TGF-β-driven Th17 differentiation, but not for late/chronic disease or for the development of alternative IL-23-driven Th17 responses. Taken together, our data indicate that MOG-induced EAE might result from different phases of inflammatory responses that are generated by distinct populations of IL-17-producing T cells. Thus, NR4A2 might prove an effective target to manipulate early/acute inflammation during autoimmune disease.

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Multiparametric analysis of Th17 and Th22 cells discloses their pathogenicity in human multiple sclerosis

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Objective: Immunological evaluation of multiple sclerosis (MS) patients suggested that several T helper (Th) subsets were involved during MS. We have already shown that Th17 and Th22 cells increased in the peripheral blood (PB) of patients with clinical signs of an MS exacerbation. However, most of the data regarding the pathogenic potential of these subsets comes from MS murine models. The aim of this study was to evaluate several molecules that identify encephalitogenic cells in human Th17 and Th22 cells.
Methods: PBMC and cerebro-spinal fluid (CSF) from Relapsing Remitting MS patient and healthy subjects (HS) were collected and analyzed for cytokine production, for self-reactivity, for the expression of chemokine and cytokine receptors and transcriptional factors, and for the response to Interferons. Th cell clones were generated from active MS patient and HS peripheral blood and evaluated for the same parameters.
Results: Th17 and Th22 cells expanded in MS were specific for the auto-antigen myelin basic protein (MBP), enlarge in the CSF, and expressed higher amount of Tβet and CCR6 compared to Th17 and Th22 cells from HS. Moreover, in MS patients, Th17 and Th22 cells co-secrete IFNγ and GM-CSF but not IL-10. Conversely to Th17 cells, Th22 cells and clones displayed lower level of IFNAR1 and were less sensitive to the inhibition exerted by IFNβ, whereas both subset expanded in MS acquire the expression of IFNγR2 that render them sensitive to IFNγ.
Conclusions: The increase in cell number, the co-expression of CCR6, Tβet, IFNγ, and GM-CSF as well as the ability to respond to MBP, all indicate the common pathogenic properties of Th17 and Th22 cells in human MS. However, whereas Th17 cell can be inhibited by IFNβ, the expansion of Th22 cells could be one of the factors that critically influence the resistance to IFNβ therapy.

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TCR-mediated cell death is differentially regulated in T helper 1 versus T helper 17 cells

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Objective: T cell immune response results from activation of naive CD4 T cells in functionally distinct T helper (Th) subsets. The control of the homeostasis of the inflammatory Th cells, and in particular of the Th17 cells, is crucial to avoid autoimmune inflammation.

Limited information is available on the homeostatic signals that regulate survival and cell death of Th cell subsets to control their expansion.

We studied the mechanisms regulating cell death sensitivity of distinct human Th subsets.
Methods: We have developed a novel strategy to obtain large numbers of clones of all Th subsets, which we expand in vitro. We analysed apoptosis induced by T cell receptor (TCR) stimulation of different Th clones. In order to investigate the mechanisms regulating cell death sensitivity, we analysed the cell death pathway and the mechanisms potentially involved, in each Th profile.
Results: We found that human Th17 clones are more resistant to apoptosis compared to other Th subsets. Th1 clones are particularly sensitive to apoptosis and they express high levels of cleaved Caspase-8 and Caspase-3, which correlates with the cleavage of its substrate, poly(ADP-ribose) polymerase-1. We found that FAS is the receptor mostly expressed by all Th profiles. Interestingly, Th1 cells upon TCR stimulation specifically express FAS ligand. We found that this process is affected in human Th17 cells. Moreover, we found that the anti-apoptotic protein caspase-8 (FLICE)-like inhibitory protein (FLIP) contribute to conferring resistance to cell death in human Th17 cells.
Conclusions: We found that human Th17 clones are more resistant to apoptosis compared to other Th subsets. Th1 clones are particularly sensitive to apoptosis and they express high levels of cleaved Caspase-8 and Caspase-3, which correlates with the cleavage of its substrate, poly(ADP-ribose) polymerase-1. We found that FAS is the receptor mostly expressed by all Th profiles. Interestingly, Th1 cells upon TCR stimulation specifically express FAS ligand. We found that this process is affected in human Th17 cells. Moreover, we found that the anti-apoptotic protein caspase-8 (FLICE)-like inhibitory protein (FLIP) contribute to conferring resistance to cell death in human Th17 cells.

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The IDH1 mutation as a potential target for immunotherapy of malignant glioma

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Objective: Gliomas are incurable brain tumors which inevitably develop resistance towards radiochemotherapy. About 80% of low-grade and anaplastic gliomas carry a mutation in the *isocitrate dehydrogenase 1* (*IDH1*) gene (IDH1R132H). With the aim of IDH1R132H-targeted vaccination therapy, we evaluated the immunogenic potential of IDH1R132H and characterized the IDH1R132H-specific cellular and humoral response.
Methods: IDH1R132H MHC-specific epitopes were identified from a peptide library in vitro and A2.DR1 mice expressing human MHCI and II were vaccinated with IDH1R132H peptide. T cell cytokine responses were assessed by IFNγ ELISpot, cytokine ELISA and intracellular flow cytometry. IgG production was detected in serum by peptide ELISA. IDH1R132H+ glioma patients were screened for humoral and cellular IDH1R132H-specific responses.
Results: IDH1R132H 10mer peptides did not bind to MHCI nor stimulated IFNγ production in PBMC from IDH1R132H+ glioma patients, but 15mer IDH1R132H MHCII – DR1 – epitopes were identified by class II binding assays. One glioma patient had a CD4+ T cell-mediated IDH1R132H-specific IFNγ response. In A2.DR1 mice, CFA-peptide vaccination with an IDH1R132H 20 mer induced a mutation-specific IFNγ response to DR1-binding peptides but not 10 mer peptides. Flow cytometric and ELISA analyses revealed a T helper response, specifically Th1, Th2, and Th17, but no induction of regulatory T cells (Treg). Using the clinical adjuvant Montanide ISA51 with TLR7 agonist Imiquimod and rmGM-CSF, IDH1R132H 20mer peptide vaccination induced CD4+ T cell IFNγ responses of the same IDH1R132H specificity. Neither Treg nor Th17 nor Th2 responses, but IDH1R132H-specific cytotoxic T cells were detected. From splenocytes of vaccinated mice, CD4+ T cell lines, containing Th1 and Th17, and one T cell clone maintaining IDH1R132H-specificity, were generated. Anti-IDH1R132H serum IgG1 was induced by vaccination. In line, IDH1R132H-specific serum IgG could be detected in IDH1R132H+ glioma patients but not in patients with histologically identical IDH1 wild-type tumors.
Conclusions: We found mutation-specific T helper and humoral responses against IDH1R132H in IDH1R132H-vaccinated MHC-humanized mice and IDH1R132H+ glioma patients. Therapeutic efficacy of peptide vaccination will be assessed in IDH1R132H+ tumor-bearing A2.DR1 mice. Detection of IDH1R132H-specific IgG in patients implies development of a serum test for diagnostics and monitoring during IDH1R132H immunotherapy.

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Immune-regulation of CD39 on Regulatory T cells

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Objective: CD39 is a membrane bound adenosine triphosphate (ATP) degrading enzyme present on various immune cells. Relatively, it is expressed in high amount on conventional regulatory T cells where in concert with CD73 produces immunosuppressive adenosine from inflammatory ATP molecule. It has been shown in various studies that regulatory T cells mediate partially their suppressive effect on target cells through CD39. However, It has not been shown whether CD39 is expressed on other regulatory T cells and how its expression is regulated. In present study we investigated CD39 expression regulation in regulatory T cells and its roles in experimental allergic encephalomyelitis (EAE).
Methods: We induced *in-vitro* Foxp3$^+$ T cells and Tr-1 (IL-10 producing cells) from naive T cells isolated from mice expressing GFP under FoxP3 or under IL-10 promoter (Tr-1). Real-time PCR was used to assess CD39 expression. We also tested CD39 expression on naturally occurring Treg (FoxP3-GFP positive cells). We crossed FoxP3-GFP knock-in mice to CD39 knockout to assess how lack of CD39 exclusively on FoxP3 positive cells will change its suppressive function on target cells. We isolated FoxP3-GFP positive T cells from CD39 knockout mice and performed suppressive assay and compared it to WT isolated FoxP3-GFP cells. To assess how CD39 expression is regulated on Treg cells, we isolated naïve T cells from interferon-gamma (IFN-ϒ) knockout mice and *in-vitro* induced them to Treg phenotype and later assessed CD39 expression level using flow cytometry. We also induced EAE in CD39 knockout, CD39 heterozygous and WT mice and followed the disease progression for 40 days for clinical score.
Results: We found that CD39 gene is expressed in high amount in both naturally occurring and *in-vitro* induced FoxP3 positive cells and surprisingly also expressed by Tr-1 regulatory T cells. We also found lack of CD39 exclusively on FoxP3 cells makes them less suppressive toward target T cells. Lack of IFN-ϒ decreases expression of CD39 on Treg cells. CD39 knockout and heterozygous mice shows heightened susceptibilities to EAE.
Conclusions: CD39 is a predictable marker for suppressive function of Treg cells (both FoxP3 and Tr-1 cells). It is also found to be important for their suppressive function as lack of CD39 on pure population of FoxP3 cells makes them less suppressive. It is interesting to find that IFN-ϒ regulate expression of CD39 on regulatory T cells. Lack of CD39 in whole or partially makes mice more susceptible to EAE.

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Differential expression of chemokine receptors and T cell localization in the CNS in EAE

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Objective: T cell mediated autoimmune responses are involved in the pathogenesis of multiple sclerosis, and its animal model experimental autoimmune encephalomyelitis (EAE). CD4+ T cells producing IFNγ (Th1), IL17 (Th17), or CD8+ T cells can mediate the pathogenic responses. The differential expression of chemokine receptors by T cell subsets might be relevant for their localization within the CNS. CD4+ T cells in EAE are organized as perivascular clusters whereas CD8+ T cells are scattered throughout the parenchyma. CD4+ T cells were more diffusely distributed in CXCR3 KO mice, whereas CD8+ T cell distribution was unaffected (Müller et al., J Immunol. 2007). The chemokine receptors expressed by CD8+ T cells in EAE are not known. Our aim was to study the expression of chemokine receptors CXCR3 and CCR6 by CD4+ and CD8+ T cells and to determine their IFNγ and IL17 production and localization in the CNS.
Methods: EAE was induced in C57BL/6 mice by immunization with p35-55 peptide of myelin oligodendrocyte glycoprotein. Flow cytometry was used to detect the expression of CCR6, CXCR3, IFNγ and IL17 by CD4+ and CD8+ T cells in the spinal cord. Immunohistochemistry was used to study the localization of CD4+ and CD8+ T cells and double immunofluorescence staining was used to study the localization of CCR6+ and CD4+ T cells.
Results: IFNγ- and IL17-producing CD4+ T cells were detected in both CXCR3+ and CCR6+, and also in chemokine receptor negative fractions. A great majority of the CD8+ T cells expressed CXCR3 and many of them produced IFNγ. CD8+ T cells from EAE spinal cord did not express CCR6 or produce IL17. CD4+ T cells were clustered as subpial perivascular infiltrates and were also scattered in

the parenchyma. CD8+ T cells did not cluster as infiltrates, but were scattered in the parenchyma. Double immunostaining showed colocalization of CCR6 with CD4+ T cells within the infiltrates and in the parenchyma.
Conclusions: Our results add to previous findings to suggest that CXCR3 and CCR6 play a complex role in directing T cell distribution in spinal cord, which differs between CD4+ and CD8+ T cells. Whether T cell subsets identified by cytokine production localize differently and how that reflects chemokine receptor expression remains to be determined.

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T-cell responses to distinct AQP4 peptides in neuromyelitis optica (NMO)

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Objective: To evaluate the T-cell responses to specific AQP4 epitopes in patients with NMO and multiple sclerosis (MS).
Methods: Peripheral blood mononuclear cells (PBMCs) were obtained from 14 patients fulfilling the criteria for definite NMO and the proliferation responses to one of 15 distinct pentadecapeptides of AQP4, spanning the whole protein except of its transmembrane parts) were tested by a standard [H3]-thymidine uptake assay and compared with those of 9 healthy controls and 7 MS patients. A cytometric bead array assay (CBA) and flow cytometry were used to evaluate cytokine (IFNγ, IL17, IL2, IL4, IL5, IL10 and TNFα) and chemokine (CXCL8, CCL5, CXCL10, CXCL9, CCL2) secretion by PHA-stimulated PBMCs and AQP4-specific T-cell lines.
Results: Four main immunodominant epitopes of the AQP4 protein (p137–151, p222–236, p217–231 and the p269–283) were identified, in the NMO group. The first two epitopes (assigned as peptides 3 and 9) showed the highest sensitivity (~60% positivity), whereas the later two (assigned as peptides 8 and 11), the higher specificity. T-cell lines specific for the AQP4 epitopes, produced from NMO patients (but not healthy donors) secreted mainly IL-17 and IL-10 and less IFNγ.
Conclusions: Our findings indicate that T-cells bearing characteristics of both TH1 and Th17 T-cells and targeting specific immunodominant epitopes of the AQP4 protein might be involved in the pathogenesis of NMO. These findings may contribute to a better understanding of the diverse immune mechanisms involved in NMO.

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A dynamics analysis of antigen specific T cells during brain autoimmunity supports the role of regulatory T cells in disease outcome

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Objective: Multiple sclerosis (MS) is considered a T-cell-mediated autoimmune disease with a prototypical oscillatory behaviour, evidenced by the presence of clinical relapses. However, the mechanisms governing such oscillatory behavior are not understood. Our aim was to analyze the dynamics of antigen specific T cells during the course of brain autoimmunity and its implications for immunotherapy.
Methods: We analyzed by flow cytometry the time behaviour of Myelin Oligodendrocyte Glycoprotein (MOG) specific effector (T_{eff}) and regulatory (T_{reg}) T cells from the spleen and CNS as well as microglia in mice (C57B6) suffering Experimental Autoimmune Encephalomyelitis (EAE). MOG specific T cells were labeled using a mouse HLA class II tetramer containing the immunodominant MOG peptide. We compared the observations with simulations from a mathematical model of the cross-regulation of T-cell dynamics in autoimmune disease. In order to model the role of T cell dynamics in immunotherapy we analyzed the effect of anti-CD20 therapy in T cell dynamics during EAE.
Results: We found that T_{eff} and T_{reg} cells specific to MOG developed coupled oscillatory dynamics with a 4 to 5-day period and irregular amplitude that is higher for the T_{eff} populations, in agreement with the mathematical model. Microglia activation followed the oscillations of MOG-specific T_{eff} cells but with slower dynamics. Interestingly, microglia activation followed MOG T cell activation in the spleen but precedes infiltration of MOG specific T cells into the CNS. Moreover, we observed that B-cell depletion by anti-CD20 therapy decreases T_{eff} and T_{reg} cells expansion, although its oscillatory behaviour persists. The C57B6 mice immunized with MOG35-55 and treated with anti-CD20 suffer a worsening of the disease. We observed that such worsening was associated with a more important effect of B cell depletion on T_{reg} cells infiltration to the CNS.
Conclusions: In summary, the oscillatory dynamics of T cells has an intrinsic origin in the regulation of the adaptive immune response, which influences disease phenotype and response to immunotherapy.

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Characterizing IL-17-producing CD8 + T cells in multiple sclerosis

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Recent findings indicate an involvement of $CD8^+$ T cells in the pathogenesis of multiple sclerosis (MS). Particularly, IL-17^+CD8^+ T cells are significantly enriched in acute compared to inactive MS lesions. IL-17 production by $CD8^+$ T cells is associated with a high expression of the surface marker CD161. In addition, it has been reported that $CD161^{high}CD8^+$ T cells belong to the mucosal-associated invariant T (MAIT) cells carrying a semi-invariant Vα7.2 T cell receptor. Here, we first analysed the frequency of $CD161^{high}CD8^+$ T cells in the peripheral blood and cerebrospinal fluid (CSF) of MS patients, patients with other neurological diseases (OND) and healthy individuals (HI). We show that there is a significant decrease in the frequency of $CD161^{high}CD8^+$ T cells in the peripheral blood of MS patients compared with HI, while the frequencies of $CD161^{int}CD8^+$ T cells and $CD161^+CD4^+$ T cells are not different. In the CSF of MS and OND patients, $CD161^{high}CD8^+$ T cells are greatly reduced in comparison to the peripheral blood, while $CD161^+CD4^+$ T cells are significantly enriched in the CSF. By simultaneous analysis of CD161 and Vα7.2 expression on peripheral blood $CD8^+$ T cells we can confirm that most, if not all, $CD161^{high}CD8^+$ T cells express the invariant Vα7.2 chain. In contrast to $CD161^{high}V\alpha7.2^+$ MAIT cells, peripheral blood CMV-, EBV- and Flu-specific $CD8^+$ T cells, identified by HLA-A2 tetramer staining, of which a fraction express CD161, do not secrete IL-17. Taking our analysis into account, CNS-infiltrating IL-17-producing $CD8^+$ T cells in MS

likely belong to MAIT cells. The presence of the MAIT T cell receptor in MS lesions has been proven by RT-PCR analysis of autopsy samples. However, the expression of CD161 and IL-17 by tissue infiltrating MAIT cells and their enrichment in acute MS lesions need further in situ characterization.

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Cytokine levels in peripheral cell culture without B cell in myasthenia gravis patients

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Objective: In acquired myasthenia gravis (MG), the differential antibody production in disease subgroups is regulated by T cells and by related cytokine activity. In this study, T cell activity mediated by cytokine secretion is compared in patients with autoantibodies (Abs) to acetylcholine receptor (AP), to muscle-specific kinase (MP) and without detectable antibodies (SN) in an in vitro cell culture with specific and non-specific T cell stimulation.
Methods: The study group of 59 MG patients (18 AP, 19 MP and 22 SN) with generalized disease (W/M: 42/17) were included. Fifty-six % of the patients were on immunosuppressives. In addition, 10 healthy controls (HC) (W/M: 6/4) were evaluated. Peripheral blood mononuclear cells without B cells were stimulated with t-AChR, recombinant-MuSK or anti-CD3 antibody. IFN-γ, IL-10, IL-2, IL-13, IL-17A and IL-21 were measured in supernatants using a microbead array system (Milliplex). Results were compared with non-parametric tests.
Results: Spontaneous IFN-γ, IL-13 and IL-2 secretion was lower in the MG group than HC ($p = 0.004$, 0.004 and 0.028). However, when the patients with or without treatment were compared, a suppression of only IFN-γ was shown in steroid-treated patients ($p = 0.02$) whereas lower IL-13 and IL-2 were not related to ongoing treatment. Spontaneous IL-10 production was not depressed in the MG group. In all groups, in vitro antigen specific stimulation with AChR or MuSK did not alter the cytokines compared to non-stimulated cells, whereas anti-CD3 stimulation induced effective secretion of all measured cytokines except IL-2. When all cytokines were compared, anti-CD3 stimulated lower levels of IL-10, IL-13, IL-17A, IL-2, IL-21 in MG compared to HC ($p = 0.019$, 0.022, 0.021, 0.002, 0.027), also in patients not-on-treatment ($p = 0.02$, 0.05, 0.03 and 0.04). CD3 stimulation was also less effective for IFN-γ in MG patients without treatment compared to HC ($p = 0.01$). The differences were also evident in all subgroup comparisons.
Conclusions: In MG, even in patients not on treatment, disease specific (AChR and MuSK) stimulations induced no cytokine secretion and non-specific (CD3) T cell stimulation induced only lower levels of cytokines in vitro. The decrease of cytokine induction in immunosuppressive naïve patients emphasizes the disease related changes in the immune response of MG patients on T cell cytokine activity.

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Therapies

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Glatiramer acetate promotes myelin development in the spinal cord of new born mice

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Objective: The aim of this study was to test whether Glatiramer acetate (GA, Copaxone), an immunomodulatory treatment that has been shown to induce neuroprotection in the inflamed CNS in multiple sclerosis (MS) and experimental autoimmune encephalomyelitis (EAE), can promote myelination in the developing CNS of healthy new born mice, in the absence of inflammation.
Methods: New born mice were injected daily with GA at days 7–21 after birth. Spinal cord were harvested at days 7, 14 and 21, and stained by antibodies to myelin basic protein (MBP) and neurofilament medium protein (NF), for myelin and axonal detection, respectively. The percentage of myelinated axons from the total number of axons was quantified in multiple ventral lumbar sections of each mouse and compared to PBS-treated siblings from the same litter. Quantification of oligodendrocyte was performed by staining for the ologodendrocyte progenitor marker NG2 in combination with proliferation markers.
Results: Three families analyzed, (2 mice per treatment group in each family for each time point) yielded similar results. On the average, in PBS-treated controls, only 41% of the axons were myelinated 7 days after birth, whereas 77% myelinated axons were found at day 14. In GA-treated mice 89% of the axons were myelinated at day 14, thus indicating that at this time point GA treatment induced 15% elevation in axonal myelination. It should be noted that at day 21, the extent of myelinated axons (97%) and their morphological appearance were identical in both the GA-treated and the PBS-treated mice, indicating that GA enhances myelin development without inducing an excessive or aberrant myelination. The amount of oligodendrocytes in spinal cords of the GA-treated mice was higher than in the PBS controls, suggesting that the enhanced myelination after GA treatment can be attributed to its effect on the oligodendrocyte population.
Conclusions: GA treatment affects myelin development even in non inflammatory conditions. The described axcelerated myelin development induced by GA treatment in new born mice coroborates its beneficial effect in promoting remyelination in EAE and MS.

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Targeting of peripheral monocytes by nasal proteasome based vaccine, to induce Amyloid beta clearance in an animal model of Alzheimer's disease

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Objective: 1) To determine the effect of nasal treatment with proteasome-based adjuvants on activation of peripheral monocytes and their ability to clear amyloid aggregates in APP-tg mice.

2) To characterize the origin of the CD11b+ cells present in the brain of Protollin-vaccinated APP-Tg mice (resident microglia versus recruited monocytes).
Methods: We generated chimeric mice by reconstitution of the BM of 20-month-old APP-Tg-mice with wild-type BM cells expressing GFP under the CX3CR1 promoter. Two months after this BM-transplantation, AD-chimera mice (age 22 mos) were treated intranasally with Protollin (1 μg/mouse) for 2 or 6 weeks. We used our novel microglia-unique antibodies to distinguish and characterize the phenotype of resident microglia versus recruited monocytes in control and Protollin-treated APP-tg mice. We used Nanostring quantitative technology for 179 inflammation-related genes to profile resident microglia and splenic monocytes.

Results: We found that nasal treatment of the AD-chimera mice with Protollin: 1) significantly reduced Aβ plaque levels accompanied by an increase in the numbers of recruited CD11b-GFP BM-derived monocytes having intracellular immunoreactivity for endogenous Aβ. CD11b was expressed by all GFP + cells adjacent to residual Aβ plaques, but by hardly any of the non-GFP + resident microglia; 2) activated splenic Ly6C^{Hi} monocytes acquired a M1 inflammation-related phenotype. Resident microglia were not affected by Protollin treatment
Conclusions: Nasal treatment with proteasome-based adjuvants targets peripheral monocytes, modulate their pro-inflammatory M1 phenotype and induces their recruitment to Aβ-plaques in the brain of APP-tg mice to facilitate in Aβ -plaque clearance.

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Low immunogenicity but reduced bioavailability of an interferon beta-1a biosimilar: Results of MATRIX, a cross-sectional, multicenter phase 4 study

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Objective: Neutralizing antibody (NAb) formation reduces the clinical activity and efficacy of interferon beta (IFNβ) therapies. Avonex® (Biogen Idec, Weston, MA, United States), an intramuscular (IM) IFNβ-1a formulation, has low immunogenicity, affecting 2%–5% of patients, while the immunogenicity of the biosimilar drug Jumtab® (Probiomed, Miguel Hidalgo, Mexico) has not been reported. The primary objective of the present study was to compare the NAb frequency in multiple sclerosis (MS) patients treated weekly with either Avonex or Jumtab.
Methods: MATRIX was a retrospective, cross-sectional phase 4 study conducted in Mexico and Colombia to determine NAb levels in MS patients with no prior disease-modifying therapy exposure who were treated with either Avonex or Jumtab (expected enrollment of n = 90 per drug) for 1–3 years prior to study enrollment. NAb levels > 100 IU as measured with a luciferase assay were considered positive. Neopterin induction, a pharmacodynamic measure of IFN response, was taken as the change in serum levels from pre-dose to 48 h post-dose. Possible relationships between patient NAb status and drug tolerability/safety were also evaluated. Data are presented as the mean ± standard deviation.
Results: Study enrollment was limited due to pharmacy substitution of branded medications with locally produced biosimilars and a lack of availability of study medications in local pharmacy markets. The Avonex (n = 36) and Jumtab (n = 29) groups differed in age (37.1 vs 44.6 years; $P<0.01$); there were no other significant baseline differences. The mean duration of IFNβ-1a therapy was 24.5 ± 7.5 months for Avonex and 22.1 ± 8.1 months for Jumtab. No patients developed NAb levels > 100 IU during the study period. Avonex patients had higher levels of neopterin induction (2.4 ± 2.2 ng/ml vs 0.7 ± 1.2 ng/ml; $P<0.01$) and flu-like symptom (FLS) incidence (80.6% vs 31.0%) than Jumtab patients. The groups did not differ significantly in either relapse rate or incidence of unexpected adverse events.
Conclusions: Both Avonex and Jumtab exhibited minimal immunogenicity. Neopterin activation and FLS were lower with Jumtab, suggesting that Jumtab has lower IFN activity than Avonex. These results underline the importance of performing a multimodal assessment to establish a biosimilar drug's immunogenicity, pharmacokinetics, and pharmacodynamics. A prospective study with a larger sample size is needed to evaluate the relative safety and efficacy of these drugs.

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Tregitope Peptides Enhance Regulatory T Cell Development and Attenuate the Autoimmune Responses in a murine model of multiple sclerosis

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Objective: The potential for harnessing the tolerance-inducing power of regulatory CD4$^+$FoxP3$^+$ T cells (Tregs) as a treatment for multiple sclerosis (MS) is gaining momentum. De Groot et al. have identified T regulatory T-cell epitopes (Tregitopes) derived from human immunoglobulins that activate Treg cells (Blood, 2008). The aim of this study is to test the ability of Tregitope peptides to regulate autoimmune responses in experimental autoimmune encephalomyelitis (EAE), the animal model of MS.
Methods: Human Tregitopes 167 and 289 (hTregitopes; 10 μg/ml) were added to primary mouse CD4 + T cell cultures from wild-type or FoxP3-GFP reporter mice. Treg cell frequency and phenotype were measured by flow cytometry; cytokine production was measured by intracellular staining and Luminex.

In vivo, hTregitope peptides were administered i.p. in C57BL/6 mice (6 injections × 200 μg/mouse) every second day starting from the day of immunization with MOG35-55/CFA. Control mice received PBS or ova peptides. Mice were monitored for EAE development. A subgroup of mice was sacrificed 10 days after the initiation of treatment to analyze Treg development and the cytokine profile.
Results: We demonstrate that hTregitopes modulate autoimmune responses in vitro and in EAE. In vitro, hTregitope peptides (10 μg/ml) enhance the conversion of naïve CD4+FoxP3-isolated from FoxP3-GFP reporter mice, into inducible Tregs (iTregs) in the presence of titrated concentrations of recombinant TGF-β1. Addition of hTregitopes to in vitro differentiated T helper (Th) 1, 2, 9 and 17 cells reduces IFNγ, but increases IL-13 and IL-9 production as measured by Luminex under Th1, Th2 and Th9 polarization conditions, respectively. In vivo administration of hTregitopes (6 × 200 μg/mouse) every second day to MOG35-55/CFA-immunized EAE mice significantly ameliorate the clinical outcome and promote the expansion of Treg cells in the peripheral compartment. This was associated with a decrease in MOG35-55-specific IFNγ + T cells and an increase in IL-9 + T cells in the peripheral lymphoid organs. Interestingly, we found increased frequency of IL-10 + CD11b+CD11c+dendritic cells and decreased frequency of IFNγ+CD11b+CD11c + in the hTregitope-treated mice compared to control mice.
Conclusions: Altogether, our findings demonstrate a protective role of Tregitopes in EAE. Understanding the mechanisms of action of Tregitopes is important as a novel approach to suppress aberrant immune activation in multiple sclerosis.

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A gene panel for screening CNS pathologies during MOG35-55-EAE in mice for use in monitoring the effectiveness of novel MS therapies

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Multiple sclerosis (MS) is a chronic, complex neurological disease in which several pathophysiological mechanisms such as axonal/neuronal damage, demyelination, and inflammation participate. Experimental autoimmune encephalomyelitis (EAE) is a mouse model that is widely used to study the mechanisms and immunepathogenesis of MS and also to test new therapeutic approaches for their efficacy. Recent work in our laboratory has focused on identifying key molecules involved in mediating neurodegeneration and neuroprotection with the aim of finding novel neuroprotective strategies for the treatment of MS. In this study we monitored the differential expression of selected genes in spinal cord samples isolated from mice at different stages of EAE compared to naïve control mice, using quantitative RT-PCR, with the aim of finding gene markers that would sensitively monitor pathology. We chose genes that are known to be involved in major pathological processes in MS and EAE, demyelination, inflammation and neuron damage, as well as genes involved in tissue repair processes. From more than 40 original gene candidates we have defined a mini-gene panel of around 10 genes that sensitively mark individual pathological events in the spinal cord during EAE. We hypothesized that this gene panel would be useful for gaining further insight into the timing and interrelationships of distinct pathological processes during the development of disease as well as for predicting the efficacy of new therapeutics for MS. Proof of principle that this gene panel has drug screening potential was obtained using a well-established MS therapeutic, glatiramer acetate (GA). The administration of GA to mice in a prophylactic protocol in mice before the onset of EAE normalized the expression of a significant number of the gene markers compared to those in non-treated mice and this correlated with the amelioration of clinical symptoms. These results suggest that the analysis of disease marker gene expression in preclinical models will be a useful approach for predicting the effectiveness and penetration of novel therapeutics aimed for the treatment of MS.

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Improving efficacy of antigen-specific therapeutic agents in an animal model of multiple sclerosis

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Objective: Our long-term aim is to develop safe and effective immunomodulatory peptides that can be used as therapeutic vaccines to specifically inhibit autoimmune responses in people with multiple sclerosis (MS); however, peptides typically do not make great vaccines. The aim of the current study was to develop ways to improve the *in vivo* efficacy of therapeutic peptides and to investigate the mechanisms responsible for their increased therapeutic efficacy.
Methods: We used the experimental autoimmune encephalomyelitis (EAE) model of MS in SJL/J mice. Two peptides (Q144 and A188) which are derived from encephalitogenic epitopes of myelin proteolipid protein (PLP) and which are known to have some *in vivo* immunoprotective effects in EAE were used as the starting point for this study. Q144/A188 and their corresponding thiopalmitoylated (S-palm) peptides were used therapeutically to treat mice with EAE induced with the relevant encephalitogenic peptides. Cells from these mice were analyzed at various time points to determine their specificity, encephalitogenic potential, and phenotype. In addition, the stability of the S-palm peptides in serum was compared with that of non-modified peptides.
Results: We found that S-palm-Q144 and S-palm-A188 had greatly improved protective effects *in vivo*, compared to Q144 and A188 alone, as evidenced by decreased incidence and severity of EAE and increased mean day of onset when peptides were administered at the time of disease induction, and by increased speed of disease resolution when peptides were administered therapeutically. The serum half-life of S-palm peptides was increased 20-fold compared to peptide alone. S-palm peptides were taken up much more rapidly than peptide alone into antigen-presenting cells and preferentially entered the MHC class II presentation pathway. S-palm peptides induced stronger immune responses, including greater numbers of regulatory T cells, and a 10-fold increase in the production of IL10, compared to peptide alone. The lipid moiety was found to be cleaved from the S-palm peptides by naturally occurring thioesterases in the endosomes of antigen-presenting cells, and thus did not interfere with the specificity of the response.
Conclusions: Thiopalmitoylation of therapeutic peptides is a simple and effective way to increase their beneficial effects. We are currently developing a series of thiopalmitoylated immunomodulatory peptides for use in MS patients.

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Efficacy of low dose, single administration of tacrolimus for ocular myasthenia gravis, a retrospective study of 8 cases

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Objective: To evaluate the efficacy of singly administrated low dose tacrolimus for ocular myasthenia gravis in a retrospective way, to relieve the ptosis and diplopia. The drug's safety and considerable side effects are also evaluated.
Methods: 8 ocular myasthenia gravis (MG) patients without systemic myasthenic symptoms were treated with low dose tacrolimus administration (3 mg/day). None of them had a previous immunological therapy, except for thymectomy. Their ptosis and diplopia are evaluated with the MG activities of daily living (MGADL) scores at following 1 month, 3 months, 12 months. At the same time, the drug's safety and existence of MG progression were evaluated.
Results: 5 out of 8 patients (63%) improved diplopia, and 5 out of 7 patients (71%) improved ptosis in a month, and it's notable that 4 out of 8 patients (50%) subjectively improved their diplopia and 5 out of 7 patients (71%) improved their ptosis in a week. The number of improved patients was gradually larger toward 12 months, but 2 patients were refractory. There are no complete remission of diplopia, but 3 out of 7 ptosis had a complete remission. As to the side effects, 2 patients with preceding type 2 diabetes had mild worsening and one patient acquired renal tubular disorder, but no other serious side effects appeared. No patients had a progression to systemic MG.
Conclusions: Single administration of low dose tacrolimus is a good option for the ocular myasthenia gravis. Ptosis was more treatable than diplopia. And more than half of patients had immediate responses subjectively, it's hard to explain only for the immunomodulation since generally it takes at least several weeks.

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Targeting B-cell survival molecules in the common marmoset EAE with anti-BLyS and anti-APRIL

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Objective: For a long time multiple sclerosis (MS) has been regarded as a mainly T-cell driven autoimmune disease with only supportive role of B-cells as producer of autoantibodies. However, using antibodies that deplete CD20+ B-cells, such as Rituximab and Ofatumumab, it was shown that B-cells have a much more prominent pathogenic role than anticipated. Remarkably, serum antibody levels were not changed in the CD20+ treated patients even though B cells had been depleted, leaving the beneficial effect of treatment without mechanistic explanation.

In the current study an alternative approach was taken to see whether a similar clinical effect could be reached by neutralization of cytokines that are needed for B cell survival and activation, e.g. BLyS/BAFF and APRIL.
Methods: To assess this, experimental autoimmune encephalomyelitis (EAE) was induced with recombinant human myelin/oligodendrocyte glycoprotein (rhMOG) with complete Freund's adjuvant (CFA) in the common marmoset (*Callithrix jacchus*). This animal model is used as a valid preclinical model for MS that can be applied for translational research into pathogenic mechanisms and therapy development. The study was performed in 18 animals, which were equally divided over 3 groups. Treatment with anti-BLyS, anti-APRIL or placebo was initiated after EAE induction, but prior to the expression of neurological signs (day 21 after immunization). During the study and after necropsy several immunological parameters were determined, including T-cell proliferation, antibody and cytokine profile. CNS pathology was assessed also by histology and magnetic resonance imaging (MRI).
Results: All animals in the control group developed clinical EAE around 37 ± 8.6 days after immunization. The anti-BLyS and anti-APRIL treated group showed a significant delay in progression to clinically evident EAE compared to control animals, respectively 60 ± 15.9 and 59 ± 23.8 days after immunization. Flow cytometry analysis showed a marked decrease of CD20+ B-cells only in the anti-BlyS group.
Conclusions: The anti-BLyS and anti-APRIL treatment had a striking effect on the onset of the clinical EAE, and anti-BLyS was associated with a significant suppression of demyelination in the spinal cord.

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Characterization of protective vitamin D effects in multiple sclerosis-like neuroinflammation

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Objective: Lack of vitamin D has been associated with higher prevalence, relapse rate and progression of multiple sclerosis (MS). We therefore sought to study the influence of vitamin D modulation on experimental autoimmune encephalomyelitis (EAE), an animal model of MS. We aimed to further characterize the effect of vitamin D on CD4 T cells during EAE induction and to define more permanent changes that can mediate therapeutic effects of vitamin D.
Methods: We modulated vitamin D levels in susceptible rats by feeding them with either vitamin D deprived food, normal food or food with fivefold higher levels of vitamin D, at *in-utero*, juvenile/adolescent and adult stage. We then assessed the impact of vitamin D intake on clinical EAE parameters, inflammation and demyelination in the central nervous system (CNS). We also investigated the impact of vitamin D intake on immune activation using flow cytometry, quantitative PCR and Elispot. Genome-wide expression and DNA methylation analyses were performed on CD4 T cells sorted from lymph nodes after EAE induction using Gene 1.0ST arrays (Affymetrix) and Comprehensive High-throughput Arrays for Relative Methylation (CHARM), respectively.
Results: Vitamin D supplementation in juvenile/adolescent rats led to a milder EAE, less severe inflammation and demyelination in the CNS, lower number of IFN-γ producing autoreactive T cells and their cytokine expression pattern reflected a shift towards an anti-inflammatory phenotype. In contrast, treatment of rats *in-utero* and during adulthood did not significantly influence EAE development. Genome-wide expression in lymph nodes after EAE induction implicated gradual change in T cell and APC functions with an increased intake of vitamin D. Preliminary data from genome-wide expression analysis in CD4 T cells indicated dramatic down-regulation of T cell activation markers upon vitamin D supplementation. Changes in expression of several genes were accompanied by more permanent changes in DNA methylation.
Conclusions: Vitamin D supplementation significantly ameliorates MS-like neuroinflammation in rats, in part through modulation of CD4 T cell activity. These positive effects on clinical disease are predominant in juvenile/adolescent period, which has important implications for vitamin D supplementation therapy.

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The effects of methadone on function of immune cells in experimental autoimmune encephalomyelitis

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Objectives: Multiple sclerosis is debilitating autoimmune neurodegenerative disease in young adults. Anti-inflammatory treatments have been used to control the exacerbation and/or stabilizing the course of a disease. Opioids have regulatory effects on inflammatory cytokines production and expression of adhesion molecules.
Methods: EAE was induced in female C57BL/6 mice and methadone was administered subcutaneously at 10 μg/kg/mouse daily from 3rd day postimmunization in half of animals. Signs of the disease was evaluated and compared between methadone and control groups from 7th day postimmunization to the end. At 21st day postimmunization, mice were sacrificed and antigen-dependent proliferation and cytokine production by splenocytes were evaluated. Mice were sacrificed at 35th day post immunization and pathological features were evaluated in spinal cord.

Results: Methadone suppressed clinical signs of EAE significantly. It also regulates inflammatory cytokines production and proliferation of splenocytes. Moreover, histopathological features, inflammatory cells infiltration and demyelination, was decreased in methadone treated group.
Conclusion: Methadone has inhibitory effects on EAE with suppressing proliferation and inflammatory function of immune cells which are responsible for EAE pathogenesis. This may indicate the regulatory effect of methadone on MS pathogenesis.

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Epidermal growth factor blockade ameliorate the severity of relapsing experimental autoimmune encephalomyelitis

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Background: Despite their abundance near MS lesions, the ability of neuronal precursor cells to differentiate into functional mature oligodendrocyte is very poor. Consider the predominant role of epidermal growth factor (EGF) in astrocytes development, and the importance of EGF inhibition in promoting oligodendroglial maturation. We studied the therapeutic effect of EGF blockade in relapsing experimental autoimmune encephalomyelitis (R-EAE) mice model.
Methods: standard R-EAE was induced in 6–8 weeks old female SJL mice ($n=36$) by immunization with PLP139-151 peptide and adjuvant. On day 9 post immunization mice were intravenously treated by either: 60 μg/100 μl/mice of anti-EGF neutralizing monoclonal antibody (mAb) ($n=12$), or 60 μg/100 μl/mice of appropriate isotype control (IC, $n=12$) or with 100 μl/mice of PBS. EAE clinical scores scale was between 0–5. Statistical differences between the groups were calculated by student t-test.
Results: treatment of R-EAE mice with anti EGF neutralizing mAb reduced the clinical scores of EAE as compared to mice that were treated with IC or PBS. The maximal average score in the PBS treated group during the first attack was 1.47 ± 0.24 and during the second attack was 1.39 ± 0.2. In the IC treated group 1.54 ± 0.26 and 1.65 ± 0.24, respectively. Whereas in the anti-EGF mAb treated group the scores much were lower: 0.88 ± 0.31 and 0.95 ± 0.29, respectively. Significant reductions in the clinical scores were found in day 11 (the first attack) and mainly in the second attack (days 25–36), in the anti-EGF mAb treated group as compared to IC or PBS treated groups ($p<0.05$). Moreover, while the first attack occurs simultaneously in all 3 groups, the second attack onset was delayed in the anti-EGF mAb treated mice (day 31) vs. day 23 in both IC and PBS treated groups. Similarly the maximal average score of the second attack was delayed anti-EGF mAb treated mice (day 41) vs. IC treated mice (day 36) and PBS treated mice (day 34).
Conclusion: Therapy with anti-EGF neutralizing mAb was found to ameliorate R-EAE and to postpone its activity, suggesting that EGF signaling blockage may have a therapeutic potential for diseases such as multiple sclerosis. We assume that the beneficial effect of anti EGF mAb therapy is via induction of oligodendrocyts diffrentialtion.

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Melatonin regulates lung associated immunity of a tropical bird Perdicula asiatica

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Objective: LAIS has been compartmentalized into bronchus associated lymphoid tissue (BALT) and non-BALT nodules, free lymphocytes and macrophages. Localization of Mel_{1a}, Mel_{1b}, AR, GR and RORα receptors suggested hormonal regulation of LAIS in *Perdicula asiatica*. Role of melatonin and photoperiod in regulation of LAIS has very less been taken into consideration.
Methods: We accessed the effects of melatonin (25 μg/100 g.B.wt./day) and different photoperiodic regimes i.e. long (LP; 20L:4D) and short (SP; 4L:20D) during reproductively inactive (RIP) and reproductively active phase (RAP) on LAIS as well as free radical load of lungs of *P. asiatica*.

We noted effect of melatonin and photoperiod on size of BALT/non-BALT nodules, proliferation of isolated lung lymphocytes in terms of % stimulation ratio, total leukocytes count (TLC) and lymphocyte count (LC). Oxidative stress of lung tissue was noted in terms of antioxidant enzymes (superoxide dismutase and catalase), lipid peroxidation in terms of malondialdehyde level and total antioxidant status (ABTS). Circulatory level of melatonin, testosterone and corticosterone was noted. Expression of Mel_{1a}, Mel_{1b}, AR, GR and RORα receptor was checked under different experimental conditions.
Results: Melatonin and SP increased nodular size, TLC, LC, plasma melatonin level, percent stimulation ratio of lymphocytes and decreased testicular activity. Steroid and LP decreased the above-mentioned immune parameters. SP and melatonin upregulated the expression of Mel_{1a} and Mel_{1b} receptor and downregulated AR/GR expression. Exogenous steroid and LP condition downregulated Mel_{1a} and Mel_{1b} receptor and upregulated AR/GR. Exogenous melatonin and SP increased circulatory melatonin and decreased steroid level whereas steroid injection and LP showed inverse effect on respective hormones.

The melatonin and SP decreased malondialdehyde level and downregulated the expression of RORα during both reproductive phases. There was higher activity of SOD and catalase as well as higher percentage of inhibition of ABTS radical cation. LP increased malondialdehyde level and upregulated the expression of RORα. There was decreased activity of SOD and catalase as well as lower percentage of inhibition of ABTS radical cation in lung tissue.
Conclusions: Results obtained reflected a parallel relationship of melatonin, photoperiod, melatonin receptors and all the immune parameters suggesting that melatonin might be immunostimulatory for LAIS. Melatonin and SP reduced free radical load of lungs which is of great adaptive significance for survival of any avian species. Thus, SP responsible for inducing melatonin secretion can be used as a mode of therapy to increase LAIS adaptability of avian species under immunosuppressed condition.

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Laquinimod reduces astrocytic NFκB activation in vitro and in vivo

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Objective: Laquinimod (LAQ) is a small, orally active and well-tolerated molecule that has been shown to reduce brain atrophy, disability progression and relapse rate in patients with relapsing-remitting multiple sclerosis. LAQ minimizes demyelination, inflammation and axonal damage in mice with cuprizone challenge and with experimental autoimmune encephalomyelitis. LAQ is known to reduce inflammation in the periphery, but clinical findings and the experimental cuprizone data suggest additional effects within the central nervous system (CNS). Since astrocytic NFκB activation plays a crucial role for cuprizone-induced demyelination, we

investigated the effects of LAQ on astrocytic NFκB activation *in vitro* and *in vivo*.
Methods: Primary mouse astrocytes pre-treated with 0, 0.25 and 2.5 μM LAQ were stimulated by pro-inflammatory cytokines including *tumor necrosis factor* α (TNFα) or the combination of *interleukin-1*β (IL-1β) and *interferon-*γ (IFNγ). A dual-luciferase reporter assay was used to measure NFκB activity. For *in vivo* experiments 10-week-old male C57BL/6 J mice were challenged with 0.25% cuprizone and treated daily with LAQ (25 mg/kg) or water. The total number of GFAP-positive astrocytes with and without p65 translocation was determined by double immunofluorescence.
Results: In vitro, both cytokine treatments significantly increased NF B activation compared to unstimulated controls in the absence of LAQ. Pre-treatment with 0.25 and 2.5 μM LAQ significantly reduced the induced NF B activity after TNFα stimulation compared to stimulated controls. Pre-treatment with 2.5 μM LAQ also significantly reduced NF B activation after stimulation with the combination of IL-1β and IFNγ compared to untreated stimulated controls. To confirm the *in vivo* relevance of these findings, we also examined astrocytic p65 translocation in mice with and without LAQ treatment after cuprizone challenge. The proportion of astrocytes with nuclear p65 immunoreactivity was significantly reduced in LAQ-treated mice (14.0%±0.9%) compared to untreated controls (25.8%±1.1%).
Conclusions: In vitro, LAQ reduced the astrocytic NFκB activation by up to 46% as evidenced by NFκB reporter assay. Similar quantitative findings were obtained *in vivo* where LAQ treatment also led to a 46% reduction of astrocytes with NFκB activation evidenced by nuclear translocation of p65. These findings indicate that targeting the astrocytic NFκB pathway might have therapeutic effects in demyelinating CNS disorders.

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Human gut-derived commensal bacteria as therapy for autoimmune diseases

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Objective: Multiple sclerosis (MS) is a chronic inflammatory, demyelinating disease of central nervous system that affects approximately 2.5 million people worldwide. MS costs the U.S. economy billions of dollars per year in medical care and indirect expenses, including lost wages and productivity. Although several therapies are in use, none of them cure the disease and many of these agents have less than optimal tolerance. Thus, there is an urgent need for new treatment(s) to cure MS. Recently, we have identified a new strain of human commensal-organism *Prevotella histicola* (*P histciola*) that appear to have potent systemic immunomodulatory effects. Understanding the mechanism by which common human commensal can provide systemic immunomodulation in the context of a severe demyelinating disease has great potential for therapeutic impact in MS.
Methods: To test therapeutic ability of human commensal to modulate disease, we used experimental autoimmune encephalomyelitis (EAE) as an experimental model of human MS. Previously we showed that PLP_{91-110} can induce EAE in humanized HLA-DR3DQ8 transgenic mice. EAE was induced in HLA-DR3DQ8 transgenic mice and day 7 post-immunization, animals were orally gavaged with *P histicola* or control bacteria on alternate day for total 7 doses and monitored for 4 weeks for disease and weight loss. Immunological and pathological parameters were compared between treated and control group to understand the mechanism of immune-modulatory action of *P histicola*.
Results: P histicola treated group showed lower disease incidence as well as severity compared to untreated or group treated with control bacteria. Pathological analysis of brain and spinal cord also showed decreased inflammation and demyelination in *P histicola* treated group compared to control or media treated group. *P histicola* suppresses disease through modulation of systemic immune response as *P histicola* treated group showed increased levels of CD4+FoxP3+ regulatory T cells (Tergs) as well as tolerogenic dendritic cells (DCs) and macrophage.
Conclusions: Our study shows that human commensal bacteria can be used as a novel therapeutic option, which might be relevant to human inflammatory and demyelination diseases such as MS. Our study also highlights importance of human gut microbiome in regulating systemic immune response and its possible role in modulation of the autoimmune diseases.

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Amelioration of Experimental Autoimmune Encephalomyelitis with Oral Administration of Traditional Japanese Herbal Medicine

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Objective: Multiple sclerosis (MS) is a chronic demyelinating disease of the central nervous system (CNS), with related neurological impairment attributed to demyelination and a neurodegenerative process as a consequence of inflammation in the CNS. Presently, immuno-modulatory and suppressive drugs remain the primary choices for MS treatment, though they provide only a modest impact on patient prognosis, while anti-inflammatory approaches may be efficacious as add-on therapy. We investigated the usefulness of Japanese traditional herbal preparations known to have anti-inflammatory effects in an animal model of MS experimental autoimmune encephalomyelitis (EAE).
Methods: C57Bl/6 J mice were immunized with 200 microgram of MOG 35–55 peptide emulsified in complete Freund's adjuvant, which was followed by intraperitoneal injections of 200 ng of *Pertussis toxin* on days 0 and 2. From day 13 after MOG injection, administrations of the Japanese herbal medicine Keishito, Saireito, or Saikokeishito were performed 3 times a week using gastric intubation. Each as an extract was diluted with distilled water (dH_2O) to make final 300-microliter preparations containing 30-, 90-, and 300-mg doses. Control mice were treated with the same amount of dH_2O. Each group consisted of 5 mice.
Results: Keishito extract was administered between days 13 and 64 (total 22 times). However, it did not show any effect on the course of EAE. In the parallel experiment with Saireito extract, administered between days 13 and 66 (total 23 times), mice in the 3 dose groups showed no symptoms or a slight decrease in tail tonicity, while control mice showed hind limbs paralysis. Moreover, Saireito-treated mice did not develop paralytic symptoms more than 3 weeks after discontinuation of treatment. Based on those results, the second experimental arm was performed using Saikokeishito extract. The drug was partially effective, as treated groups showed tail weakness and control mice displayed hind limb paralysis.
Conclusions: The composition of Saireito extract is partially shared by Keishito and Saikokeishito extracts. Our results suggest that the

materials used to produce Saireito extract but not those used for Keishito extract have a pivotal role in largely abrogating EAE. If smaller doses of oral Saireito extract are able to suppress EAE similarly to the doses used in the present experiment, it may be a candidate future treatment option for MS.

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Prevention and treatment of toxicity in the holistic approach: Population-based observations among the residents of Barguna district in Bangladesh

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Objective: Plants are in great demand in both developed and developing countries in primary health care because of their great efficacy and no side effect. The observations were conducted among the Barguna district of Bangladesh during May 2008 to April 2012, which area because of its proximity to the Sunderbans forest region contains a diversity of plants.
Methods: First-hand information was collected from old and experienced medicine men and women of Magh ethnic group and rural people. The information regarding the dosage of crude drug, purpose of usage, mode of preparation, and administration was carefully recorded in audiotapes as well as field notebooks. The voucher specimens for each plants was collected in quadruplicates, which were carefully tagged with field numbers after making a critical observation on the habit, habitat, color and odor of flowers, phyto-association, occurrence and other relevant ecological features, which cannot be discerned from dried herbarium specimens.
Results: The observations consist 33 plants are used for wide range of venomous insects, arthropod, reptiles, and mammals. 13 plants are used against scorpion sting, 12 plants are used against dog bite, 09 plants are used against snake bite, 05 plants are used against honey bee sting, 03 plants are used against rat bite, 02 plants are used against lizard, and 01 plant is used for centipede poisoning. Of these, 21 uses of 15 plants (asterisked in enumeration) form new reports of Bangladesh after comparing with the classical literature and recent research papers on the subject matter. This report lime lights the secret folklore of the people of Barguna district. These represent unexplored potential. They are administered in various forms e.g. infusion, juice, extract, powder, paste, latex, and oil.

They are supplemented by other botanicals or domestic substances such as honey, common salt, and cow milk. Thus sole drug or mixed with other resources are in vogue.
Conclusions: On account of poor financial position of these people, they prefer for low cast holistic treatment offered by medicine men. Further studies for their chemical contents and toxicity, if any, may help to increase efficacy vis-à-vis authenticity of the claims.

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Methylthioadenosine promotes neuroprotection for inflammatory and excitotoxic brain diseases

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Objective: Methylthioadenosine (MTA) is a metabolite of the polyamine pathway with anti-oxidant, anti-proliferative and immunomodulatory properties. Here, we report that MTA displays a wide array of neuroprotective activities against different insults.
Results: As such, MTA is able to protect neurons and myelin-forming cells from toxicity and inflammation in vitro, promoting neuronal differentiation and remyelination. In vivo, MTA reduced axonal loss and also protected neurons from death in animal models of brain ischemia, Parkinson disease and epilepsy. In addition, MTA is elevated in the brain of patients with multiple sclerosis with active inflammation. MTA prevents microglia activation through modulation of different pathways. Specifically, it increased the expression of transcription factors (STAT3, ATF2 and NR4A) and promoted the release of a trophic factor (CNTF), all of which are pathways associated with neuroprotection.
Conclusions: Our findings point to the polyamine pathway as part of the natural response to cell damage and suggest MTA as a new neuroprotective therapy.

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Preventive treatment with laquinimod reduces myeloid dendritic cells and shifts pro-inflammatory to regulatory T cells in experimental autoimmune encephalomyelitis

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Objective: Laquinimod (LAQ) is a new orally active, well tolerated drug which significantly reduces the annualized relapse rate, brain volume loss and disability progression in multiple sclerosis (MS). In animals LAQ inhibits acute experimental autoimmune encephalomyelitis (EAE) and suppresses relapses in chronic EAE. Central inflammation is reduced and the cytokine balance is shifted in favor of Th2/Th3 cytokines.

The aim of this study was to examine the effects of LAQ on antigen presenting cells and T subpopulations during the priming phase of EAE.
Methods: C57BL/6 mice with MOG_{35-55}-induced EAE were treated daily with 0 or 25 mg/kg LAQ. To investigate effects on the priming phase, LAQ was administered from the day of immunization until day eleven. Single cell suspensions from lymph nodes and spleen were obtained and analyzed by flow cytometry and ELISA.
Results: Antigen presenting cells were affected by preventive LAQ treatment. FACS data revealed a significant decrease of $CD11c^{high}$ dendritic cells (DC) ($p = 0.0013$) in the spleen of LAQ treated animals. Within the DC population CD11b myeloid DC were significantly reduced ($p = 0.0001$). In contrast, the frequency of CD8α DEC205 DC, previously described as regulatory subtype, remained unaltered.

Treatment with LAQ significantly reduced the percentage of Th17 ($p = 0.0022$) and Th1 T cells ($p = 0.026$) among the CD4 T cell

population. The absolute number of CD4 T cells producing IL-17 or IFN-γ in response to PMA/Ionomycin was also reduced after therapy. Spleen and lymph node cells of treated animals produced less of these cytokines in a recall assay with MOG_{35-55} compared to controls. Moreover, the percentage of FoxP3 regulatory T cells increased after LAQ treatment in the spleen ($p = 0.0022$).
Conclusions: In conclusion, preventive treatment with LAQ inhibits the pro-inflammatory autoimmune T cell response after immunization with MOG_{35-55}. These findings indicate that LAQ interferes with the early phase of priming and activation of immune cells and may therefore be beneficial in future treatment of MS.

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Dimethyl fumarate inhibits dendritic cell maturation via NF-kB and ERK1/2-MSK1 signaling

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Background: Dimethyl fumarate (DMF) is an effective novel treatment for multiple sclerosis (MS) in clinical trials. A reduction of IFN-γ-producing $CD4^+$ T cells is observed in DMF-treated patients and may contribute to its clinical efficacy. However, the cellular and molecular mechanisms behind this clinical observation are unclear. In this study, we investigated the effects of DMF on dendritic cell (DC) maturation and subsequent DC-mediated T cell responses.
Methods: To determine if DMF affects the maturation of DCs, *in vivo* generated DCs were purified and stimulated by LPS (100 ng/ml) in the presence or absence of DMF (70 μM) for 24 h. Proinflammatory cytokine production and expression of maturation markers were analyzed by ELISA and flow cytometry. To further determine if DMF-DCs have impaired capacity for driving T cell responses, a co-culture system was utilized. Purified DCs were stimulated by LPS in the presence or absence of DMF for 24 h, then supernatants were removed and cells were washed with warm PBS for twice. MOG_{35-55} specific T cells were then added to the conditioned DC culture with the addition of MOG_{35-55} (2 μg/ml). T cell activation and cytokine production were evaluated at 48 h and 72 h. To enhance our understanding of the effect of DMF on DCs, the molecular mechanisms were also explored. Bone marrow derived DCs were stimulated with LPS in the presence or absence of DMF (70 μM). To determine the distribution of p65, cells were then fixed by 2% PFA and stained by p65 antibody. In addition, whole cell protein was extracted in each condition for analyzing the activation of p65 and MAPKs.
Results: DMF inhibits DC maturation by reducing cytokine production (IL-6 and IL-12) and the expression of MHC II, CD80 and CD86. Importantly, this immature DC phenotype generated less activated T cells that were characterized by decreased IFN-γ and IL-17 production. Further molecular studies demonstrated that DMF impaired NF-κB signaling via reduced p65 nuclear localization and phosphorylation. NF-κB signaling was further decreased by DMF-mediated suppression of Extracellular signal-Regulated Kinase 1 and 2 ($ERK_{1/2}$) and its downstream kinase Mitogen Stress activated Kinase 1 (MSK1). MSK1 suppression resulted in decreased p65 phosphorylation at serine 276 and reduced histone phosphorylation at serine 10. As a consequence, p65 transcriptional activity and p65 transcriptional-prone chromatin environment at gene promoters are impaired. Finally, treatment of DC with the MSK1 inhibitor H89, partially mimics the effects of DMF on the DC signaling pathway and impaired DC maturation.
Conclusion: By suppression of both NF-κB and $ERK_{1/2}$-MSK1 signaling, DMF inhibits maturation of DCs and subsequently impairs IFN-γ and IL-17 producing $CD4^+$ T cells differentiation.

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Improved drugability of vasoactive intestinal peptide (VIP) by nanotechnology for therapeutic selective targeting and immunomodulation

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Nanotechnology can address key bottlenecks hindering successful bench to bedside translation of recent research in the development of neuropeptide-based drugs. This is the case for vasoactive intestinal peptide (VIP), where sustained interest in its therapeutic applications needs to devise new methodologies to improve its drugability or to convey innovation to diagnostics to identify cells that result from conditions such as carcinoid metastasis in which VPAC receptors are involved. Here we present our results covering the chemical synthesis and functional characterisation of VIP Au/Ag nanoparticles, the protective effects on protease-based degradation, and the specific target to tumor cells or dendritic cells as an approach to cell therapy by using VIP-nanoliposomes.

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Cannabinoids affect mitochondrial targets in BV-2 microglial cells

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Objective: Cannabidiol (CBD) is a non psychoactive plant cannabinoid that is clinically used (as Sativex) in a 1:1 mixture with the psychoactive cannabinoid Δ^9- tetrahydrocannabinol (THC) for the treatment of neuropathic pain and spasticity in multiple sclerosis. In animal models of multiple sclerosis such as the experimental autoimmune encephalomyelitis model, CBD ameliorates the pathological symptoms and significantly reduces the disease-induced immune cell infiltration and microglial activation. The mechanisms by which CBD acts to reduce inflammation remain unclear. Among other cellular pathways, CBD may affect mitochondrial molecular targets in immune cells including microglia.
Methods: Here, we studied the effects of CBD and THC on various mitochondrial functions. These included: formation of reactive oxygen species (ROS), mitochondrial permeability transition pore opening, mitochondrial membrane potential (tested using live cell imaging flow cytometry), changes in mitochondrial morphology (studied using electron microscopy), and interaction with mitochondrial proteins (studied using density gradients and Western blotting).
Results: We found that CBD treatment led to significant changes in mitochondrial morphology (mainly swelling). In addition, density gradient analysis of detergent-free BV-2 microglial membrane fractions showed co-localization of CBD with markers of mitochondrial proteins.

Furthermore, CBD increased ROS production and affected mitochondrial membrane potential in a dose dependent manner. Finally, CBD interacted with the outer-mitochondrial membrane protein, the voltage-dependent anion channel (VDAC1) and decreased its channel conductance.
Conclusions: To conclude, CBD shows affinity to BV-2 mitochondrial membrane fractions and affects mitochondrial function and morphology. The functions of CBD at specific targets compared with THC will be discussed.

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Characterizing the role of CD200R1-expressing leukocyte populations in the inflammatory response to stroke: Defining a cellular target for immune inhibitory-based therapies

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Objective: Cell-surface receptors (e.g. CD200R1) on microglia and other myeloid-derived cells directly interact with specific endogenous ligands (e.g. CD200) expressed on neurons and act to suppress pro-inflammatory signaling by maintaining microglia in an "inactive state". We have previously shown that total CD200 expression levels decrease, while CD200R1 levels increase in the brain at 72 h post-reperfusion after experimental stroke. To determine whether stroke-enhanced expression of CD200R1 is associated with the endogenous microglial response or the influx of peripheral myeloid cells, we characterized $CD200R1^+$ immune cell populations at two timepoints afters stroke using flow cytometry.
Methods: Young male C57Bl/6 mice (N = 5/group) were subject to sham surgery or 90 min right middle cerebral artery occlusion and sacrificed 8- and 72 h post-reperfusion. Following perfusion with PBS, the stroke-side brain hemisphere, spleen, and blood were harvested for each mouse and processed into single cell suspensions for analysis by flow cytometry.
Results: At 8 hrs post-reperfusion, there is a robust increase in the number of $CD45^{hi}CD11b^+Ly6C^{hi}CD200R1^+$ cells in the spleen compared to sham. There is a concurrent increase in the number and mean fluorescence intensity of $CD45^{hi}CD11b^+Ly6C^{hi}CD200R1^+$ cells in the blood. At 72 h, 95% of $CD45^{hi}CD11b^+CD11c^+$ cells in the ischemic brain expressed CD200R1 (compared to 85% of $CD45^{int}CD11b^+$ microglia), and have a relative 2-fold increased shift in CD200R1 fluorescence intensity compared to microglia.
Conclusions: We hypothesize that the early increase in circulating $CD200R1^+$ cells results from an activated splenic response characterized by an enhanced production and release of $CD11b^+Ly6C^{hi}$ inflammatory monocytes. These subsequently migrate to ischemic regions of the brain and give rise to a subset of $CD11b^+CD11c^+CD200R1^+$ dendritic cells. The presence of $CD200R1^+$ inflammatory monocytes in the blood 8 h after stroke suggests that this population may represent an important cellular target for CD200-antibody-based therapies that could downregulate the activation state of immune cells that contribute to ischemic injury.

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Deficiency of natural killer cells in patients with multiple sclerosis is associated with aberrance of interleukin-7

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An aberrance of the immune system to discriminate self from non-self is believed to contribute to the emergence of autoimmune diseases such as multiple sclerosis (MS), a condition-derived from the immune-mediated damage to myelin and other components of the brain and spinal cord. Natural killer (NK) cells are significant players in the immune system; expansions of NK cells via engagement of common γ-chain cytokine IL-2 receptor reduce disease activities of MS and in the animal model experimental autoimmune encephalomyelitis. These observations prompted us to explore the immune phenotypes and functions of NK cells in MS. Here, we demonstrated that the numbers of NK cells are reduced and their cytolytic activities are compromised in patients with MS. This deficient phenotype of NK cells is not due to their intrinsic features, because these cells isolated from patients with MS can proliferate equally well as NK cells from normal subjects in NK-cell deficient mice upon cell transfer. Furthermore, we found that NK cells express the IL-7 receptor CD127 and a reduction of NK cells appears to correlate with a low level of IL-7 and in patients with MS, whereas other NK cell growth factors IL-2, IL-15 and IL-21 were elevated. Therefore, this study reveals the aberrance of NK cell in MS and its potential underlying mechanisms and further establishes that NK cells serve as an important disease player as well as a therapeutic target in MS and other autoimmune disorders.

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Human monocytes promote recovery from an animal model of spinal cord injury

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Objective: Increasing evidence shows that peripheral blood leukocytes, specifically monocyte-derived macrophages, are required for the healing process of injured tissue. These cells play a critical role in the innate immune response and in the regulation of local inflammation. Here, we investigated the potential therapeutic role of human monocytes administered by intracerebral-ventricular (ICV) transplantation in a murine model of severe spinal cord injury (SCI).
Methods: Human monocytes were isolated from peripheral blood and injected stereotactically ICV into immunodeficient mice, to avoid their rejection by the host, after contusive thoracic SCI. The functional motor recovery was determined following administration of increasing doses of monocytes; cell distribution throughout the central nervous system (CNS) was assessed using flow cytometry and histopathological techniques. Finally, specific gene expression was investigated by RT-PCR to gain insights into the mechanism of functional recovery after SCI.
Results: The transplantation of human monocyte-derived macrophages enhanced the recovery of locomotor functions in injured mice. These cells, injected in the lateral ventricle, homed to the site of injury but did not home to uninjured parts of the CNS parenchyma. At 7 days post-injury, the transplanted monocytes significantly down-regulated the expression levels of major inflammatory cytokines such as IL-6 and IL-1β, in the lesioned spinal cord. Moreover, these cells could locally release human IL-10.
Conclusion: The sub-acute administration of human blood-derived monocytes into the cerebrospinal fluid of injured immunodeficient mice promotes functional motor recovery in a dose-dependent manner. Monocytes migrate to the injury site and modulate the local

environment, by decreasing the level of pro-inflammatory cytokines and promoting the release of anti-inflammatory cytokines, such as IL-10. The model developed here for testing human monocytes in rodents provides an in vivo system for translation of a monocyte-based treatment protocol from bench to bed-side.

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Immunotherapy of experimental autoimmune encephalomyelitis using APC-targeted myelin peptides in prophylactic and therapeutic vaccines

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Objective: Antigen-presenting cells (APC), particularly dendritic cells (DC), are essential for priming T cell responses to antigen and also important for the maintenance of peripheral T cell tolerance. We tested mannan-peptide conjugation as a method for directly targeting myelin peptides to APC with the aim of inducing peripheral T cell tolerance and resistance to experimental autoimmune encephalomyelitis (EAE).
Methods: We performed repeated vaccinations of mice with myelin conjugated peptides in soluble form prior to induction of MOG_{35-55}-EAE. Clinical scores of mice were recorded and histopathological analysis of CNS tissues for demeylination and inflammation were performed. Various aspects of T lymphocyte maturation and effector functions were analyzed and dendritic cell function was assessed.
Results: Myelin peptides (MOG35-55, PLP139-151) synthesized with a (Lys-Gly)5 linker and conjugated to mannan in its oxidized (OM) or reduced (RM) forms, but not unconjugated peptides or mannan, strongly protected mice against EAE symptoms and neuropathology when administered in prophylactic or therapeutic protocols, with OM-conjugated peptides giving best results. Protection was peptide-specific and associated with impaired antigen-specific T cell proliferation and cytokine responses but not with depletion of $CD4^+$ T cells. Based on results from previous studies of APC-directed autoantigens we investigated several mechanisms of peripheral T cell tolerance for their possible contribution to the effects of OM-MOG. Reduced secretion of encephalitogenic T cell cytokines (IFN-g, TNF, IL-17) and normal secretion of immunodulatory cytokines (IL-4, IL-5) were seen in lymph node cells isolated from OM-MOG-, RM-MOG- and MOG- compared to PBS-vaccinated mice before and after the development of EAE. The proportions of $CD4^+FoxP3^+$ lymph node cells were equivalent in all groups of vaccinated mice during EAE development, while $CD4^+IL\text{-}10^+$ lymph node cells were reduced by therapeutic administration of OM and OM-MOG. Further, the proportions of $CD4^+$ lymph node cells expressing CD5, a co-receptor that regulates T cell tolerance, were increased by therapeutic administration of either OM or OM-MOG.
Conclusions: These results show that APC-targeting of peptide antigens by mannan conjugation is an efficient method for inducing peripheral T cell tolerance and reducing sensitivity to EAE in prophylactic and therapeutic protocols. They also show that tolerance mechanisms involving as Th1/Th2 shift in effector T cell responses, regulatory T cells and tolerogenic $CD4^+CD5^+$ T cells are not specifically induced by OM-MOG and suggest that a novel mechanism of tolerance is involved.

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Therapeutic treatment with an anti-mouse CD52 antibody reverses disease symptoms in a murine EAE model of multiple sclerosis

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Objective: Alemtuzumab is an anti-human CD52 humanized monoclonal antibody that alters the circulating lymphocyte pool. Alemtuzumab has demonstrated superior clinical efficacy compared to interferon-beta in relapsing-remitting multiple sclerosis patients. The goal of these studies was to characterize the effects of anti-mouse CD52 (muCD52) treatment in an experimental autoimmune encephalomyelitis (EAE) mouse model of multiple sclerosis.
Methods: A depleting antibody against muCD52 was generated for exploratory studies in an EAE model. C57BL/6 mice were immunized with MOG_{35-55} peptide to induce EAE and treated therapeutically with anti-muCD52 antibody. Cohorts were evaluated for the development and severity of disease symptoms and polychromatic flow cytometric analysis was used to determine the impact of anti-muCD52 therapy on various lymphocyte populations in both the spleen and central nervous system (CNS). Histological readouts were used to evaluate the level of cellular infiltrate and myelination within the CNS.
Results: Administration of the anti-muCD52 antibody to C57BL/6 mice resulted in lymphocyte depletion and subsequent repopulation with blood counts returning to baseline approximately 40 days post dosing. Therapeutic administration in an EAE model reversed existing paralytic symptoms and inhibited disease progression. Longitudinal flow cytometric evaluation showed a decrease in both the total number of circulating CD4 T cells as well as MOG_{35-55} specific CD4 T cells as measured by intracellular cytokine staining for IFN-gamma and IL-17. Histological evaluation showed a reduction in the level of lymphocytic infiltrate in the CNS of anti-muCD52 treated animals compared to control animals treated with vehicle.
Conclusions: Treatment with a depleting anti-muCD52 antibody in the MOG induced EAE model was effective in reversing paralytic disease. The effect was associated with a reduction in autoreactive MOG-specific T cells and diminished lymphocyte infiltration into the CNS.

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Suppression of experimental autoimmune encephalomyelitis with nanoparticles carrying a central nervous system antigen and a non-toxic aryl hydrocarbon receptor ligand

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Objective: To study the use of nanoparticles for the induction of antigen specific tolerance and the arrest of central nervous system (CNS) inflammation.
Methods: Multiple sclerosis (MS) is caused by an autoimmune response against the central nervous system (CNS). The immune response is normally controlled by regulatory T cells (Tregs), but Treg deficits characterize autoimmune diseases like MS. Thus the induction of antigen-specific Tregs is a potential therapeutic approach for autoimmune disorders. We found that the ligand activated transcription factor aryl hydrocarbon receptor (AHR) controls the differentiation of Treg and pro-inflammatory IL-17-producing Th17 cells. AHR activation by its mucosal ligand 2-(1′*H*-indole-3′-carbonyl)-thiazole-4-carboxylic acid methyl ester (ITE) triggers dendritic cells to promote the differentiation of Tregs that suppress experimental autoimmune encephalomyelitis (EAE), a murine model of MS. Here we report the use of nanoparticles to co-administer ITE and a peptide containing the encephalitogenic epitope $MOG_{35-55,}$ to induce tolerogenic DCs and generate CNS-specific Tregs that suppress EAE. We constructed Pegylated gold nanoparticles containing the non-toxic AHR ligand ITE and a peptide coding for the encephalytogenic peptide MOG_{35-55}. We

studied the effects of the nanoparticles on DCs in culture, and also on the recall response and the development of EAE in mice immunized with MOG_{35-55}.
Results: Nanoparticles containing ITE and MOG_{35-55} induced tolerogenic DCs that showed a reduced ability to drive the polarization of Th1 and Th17 cells, while they promoted the development of FoxP3 + Tregs. In vivo, nanoparticles containing ITE and MOG_{35-55} suppressed the recall response to MOG_{35-55}, interfering with the differentiation of Th1 and Th17 cells and promoting the differentiation of FoxP3 + Tregs. Moreover, the administration of nanoparticles containing ITE and MOG_{35-55} suppressed the development of EAE.
Conclusions: Nanoparticles are a new tool to reestablish antigen-specific tolerance in MS and other autoimmune disorders.

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Stat6 and RARβ signaling synergistically induces tolerogenicity in inflammatory dendritic cells

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Objective: While activated Ly-$6C^{hi}$ inflammatory monocytes (IMCs) and dendritic cells (IDCs) are potent T cell suppressors producing nitric oxide, non-activated IMCs and IDCs promote T cell activation and Th1/Th17 differentiation, and thus play a pathogenic function in EAE and other autoimmune disease models. In this study, we have explored a novel strategy that converts pro-inflammatory IDCs to tolerogenic dendritic cells.
Methods: C57BL/6 mice were immunized with complete Freund's adjuvant and pertussis toxin, and $CD11b^{+}$Ly-$6C^{hi}$ IMCs were purified from the spleens on day 11. IMCs were treated with GM-CSF for 24 h, and then IL-4 and retinoic acid for another 24 h. Gene expression was monitored by real time PCR and Western blot, and cell phenotype was analyzed by flow cytometry. The immune functions of treated IMCs were studied by co-culture with CD4 T cells isolated from MOG TCR transgenic 2D2 mice in vitro, and by adoptive transfer in vivo. Signaling events were studied by luciferase assay and chromatin immunoprecipitation.
Results: We show that IL-4 and retinoic acid (RA) treatment of IDCs synergistically induces the expression of aldehyde dehydrogenase 1a2 (Aldh1a2), a rate-limiting enzyme in RA synthesis in DCs. IL-4/RA treated IDCs upregulate CD103 expression and have a mature DC phenotype. They are functional in antigen presentation, but strongly induce Treg expansion and the conversion from non-Treg cells, while reducing Th1 and Th17 differentiation. These effects are dependent on RA production by treated IDCs. Mechanistically, Aldh1a2 induction by IL-4/RA treatment is dependent on Stat6 activation, and Stat6 binds directly to the Aldh1a2 promoter. In addition, this treatment synergistically induces RARβ expression. Using a luciferase assay, we show that Stat6 and RARβ overexpression drives Aldh1a2 promoter activity. Furthermore, our preliminary data show that adoptive transfer of IL-4/RA treated IDCs increases Treg frequency in vivo, and transfering at EAE peak promotes disease recovery.
Conclusions: Our data demonstrate the critical role of Stat6 and RARβ signaling in inducing a tolerogenic phenotype in IDCs, providing a potential treatment strategy for various autoimmune diseases including multiple sclerosis.

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Human IgG but not liposomal prednisolone improves the early clinical course of mdx mice as model for Duchenne muscular dystrophy

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Objective: In Duchenne muscular dystrophy (DMD), a gene replacement therapy is not yet available and the current standard treatment with oral prednisolone (PRED) is associated with considerable side-effects. Drug targeting by liposomal encapsulation of prednisolone (PL) or immunomodulation by immunoglobulin G (IgG) could be suitable alternatives for treatment.
Methods/results: 1. Using a computerized 24-hour detection system of voluntary wheel-running with separated cages for each mouse, a significantly impaired running performance was detected in mdx mice compared with C57black/10 controls aged 6 weeks.

2. In three week old mdx mice, intraperitoneal (i.p.) PL was tested at a dose of 1 mg/kg every other day and compared with i.p. PRED at a dose of 1 mg/kg every day or i.p. sham injections with saline or empty liposomes for 3 weeks (n = 12 each group). PL or PRED failed to improve the running performance of mdx mice and the muscle histopathology and real-time PCR of relevant inflammatory mediators such as TGF-beta and CCL-2 remained unaltered although the bioactivity in skeletal muscle of PL and free prednisolone was demonstrated by elevated mRNA expression of muscle ring finger protein 1 (MuRF1), and its forkheadbox transcription factors (Foxo1/3).

3. Three week old mdx mice received i.p. human IgG (Sandoglobulin Liquid) at a dose of 2 g/kg or an equal amount of human albumin or saline as controls every four weeks (total of two injections over 8 weeks; n = 10 each group). IgG compared to sham treatment significantly ameliorated the running parameters such as maximum velocity, distance and number of runs. In an *ex vivo* muscle contraction test, IgG significantly improved fatigability. The mRNA levels of TGF-beta, CCL-2 and secreted phosphoprotein 1 (osteopontin) were reduced in the diaphragm and skeletal muscle. Upon IgG, myopathic changes including the number of fibers with central nuclei were significantly reduced in lower limb muscles. Serum-CK, bodyweight and grip-strength of the forelimbs remained unchanged.
Conclusions: Our results support the validity of the computerized running wheel system for evaluation of treatment studies in mdx mice. Whereas conventional PRED as well as PL did not produce significant changes during the early disease course of mdx mice, IgG treatment profoundly improved the running performance and reduced myopathic changes as well as muscle inflammation. Thus, IgG may serve as a promising therapeutic agent for DMD.

Indice degli autori

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