Identification of gene expression patterns critically involved in experimental autoimmune encephalomyelitis and multiple sclerosis

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ABSTRACT

After encounter with central nervous system (CNS)- derived autoantigen, lymphocytes leave the lymph nodes and enter the CNS. This event leads only rarely to subsequent tissue damage. Genes relevant in CNS- infiltrating cells leading to subsequent CNS pathology are largely undefined. Myelin-oligodendrocyte-glycoprotein (MOG)- induced experimental autoimmune encephalomyelitis (EAE) is an animal model of multiple sclerosis (MS), a chronic autoimmune disease of the central nervous system (CNS), resulting in disability. To assess genes which are involved in encephalitogenicity and subsequent tissue damage mediated by CNS infiltrating cells we performed a DNA microarray analysis from cells derived from lymph nodes and eluted from CNS in LEW.1AV1 (RT1[av1]) rats immunized with MOG 91-108. The data was compared to immunizations with adjuvant alone or naïve rats and to immunizations with the immunogenic but not encephalitogenic MOG 73-90 peptide. Here we show involvement of *Cd38*, *Cxcr4* and *Akt* and confirm these findings employing CD38 knock-out (B6.129P2-Cd38[tm1Lnd]/J) mice, S1P-receptor modulation during EAE and quantitative expression analysis in patients with MS. The hereby defined underlying pathways indicate cellular activation and migration pathways mediated by G-protein coupled receptors as critical events in CNS tissue damage. These pathways can be further explored for novel therapeutic interventions.

INTRODUCTION

Multiple sclerosis (MS) is a disease of the central nervous system (CNS) which leads to chronic inflammation, demyelination, axonal and neuronal loss resulting in disability (Noseworthy et al., 2000, Weissert, 2013). Myelin-oligodendrocyte-glycoprotein (MOG)-induced experimental autoimmune encephalomyelitis (EAE) in rats reproduces major aspects of the human pathology (Weissert et al., 1998b, Storch et al., 1998, Kornek et al., 2000, Weissert, 2016). MOG is expressed on the outer surface of the myelin sheath. In contrast to merely T cell-mediated animal models, the pathogenesis of MOG-induced EAE in the rat involves the combined action of T and B cells, antibodies as well as macrophages, mimicking type II lesions in MS (Genain et al., 1995, Mathey et al., 2004, Lucchinetti et al., 2000). Encephalitogenic peptides presented on MHC class II molecules to T cells lead to a program which forces lymphocytes to be activated and migrate towards the CNS (Riedhammer and Weissert, 2015). Adjuvant contributes by affecting multiple signalling pathways in lymphocytes as well as in organ resident cells like the CNS. We have previously demonstrated that MOG 91-108 is the major determinant to trigger disease in rats expressing $RT1^{av1}$ or $RT1^n$ haplotypes (Weissert et al., 2001). Interestingly, the capacity of MOG 91-108 to induce EAE was dissociated in regard to Th1 or Th2 cytokine expression in lymphoid tissue compared to CNS. Moreover, different MOG 1-125- derived peptides, such as MOG 73-90, were immunogenic showing strong Th1 responses but were not encephalitogenic. The induction of active EAE in LEW MHC congenic rat strains and DA ($RT1^{av1}$) rats does not require the application of pertussis toxin like in mice. This is an advantage since the exact role of pertussis toxin in EAE induction is not clear so far. Pertussis toxin inhibits Gi proteins and influences by this multiple cellular processes and pathways (Dumas et al., 2014). Active EAE in susceptible rat strains is induced by immunization with an encephalitogenic peptide mixed with mineral oil (incomplete Freund's adjuvant) with the addition of heat-inactivated

mycobacterium tuberculosis (MT) as adjuvant (complete Freund's adjuvant). MT leads by binding and signalling through Toll-like receptors (TLR) to an activation program in a number of cell types and is also a systemic 'danger signal' (Mills, 2011).

In regard to susceptibility to EAE and MS gene expression profiling studies were performed to elucidate genes which are involved in disease pathogenesis. A number of interesting genes were described like osteopontin (Hur et al., 2007). In no study a systematic comparison of gene expression profiles was performed in EAE in which the influence of adjuvant and antigen was systematically compared on the expression profile of lymph node derived cells or cells eluted from CNS of diseased animals. In this study we systematically compared the gene expression profiles of cells from draining lymph nodes and CNS infiltrating cells which were eluted in LEW.1AV1 (RT1[av1]) rats immunized with MOG91-108 in CFA, CFA alone and naïve rats. Moreover we compared the gene expression profile of rats immunized with the encephalitogenic MOG peptide 91-108 with rats immunized with the non-encephalitogenic MOG peptide 73-90. We found differentially expressed genes which are of major importance for encephalitogenicity. The influence of these genes was subsequently verified by different means.

RESULTS

Gene expression after immunization with encephalitogenic and non-encephalitogenic peptides

One of the important questions in MS and other inflammatory diseases of the CNS is to understand the requisites of autoantigenic peptides to induce CNS inflammation (Riedhammer and Weissert, 2015). Beside presentation of autoantigen-derived peptides on MHC molecules and the availability of reactive T cell and B cell repertoires as well as the presence of the target antigen in the CNS, pathways of cellular activation exist that allow disease development. These pathways are presently only partly elucidated. We used MOG-induced EAE in LEW.1AV1 (RT1[av1]) rats as a model system for CNS inflammation. In this EAE model the determinant MOG 91-108 is immunogenic and encephalitogenic. In contrast the determinant MOG 73-90 is immunogenic but not encephalitogenic. We assessed the gene expression profiles by gene arrays of lymphocytes from draining lymph nodes and from lymphocytes eluted from the CNS.

To focus on genes which are truly relevant to encephalitogenicity and not simply involved in general inflammatory responses, we compared gene arrays of LEW.1AV1 (RT1[av1]) rats immunized with the encephalitogenic MOG stretch MOG 91-108 to naïve LEW.1AV1 (RT1[av1]) rats and rats immunized with the adjuvant CFA alone as well as rats immunized with the non-encephalitogenic MOG 73-90 determinant. We analyzed ten comparisons for each of the naïve and CFA groups versus MOG 91-108 and five comparisons for MOG 73-90 versus MOG 91-108. The number of comparisons in which a given gene had a signal log ratio (SLR) of above 1 were counted. In table 1, we show genes which are upregulated in at least half of the comparisons (=50%). Besides *Cxcr4* and *Cd38*, which were subsequently analysed in greater detail, many genes with a known function in EAE and MS pathology were found to have an increased expression in MOG 91-108 immunized rats as compared to

controls. This validates our gene list and supports the relevance of the genes not previously described in EAE. In table S1, genes with decreased expression in EAE are listed. In this analysis, the variability between gene arrays was much higher and less genes were found to be regulated with a clear pattern according to our criteria.

Comparisons of microarrays of CNS-infiltrating lymphocytes derived from LEW.1AV1 (RT1^{av1}) rats after immunization with MOG 91-108, MOG 73-90 and CFA alone resulted in many more genes being differentially expressed as compared to the analysis of lymph node cells (Table S2 and Table S3). This could mirror the influx of different cell populations into the CNS during an inflammatory attack. Similar to the analysis of lymph node cells we found that *Cd38* and *Cxcr4* mRNA was strongly increased in CNS-infiltrating cells.

Subsequently, we analysed purified CD4+ cells from lymph nodes and CNS of MOG 91-108 and MOG 73-90 immunized rats. To some extent similar gene expression profiles were found in the purified CD4+ cell population compared to non-separated lymph node cells (Table 1, Table S4, Table S5).

Due to their strong expression in MOG 91-108-immunized rats compared to naïve, CFA-immunized and MOG 73-90-immunized rats, we chose *Cxcr4* and *Cd38* for further analysis.

Cxcr4 and *Cd38* in EAE in LEW.1AV1 (RT1^{av1}) rats

Confirming our microarray results by quantitative PCR we found a significant upregulation of *Cxcr4* expression in lymph node cells of MOG 91-108-immunized rats (n=8) as compared to CFA-immunized (n=8, ANOVA, *P*<0.0001) and naïve (n=6, ANOVA, *P*<0.0001) LEW.1AV1 (RT1^{av1}) rats (Fig. 1A). Also an increased expression of *Cd38* was measured (MOG 91-108-immunized rats [n=8] as compared to CFA-immunized [n=8, ANOVA, *P*<0.05] and naïve [n=6, ANOVA, *P*<0.05] LEW.1AV1 [RT1^{av1}] rats).

In cells eluted from CNS we found upregulation of *Cxcr4* (Fig. 1B) and *CD38* (Fig. 1C) in MOG 91-108 immunized LEW.1AV1 (RT1^av1) rats (n=6) compared to rats immunized with MOG 73-90 (n=6, ANOVA *Cxcr4* and *Cd38* each *P*<0.001) or CFA alone (n=6, ANOVA, *Cxcr4* and *Cd38* each *P*<0.001).

Cxcr4 and *Cxcl12* expression in spinal cord of DA (RT1^av1) rats

Next we assessed the mRNA expression of *Cxcr4* (Fig. 2A) and its ligand *Cxcl12* (Fig. 2B) in spinal cord of either naïve DA (RT1^av1) rats or DA (RT1^av1) rats immunized with IFA or CFA alone or MOG 1-125 in IFA or MOG 1-125 in CFA (each n=4). Upregulation of both *Cxcl12* and *Cxcr4* mRNA expression was observed in CFA and MOG 1-125 in CFA immunized DA (RT1^av1) rats in spinal cord compared to naïve rats, IFA injected or MOG 1-125 in IFA immunized DA (RT1^av1) rats (ANOVA, *P*<0.001). Increased *Cxcl12* and *Cxcr4* mRNA expression was observed in MOG 1-125 in CFA compared to CFA immunized DA (RT1^av1) rats (ANOVA, *P*<0.01).

Cxcr4 and CXCL12 in patients with MS

Upregulation of mRNA of *Cxcr4* was also observed in white blood cells of MS patients with a relapsing-remitting disease course (RRMS, n=32) and a secondary chronic progressive disease course (SPMS, n=22) compared to controls (n=25, ANOVA for RRMS and SPMS each *P*<0.05) (Fig. 3A, Table S6, Table S7). Also, we detected increased protein CXCL-12 serum levels in both patients with RRMS (n=24) and SPMS (n=28) compared to controls (n=21, ANOVA for RRMS and SPMS each *P*<0.05) (Fig. 3B, Table S6, Table S7).

EAE in B6.129P2-Cd38[tm1Lnd]/J mice

Cd38 was strongly upregulated in encephalitogenic lymph node cells. To functionally validate our data and to elucidate the role of CD38 in EAE, we induced disease with the extracellular domain of MOG (MOG 1-125) in CD38 knock out (B6.129P2-Cd38[tm1Lnd]/J) mice and appropriate controls. We found reduced disease severity in MOG 1-125-immunized B6.129P2-Cd38[tm1Lnd]/J mice (n=22) compared to wild type control mice (n=21, t-test, cumulative disease score $P<0.01$) (Fig. 4A). Next we determined the height of the antibody response to MOG 1-125. Reduced anti-MOG IgG autoantibody responses in B6.129P2-Cd38[tm1Lnd]/J mice (n=4) compared to wild type control mice (n=4, ANOVA, $P<0.05$)) after immunization with MOG 1-125 were seen (Fig. 4B) on day 12 p.i. Furthermore, also T cell responses upon restimulation with MOG 1-125 were reduced in the B6.129P2-Cd38[tm1Lnd]/J mice (n=4) on day 12 p.i. compared to control mice (n=4, t-test for stimulation with 50μg/ml MOG 1-125, $P<0.05$) as measured in a proliferation assay indicating in addition a T cell priming or expansion defect in B6.129P2-Cd38[tm1Lnd]/J mice (Fig. 4C).

Targeting cellular migration by FTY720

We found an upregulation of *Akt* in our differential gene expression studies (Table 1). FTY720 is a S1P-receptor modulator known to influence T cell trafficking by an *Akt*-dependent mechanism, as does CXCR4 (Mandala et al., 2002, Cyster, 2005, Lee et al., 2001, Brinkmann et al., 2002, Matloubian et al., 2004). We evaluated inhibition of *Akt*-dependent cell trafficking in MOG 91-108-immunized LEW.1AV1 (RT1[av1]) rats. Disease was completely inhibited in rats treated from day 0 p.i. with FTY720 (n=10) as compared to the vehicle treated controls (n=10, t-test, cumulative disease score $P<0.0001$) (Fig. 5A). Next, we tested the efficacy of FTY720 to treat established relapsing-remitting MOG 1-125-induced

EAE in the DA (RT1^{av1}) rat. FTY720 treatment was started on day 21 p.i., after the first bout of disease (n=8), and showed a significant effect on the disease course as compared to vehicle treatment (n=8, t-test, cumulative disease score day 21-44 p.i., *P*<0.01) (Fig. 5B). To further examine the beneficial effect of FTY720 treatment on EAE, we boosted the rats with MOG 1-125 on day 44 after the first immunization. Although a relapse was induced in both groups, the DA rats under FTY720 treatment had a better clinical outcome compared to the vehicle treated animals (t-test, cumulative disease score day 45-56 p.i. *P*<0.0001). On day 14 p.i. FTY720 treatment led to an increase of the relative size of the CD4 T cell compartment (ANOVA, *P*<0.01) in the treated LEW.1AV1 (RT1^{av1}) rats (n=10) compared to controls (n=9) concurrent with a decrease in the CD8 T cell (ANOVA, *P*<0.01) and the B cell (ANOVA, *P*<0.01) compartment (Fig. 5C; Fig. S1). FTY720 treatment (n=9) compared to controls (n=9) led to down-regulation of mRNA expression of its receptor *S1p1* (ANOVA, *P*<0.0001) and of *Akt2* (ANOVA, *P*<0.0001), one of the genes involved in the intracellular signalling cascade connected to S1P1 and CXCR4 on day 14 p.i. Treatment also had a negative effect on the expression levels of *Cd38*, (ANOVA, *P*<0.0001). In contrast, the expression of *Cxcr4* was not altered (ANOVA, not significant) (Fig. 5D).

DISCUSSION

In this study we identified gene networks that are critically involved not only in raising an autoantigen- specific immune response but also constituting encephalitogenicity. We analysed the expression and functional relevance of genes and their products expressed on lymphocytes after immunization with the encephalitogenic MOG91-108 peptide in adjuvant, the non-encephalitogenic MOG73-90 peptide in adjuvant, adjuvant alone or naïve rats. Most interestingly in the comparison of MOG91-108 in adjuvant to MOG73-90 in adjuvant immunized rats, compared to the other analyses, only a small number of genes were differentially expressed. These genes seem to be of major importance since they are genes involved in the encephalitogenic response leading to disease manifestation. From the overall expression data, we selected three genes which were upregulated in many comparison: *Cxcr4*, *Cd38* and *Akt*. We performed functional studies regarding these genes in EAE and analysed tissue samples from MS patients.

CXCR4 (CD184) is a seven transmembrane G-coupled receptor expressed by a number of tissues including cells of the immune system (Campbell et al., 2003). CXCR4 knock-out mice die in utero or perinatally and do not only have defects in the hematopoietic system (impairment of myeloid and B cell generation, reduced proliferation of triple-negative and double-positive lymphocytes), but also in the circulatory system and in the CNS (Zou et al., 1998, Tachibana et al., 1998). Overexpression of CXCR4 on T cells induces their accumulation in the bone marrow and reduction of these cells in the peripheral blood. CXCR4 signalling leads to a prolonged protein kinase B (AKT) and extracellular signal-regulated kinase 2 activation in T cells. AKT activation promotes cell survival and can act as costimulation for T cell activation (Tilton et al., 2000). CXCR4 has only one known cognate ligand which is CXCL12. CXCL12 is constitutively produced by stromal and endothelial cells. CXCL12 activates numerous signalling pathways like receptor-associated trimeric G

proteins, phospholipase Cγ, PI3K and small G proteins (Pawig et al., 2015). Signalling through these receptors leads to an increase in the intracellular calcium concentration, cytoskeleton reorganization and cellular migration. Several modulating factors such as phosphatases, regulator of G-protein signalling, adaptor proteins and ubiquitin may affect signalling and/or chemotactic response of CXCR4 to its ligand. An important function of CXCR4/CXCL12 is the regulation of bone-marrow homeostasis and lymphocyte trafficking. Chemotaxis and integrin-mediated adhesion are the main cellular responses to CXCL12. CXCL12 knock-out mice display the same phenotype as CXCR4 knock-out mice (Nagasawa et al., 1996).

In autoimmunity there are indications that the interaction of CXCR4 and CXCL12 could be important. CXCL12 recruits B cells to inflamed glomeruli in which these cells my produce autoantibodies (Balabanian et al., 2003). Also in rheumatoid arthritis CXCR4 and CXCL12 have been proposed to be important in the disease precipitation (Zhang et al., 2005). A role of CXCR4 and CXCL12 in EAE (Meiron et al., 2008, Kohler et al., 2008, McCandless et al., 2006) as well as in MS has been described (Azin et al., 2012, Krumbholz et al., 2006).

Our data indicates that also in MOG-induced EAE and possibly MS the interaction of CXCR4 and CXCL12 is of paramount importance for disease development. We found specific upregulation of *Cxcr4* on cells derived from lymph nodes and eluted from CNS of rats immunized with encephalitogenic MOG91-108 peptide in comparison to controls. In addition, we measured upregulation of *Cxcl12* and *Cxcr4* in spinal cords in EAE compared to controls. CXCL12 is upregulated in the CNS of MS patients (Krumbholz et al. 2006 and own unpublished observations). Together the presented data would argue for a scenario in which lymphnode- derived cells are in the context of an encounter with an encephalitogenic antigen activated and migrate towards CXCL12 in the CNS. This is further underscored by the fact

that nitric oxide enhances LPS-induced expression of CXCR4 and migration towards CXCL12 (Giordano et al., 2006).

CD38 is a membrane associated type II glycoprotein which acts both as an receptor and enzyme (Cockayne et al., 1998, Kato et al., 1999, Salmi and Jalkanen, 2005). As an enzyme it catalyzes NAD+ into cyclic ADP-ribose and further into ADP-ribose. It also regulates Ca^{2+} levels from ryanodine receptor stores. CD38 is expressed on a variety of myeloid and lymphoid cells. CD38 ligation in B cells leads to tyrosine phosphorylation of several intracellular proteins such as Syk, p85 of phophatidylinositol-3 kinase and phospholipase C-γ (Silvennoinen et al., 1996). CD38 ligation in T cells results in phosphorylation of the Raf-1/MAP kinase and CD3-ζ/ZAP-70 signalling pathway (Zubiaur et al., 1997). Furthermore it is involved in dendritic cell migration and adhesion between lymphocytes and endothelial cells (Partida-Sanchez et al., 2004). B6.129P2-Cd38[tm1Lnd]/J mice show slight reduction of antibody titers to T cell-dependent antigens (Cockayne et al., 1998). Additionally, these mice had increased susceptibility to bacterial infections, which is thought to be caused by a defective chemotactic response of neutrophils towards bacteria underscoring a role in innate immunity as well (Partida-Sanchez et al., 2001).

We induced EAE in CD38 knock out mice (B6.129P2-Cd38[tm1Lnd]/J) and controls. CD38 knock out mice had a reduced disease severity and lower autoantibody and T cell responses as compared to the controls. These finding show the importance of CD38 in EAE and possibly MS. This work lies the basis for further analysis of the involved cellular compartments and regulation of human disease (Mayo et al., 2008, Lischke et al., 2013).

FTY720 is a potent drug that affects lymphocyte trafficking and homing. Our studies and studies by others (Brinkmann et al., 2002, Balatoni et al., 2007) showed a strong beneficial effect of FTY720 on EAE under various experimental settings. The drug was approved for treatment of MS (Kappos et al., 2010). We demonstrate that treatment of MOG-EAE with

FTY720 impacts the gene expression of the genes that are involved in encephalitogenic immune responses. This further validates our approach. We measured changes in expression of *S1P1*, *Akt2* and *Cd38*. Interestingly, no changes in expression of *Cxcr4* was observed. The reason for this is not fully understood and deserves further investigations.

In conclusion in this study we have identified genes which are involved not only in raising an autoantigen-specific immune responses but constitute encephalitogenicity. The immunization with encephalitogenic peptides induces a network of genes involved in activation and migration of lymphocytes. Based on this platform we have established the paramount importance of G-coupled proteins in encephalitogenicity of adaptive immune responses. We speculate that similar involvement might operate also in other autoimmune diseases and possibly transplant rejection thereby establishing common mechanisms. These pathways might be valuable targets of therapeutic approaches as we have shown attenuation of EAE after treatment with FTY720 or reduced EAE severity in CD38-deficient mice. These findings not only validate our gene expression data, but also underscore the importance of the rat EAE model in translational medicine.

MATERIALS AND METHODS

Animals and EAE induction

Female rats or mice, 10-14 weeks of age, were used in all experiments. LEW.1AV1 (RT1^{av1}) rats were obtained from Hans Hedrich (Central Animal Laboratory, Hannover Medical School, Hannover, Germany) and DA (RT1^{av1}) rats were obtained from Harlan Winkelmann (Borchen, Germany). Female B6.129P2-CD38^{tm1Lnd}/J mice and the appropriate controls C57BL/6J 000664 were purchased from the Jackson Laboratory (Bar Harbor, USA).

Animals were bred and kept under specific pathogen-free conditions. Animals were injected intradermally at the base of the tail (rats) or both flanks (mice) with 100 µg of MOG 91–108 (rats) or 50 µg of rat recombinant MOG 1-125 (rats) or 100 µg MOG 35-55 (mice) or 20 µg rat recombinant MOG 1-125 (mice). The antigens in a total volume of 100 µl were mixed with 100 µl of CFA (1:1). A total of 100 µl of CFA consisted of IFA (Sigma-Aldrich, St. Louis, MO) and 500 µg for rats or 400 µg for mice of heat-inactivated *Mycobacterium tuberculosis* (strain H37 RA; Difco Laboratories, Detroit, MI) (Weissert, 2016). Mice additionally received 100 ng of pertussis toxin (Calbiochem, Darmstadt, Germany) on day 0 and 2 intraveniously. Some groups of rats were also injected with 100 µl IFA mixed with 100 µl MOG 1-125 in PBS (50 µg) without the addition of *Mycobacterium tuberculosis* or with IFA or CFA alone. The clinical scoring was as follows: 0 = no illness; 1 = tail weakness or paralysis; 2 = hind leg paraparesis or hemiparesis; 3 = hind leg paralysis or hemiparalysis; 4 = tetraparesis or moribund. All experiments were approved by the regional board in Tübingen, Germany.

Human samples

Blood samples were obtained after consent from patients with MS and controls. The characteristics of the MS patients and controls are indicated in Table S6 and Table S7. The research was approved by the Ethics committee of the University of Tuebingen in Germany (Permission 125/2001).

Isolation of CNS infiltrating cells

Infiltrating cells from the CNS were prepared as described before (Weissert et al., 1998a, Weissert et al., 2001). In brief, rats were perfused with cold PBS, and brains and spinal cords were dissected out on day 12 p.i. Subsequently, brains and spinal cords were homogenized in 10 ml 50% Percoll/0.1% BSA/1% glucose (Amersham Pharmacia Biotech) containing 500 U DNase type I (Life Technologies). Ten ml of 50% Percoll were added to each sample after homogenization. A discontinuous Percoll gradient was obtained by adding seven ml of 63% Percoll below and 20 ml of 30% Percoll above the sample. Samples were centrifuged for 40 min at 1000 x g at 4°C. Lymphocytes were collected from the 63/50% Percoll interface. The cells were subsequently washed twice in 15–25 ml PBS with centrifugation at 600 x g for 15 min at 4°C.

Isolation of mononuclear cells (MNC) from lymph nodes (LN) and spleens

Draining inguinal LN and spleens were dissected out under deep anaesthesia. LN were disrupted and MNC washed twice in Dulbecco's modified eagle medium (DMEM, Life Technologies, Paisley, U.K.), resuspended in complete medium (CM) containing DMEM supplemented with 5% fetal calf serum (PAA Laboratories Linz, Austria), 1% penicillin/streptomycin (Life Technologies), 1% glutamine (Life Technologies), and 50 µM

2-mercaptoethanol (Life Technologies) and flushed through a 70-µm plastic strainer (Falcon; BD Biosciences, Franklin Lakes, NJ). MNC from spleen were prepared in the same way as from LN with the difference that red blood cells (RBC) were lysed with lysis buffer consisting of 0.15 M NH_4Cl, 10 mM $KHCO_3$, and 0.1 mM Na_2 EDTA adjusted to pH 7.4. CD4+ cells were isolated by anti- rat CD4 microbeads using MACS technology (Miltenyi Biotech, Bergisch Gladbach, Germany) according to the manufacturers instruction.

CD4+ cell purification

CD4+ cells from the lymph nodes were purified by MACS (Miltenyi Biotech, Bergisch Gladbach, Germany) and subsequently analyzed by Affymetrix gene array for differential gene expression.

Spinal cord tissue

From PBS- perfused naïve or in either IFA, CFA, MOG 1-125 in IFA or MOG 1-125 in CFA DA immunized rats, spinal cord tissues was dissected and homogenized and subsequently assessed for mRNA expression.

RNA preparation

Total RNA of brain infiltrating leukocytes or lymphocytes or spinal cord tissue was isolated by using a RNeasy kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The RNA quality was analysed with a Bioanalyser 2100 (Agilent, Palo Alto, USA).

Microarrays

Affymetrix microarrays of the type RG U34 A (Affymetrix Inc., Santa Clara, USA) representing approximately 7000 full length genes and 1000 EST clusters were used. For the purified CD4+ cells the rat expression set 230A (Affymetrix) containing about 30.000 features was used. For each array, samples from at least three rats were pooled. Biotin labelled cRNA was prepared and hybridized to the arrays. In brief, double stranded cDNA was synthesized from whole RNA using a superscript choice kit (Invitrogen) with a T7-(dT)24 primer (Metabion) and in vitro transcribed into biotin-labelled cRNA. After hybridization gene arrays were washed and stained by a fluidics station (Affymetrix) and scanned by a confocal laser scanning microscope (Agilent).

The data was analyzed using the microarray suite software, micro DB, and data mining tool (Affymetrix). Only genes and EST were included in further analysis which were "present" and gave a difference call of either "increase" or "decrease" according to the Affymetrix software. The extend of differential expression is expressed as signal log ratio (SLR). For the tables the cut off was set to a SLR of 1 signifying an 2-fold change in expression. Doubles, ESTs, and sequences not corresponding to a gene were not included in the tables.

Real time PCR

To avoid amplification/detection of contaminating genomic DNA, extracted RNA was treated with Rnase free DNase (Promega, Madison, WI). Subsequently, cDNA was synthesized by reverse transcription with Moloney murine leukemia virus reverse transcriptase and random pdN6 primers in the presence of RNase inhibitor (Promega). Amplification was performed on an Applied Biosystems Prism 7000 Sequence Detection System (Applied Biosystems, Foster

City, CA) using a SYBR green protocol. Results were expressed as $2^{-\Delta\Delta CT}$ values. The primer sequences 5´ to 3´ were as follows:

<u>Rat primers</u>

Gapdh:	forward: GGTTGTCTCCTGTGACTTCAA
	reverse: CATACCAGGAAATGAGCTTCAC
S1p1:	forward: TAGCCGCAGCAAATCAGAC
	reverse: GCAGCAGTGGAGAAAGAGAGA
Cxcr4:	forward: GATGGTGGTGTTCCAGTTCC
	reverse: CAGCTTGGAGATGATGATGC
Cxcl12:	forward: CGATTCTTTGAGAGCCATGT
	reverse: AGGGCACAGTTTGGAGTGTT
Cd38:	forward: AGGACACACTGCTGGGCTAT
	reverse: CAGGGTTGTTGGGACAATTT
Akt2:	forward: GAAGACTGAGAGGCCACGAC
	reverse: GGGAGCCACACTTGTAATCC

<u>Human primers</u>

h18s:	forward: CGGCTACCACATCCAAGGAA
	reverse: GCTGGAATTACCGCGGCT
Cxcr4:	forward: CATCAGTCTGGACCGCTACC,
	reverse: GGATCCAGACGCCAACATAG.

FACS

FITC conjugated mAb against CD45RA (OX-33) and TCRAB (R73) and PE conjugated mAb against CD4 (OX-35) and MHC II (OX-6) and appropriate isotype controls were purchased from Becton Dickinson (Heidelberg, Germany). Flow cytometry was performed on a FACScalibur running with Cellquest software (Becton Dickinson). Cells were gated on the lymphocyte population in the FSC-SSC dot plot. For data analysis also Flowing Software 2.5.1 (Turku Center for Biotechnology, Finland) was used.

ELISA

Serum taken at the time point of euthanasia was subject to an anti-MOG autoantibody ELISA. ELISA plates (96 well; Nunc, Roskilde, Denmark) were coated with 2.5 µg/ml (100 µl/well) MOG 1-125 overnight at 4°C. Plates were washed with PBS/0.05% Tween 20 and blocked with milk powder for 1 h at room temperature. After washing, diluted serum samples were added and plates were incubated for 1 h at room temperature. Then, plates were washed and rabbit anti mouse antiserum (IgG, IgG1, IgG2a, IgG2b, IgG3, IgM Nordic, Tilburg, The Netherlands) was added and incubated for 1 h at room temperature. Plates were washed prior to the addition of peroxidase conjugated goat anti rabbit antiserum (Nordic) diluted in PBS/0.05% Tween 20. After 30 min incubation, plates were washed and bound Abs were visualized by addition of ABTS (Roche Diagnostics Mannheim, Germany). After 15 minutes of incubation optical density was read at 405 nm.

ELISA with human serum was performed according to the instructions of the manufacturer.

Proliferation assay

Cells from draining LN were prepared as described above and cultured in the presence of rrMOG in 96 well plates as described in the Elispot section. Cells were cultured for 48h and pulsed with 1µCi [^{3}H]thymidine for the last 18 h. The incorporation of [^{3}H]thymidine was measured using a beta-scintillation counter.

FTY720 treatment

Rats were treated with FTY720 (generous gift of Novartis AG, Switzerland) and received a daily dose of 0,4 mg/kg in sterile water by oral gavage as described (Balatoni et al., 2007).

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Competing interests

There are no competing interests

Author contribution

M.M.H. performed the gene arrays, data analysis, the target validation in rats and revised the paper. S.B. performed the gene arrays, data analysis and the target validation. B.G. did the work on CD38 in mice. K.M.S. performed target validation studies in rats and analysed the data. A.B. performed studies in MS patients and controls. R.W. designed the study, raised the funding, performed the data analysis and wrote and revised the paper.

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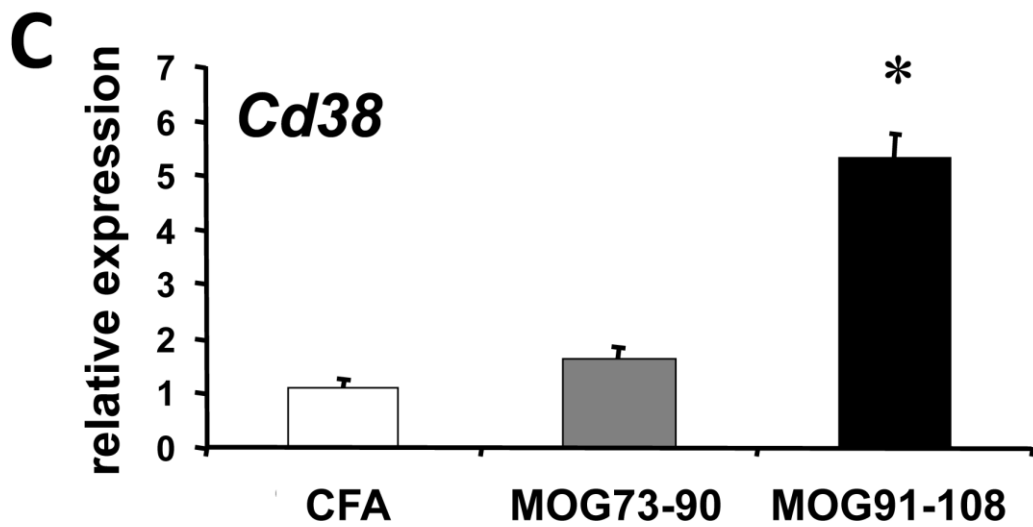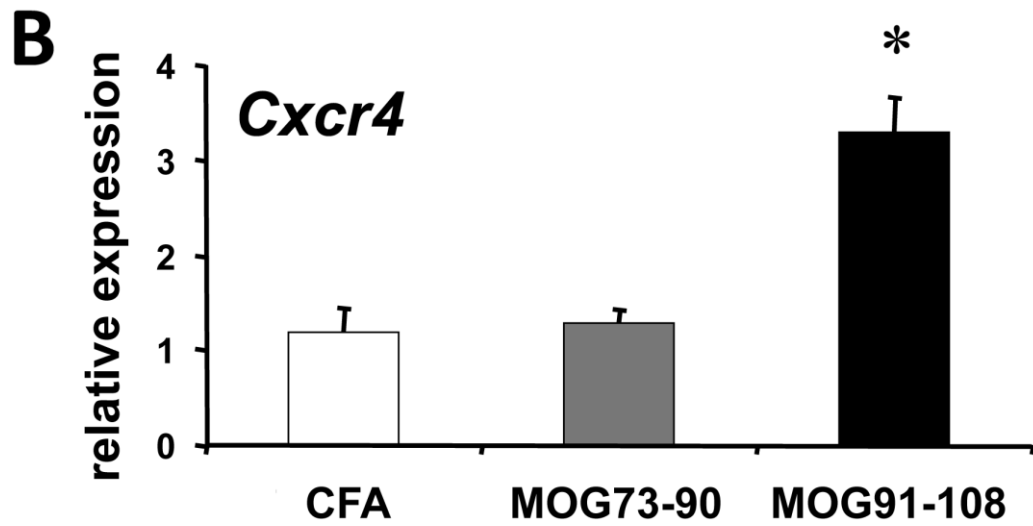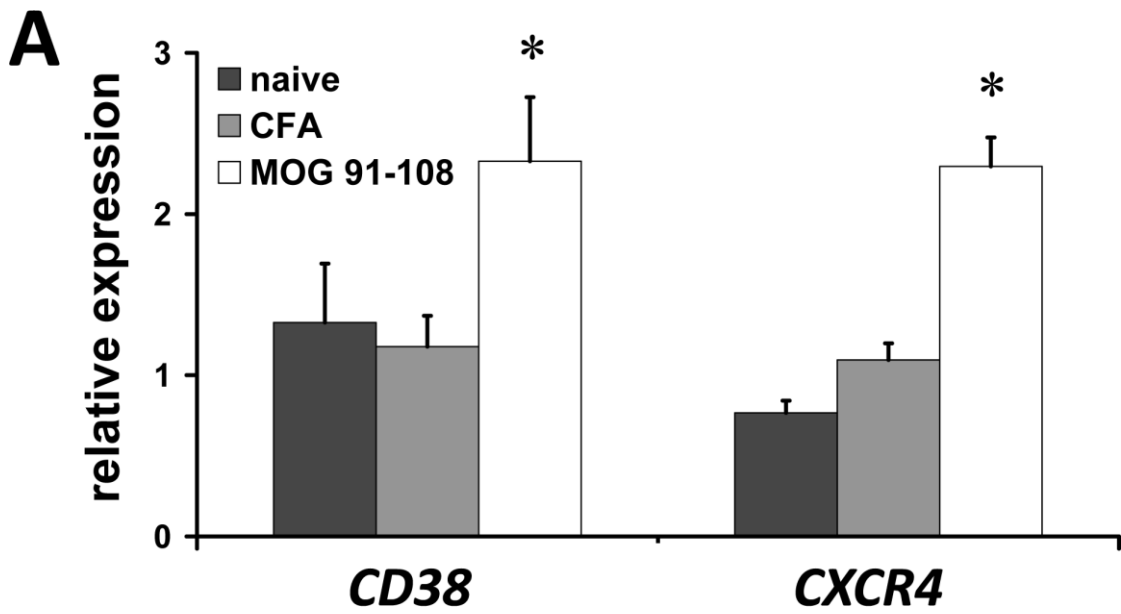
Fig. 1. *Cxcr4* **and** *Cd38* **expression in lymph node cells and CNS.**

A, Quantitative SYBR green real time PCR was performed for *Cxcr4* and *Cd38* in lymph node cells from naïve (black bars, n=6), CFA (grey bars, n=8) or MOG 91-108 in CFA (white bars, n=8)- immunized LEW.1AV1 (RT1^{av1}) rats on day 12 p.i.. Increased *Cxcr4* (*ANOVA, *P*<0.0001) and *Cd38* (*ANOVA, *P*<0.05) expression was found in MOG 91-108 in CFA- immunized rats compared to naïve and CFA- alone- immunized rats. **B**, Quantitative expression of *Cxcr4* in cells eluted from CNS of CFA (white bars, n=6), MOG 73-90 in CFA (grey bars, n=6) and MOG 91-108 in CFA (n=6) immunized LEW.1AV1 (RT1^{av1}) rats. *Cxcr4* was upregulated in MOG 91-108 in CFA-immunized rats compared to the other groups (*ANOVA, *P*<0.001) on day 12 p.i.. **C**, Quantitative expression of *Cd38* of lymphocytes eluted from CNS of CFA (white bars, n=6), MOG 73-90 in CFA (grey bars, n=6) and MOG 91-108 in CFA (n=6) immunized LEW.1AV1 (RT1^{av1}) rats on day 12 p.i. *Cd38* was upregulated in MOG 91-108 in CFA-immunized rats compared to the other groups (*ANOVA, *P*<0.001). Results are expressed as 2$^{-\Delta\Delta\ CT}$ values. Numbers are mean +/- SEM.
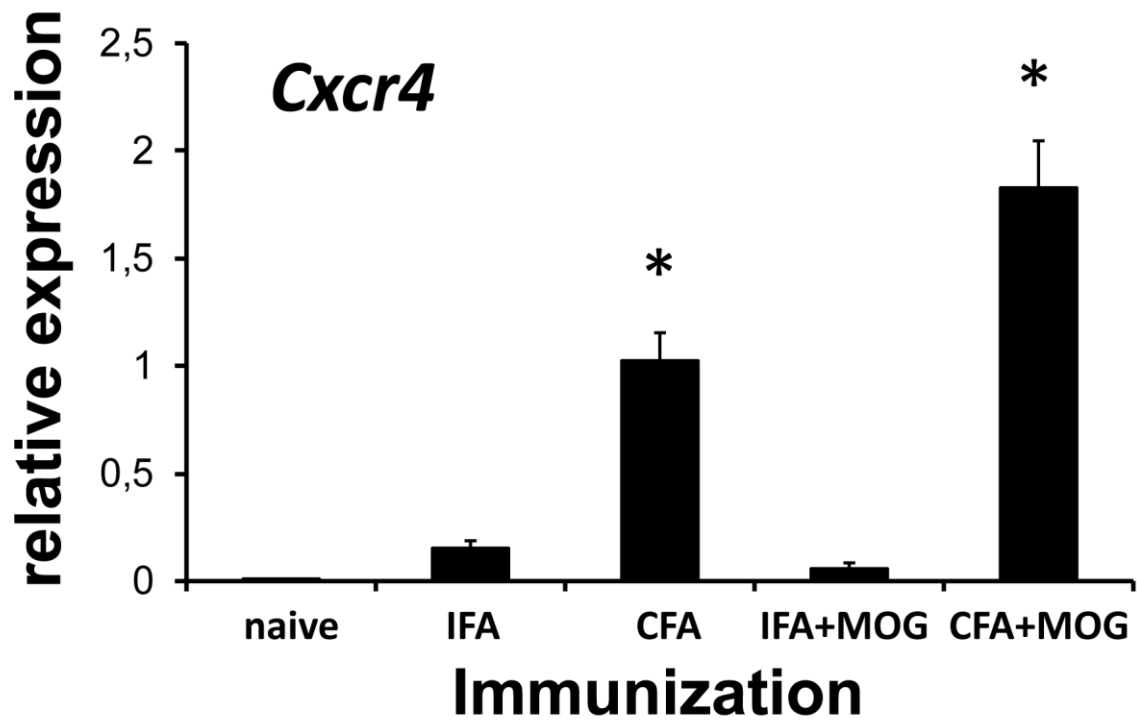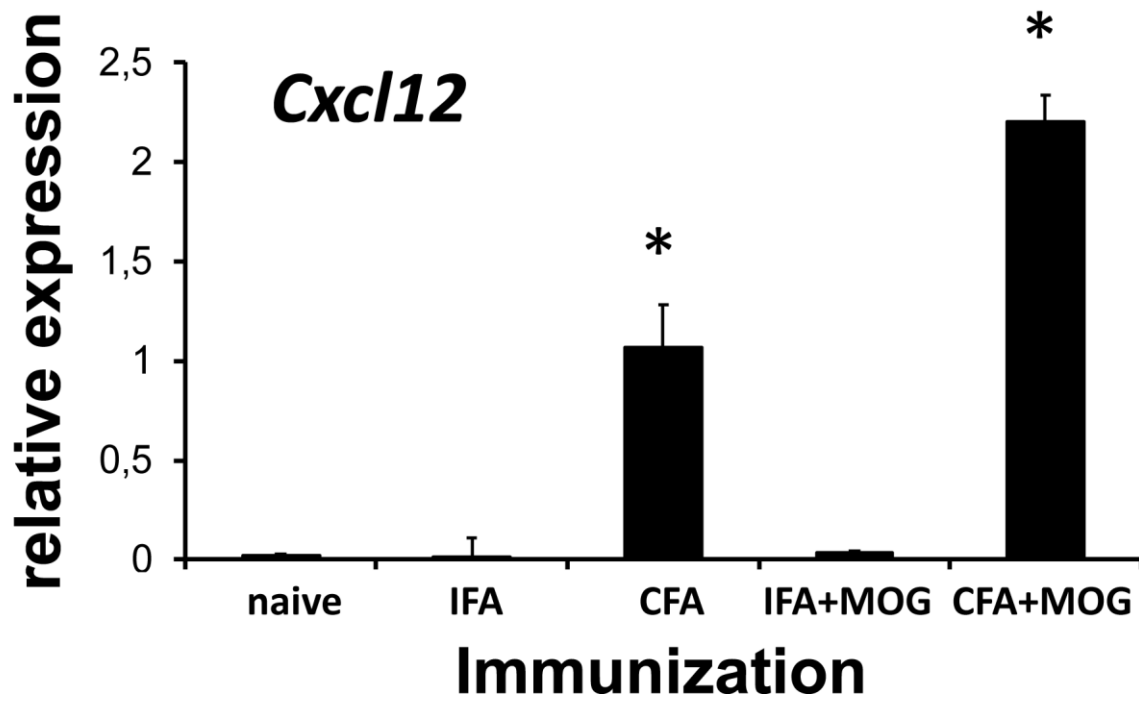
Fig 2. *Cxr4* and *Cxcl12* expression in spinal cord of DA (RT1^{av1}) rats.

Quantitative SYBR green real time PCR was performed for *Cxcr4* (**A**) and *Cxcl12* (**B**) from PBS- perfused spinal cord tissue of naïve DA (RT1^{av1}) rats (n=4), rats immunized with IFA (n=4) or CFA alone (n=4) and DA rats immunized with MOG 1-125 in IFA (n=4) or MOG 1-125 in CFA (n=4) on day 12 p.i. Increased *Cxcr4* and *Cxcl12* expression was observed in spinal cord in DA (RT1^{av1}) rats immunized with CFA and MOG1-125 in CFA (*ANOVA, *P*<0.001). There was an upregulation of *Cxcr4* and *Cxcl12* mRNA in DA (RT1^{av1}) rats immunized with MOG 1-125 in CFA compared to DA (RT1^{av1}) rats immunized with CFA alone (*ANOVA, *P*<0.01). Quantitative PCR results are expressed as $2^{-\Delta\Delta\ CT}$ values. Numbers are mean +/- SEM.
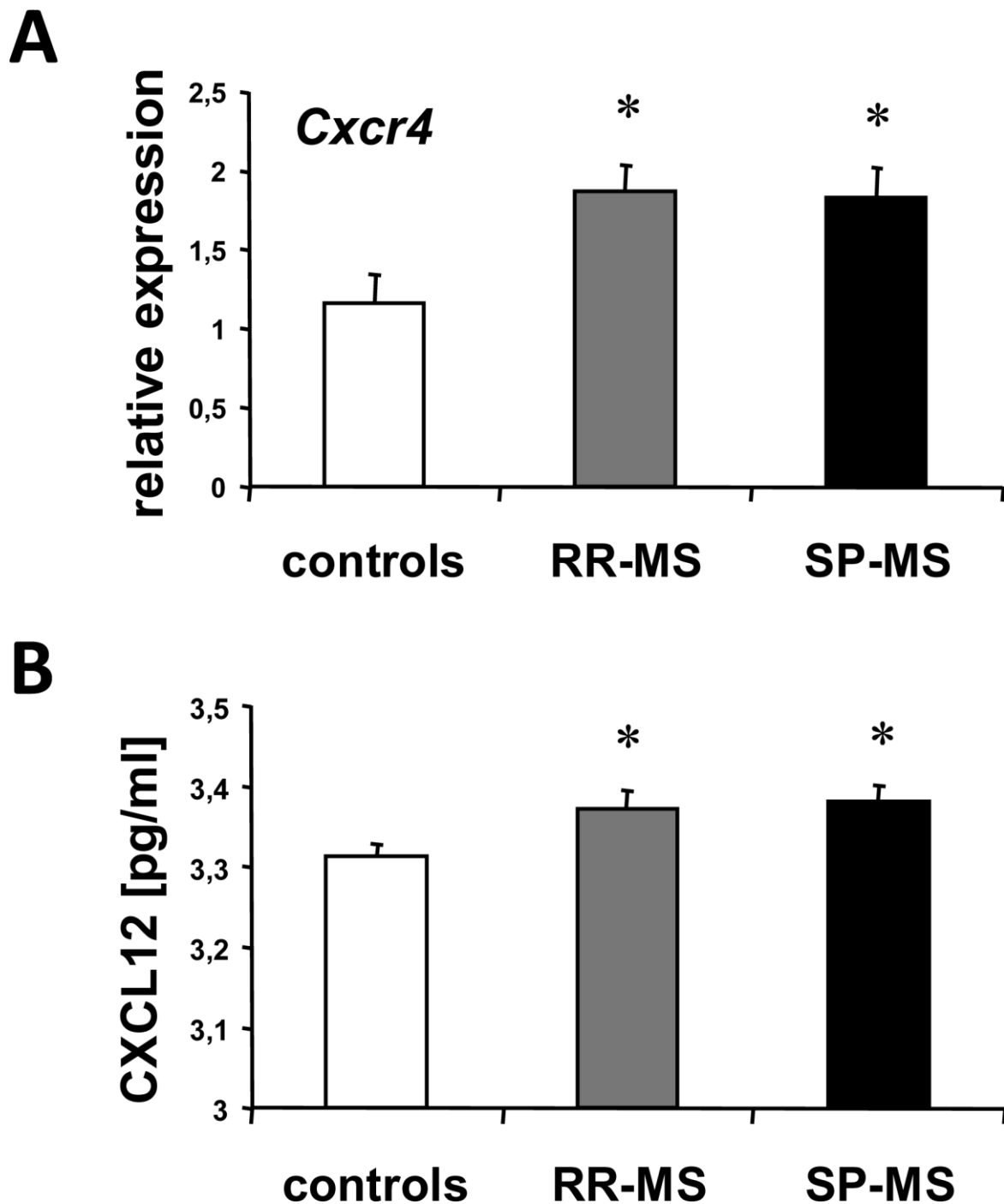
Fig. 3. *Cxcr4* **expression in white blood cells and CXCL12 protein in serum of patients with MS.**

A, *Cxcr4* mRNA was quantified by real time PCR from white blood cells of patients with relapsing remitting MS (grey bars, n=32), secondary chronic- progressive MS (black bars, n=22) and controls (white bars, n=25). Upregulation of *Cxcr4* in both patient groups compared to controls was observed (*ANOVA, *P*<0.05). **B**, CXCL12 serum levels were

assessed by ELISA in patients with relapsing-remitting MS (grey bars, n=24), secondary chronic progressive MS (black bars, n=28) and controls (white bars, n=21). Increased serum levels of CXCL12 were measured in both MS groups compared to controls (*ANOVA, P<0.05). Quantitative PCR results are expressed as $2^{-\Delta\Delta\ CT}$ values. Numbers are mean +/- SEM.
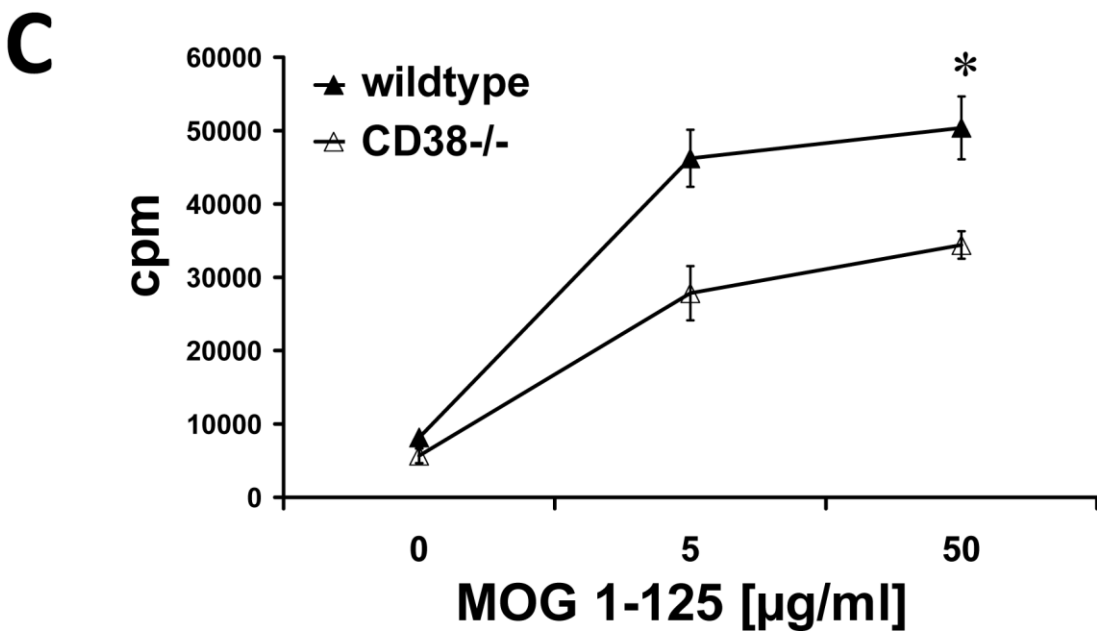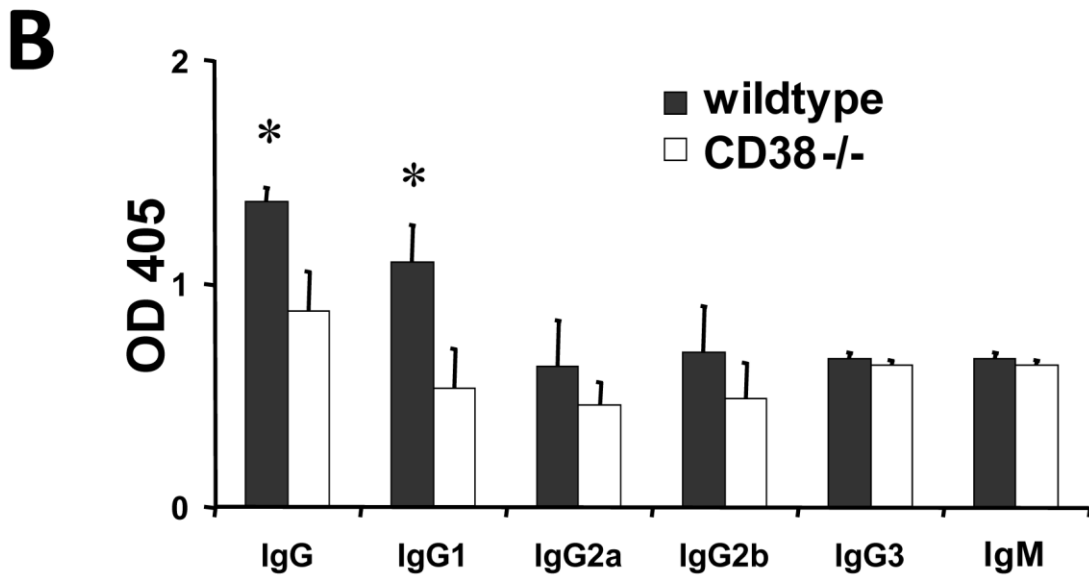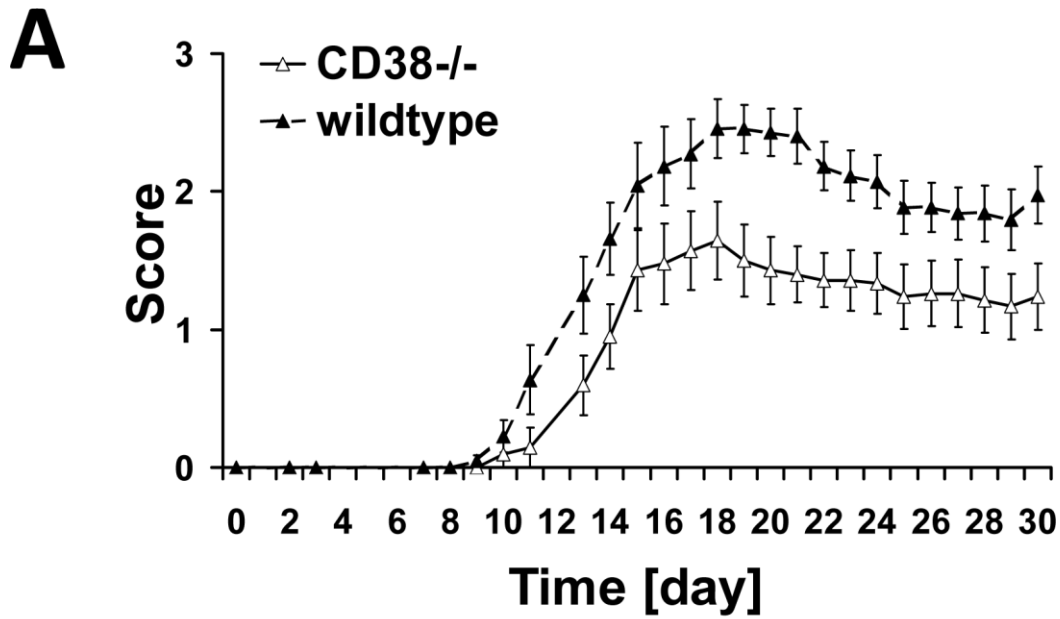
Fig. 4. EAE in B6.129P2-Cd38^{tm1Lnd}/J mice.

A, EAE was induced in female B6.129P2-Cd38^{tm1Lnd}/J mice (open triangles) and C57BL/6J 000664 controls (closed triangles) with MOG 1-125 in CFA. On day 0 and 2 p.i. mice received an i.v. injection of 150 ng pertussis toxin. EAE was scored as follows: 0, no disease; 1 tail paralysis; 2, paraparesis; 3, paraplegia; 4, tetraparalysis; 5, moribund or dead. Immunization with the extracellular domain of MOG, MOG 1-125 lead to disease reduction in B6.129P2-Cd38^{tm1Lnd}/J (n=22) compared to C57BL/6J 000664 controls (n=21) mice (t-test, cumulative disease score, *P*=0.01). **B**, Antibodies against MOG 1-125 were measured by ELISA as described (Weissert et al., 2001). B6.129P2-Cd38^{tm1Lnd}/J (white bars, n=4) mice had reduced IgG and IgG1 antibodies compared to C57BL/6J 000664 mice (black bars, n=4) (*ANOVA, *P*<0.05) on day 12 p.i. **c**, T cell responses upon restimulation against MOG 1-125 from draining lymph nodes on day 12 p.i. were reduced in B6.129P2-Cd38^{tm1Lnd}/J (open triangles, n=4) compared to C57BL/6J 000664 (closed triangles, n=4) mice (*t-test for stimulation with 50µg/ml MOG 1-125, *P*=0.05). Numbers are mean +/- SEM.
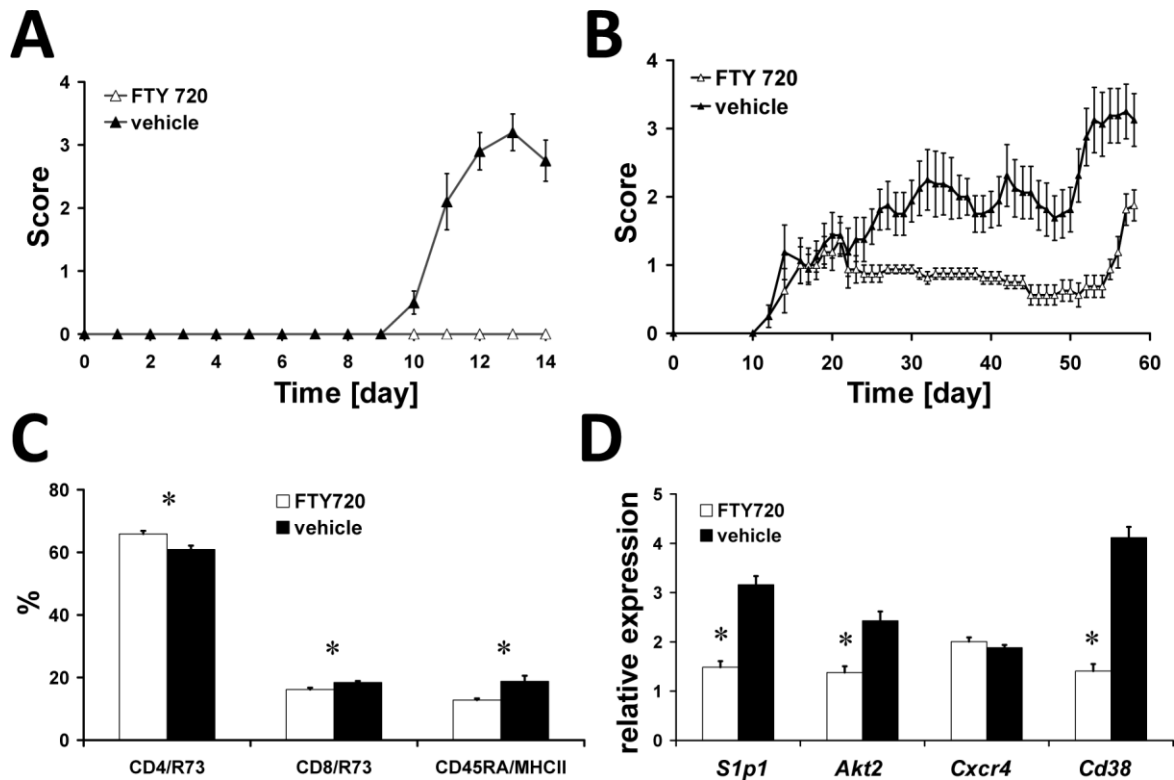
Fig. 5. Influence of FTY720 in EAE.

A, Female LEW.1AV1 (RT1[av1]) rats were immunized with MOG 1-125 in CFA. EAE was scored as follows: 0, no disease; 1 tail paralysis; 2, paraparesis; 3, paraplegia; 4, tetraparalysis; 5, moribund or dead. FTY720 completely inhibited EAE in rats treated orally with 0.4 mg/kg from day 0 p.i. (open triangles, n=10) as compared to vehicle (PBS)- treated controls (closed triangles, n=10) (t-test, cumulative disease score *P*<0.0001). **B**, Female DA (RT1[av1]) rats treated orally with 0.4 mg/kg FTY720 starting on day 21 p.i. after a first bout of disease (open triangles, n=8) showed a disease course compared to vehicle (PBS)- treated controls (closed triangles, n=8) (t-test, cumulative disease score day 21-44 p.i., *P*=0.01). On day 44 p.i. rats were boosted with MOG 1-125 in CFA. Both groups relapsed but with a better outcome of the FTY720 treated group (t-test, cumulative disease score day 45-56 p.i., *P*<0.0001). **C**, FACS analysis of cells from draining lymph nodes demonstrated an increase in relative size of the CD4 T cell compartment and a decrease in the size of the CD8 and B cell compartment in FTY720 treated (white bars, n=10) rats compared to controls (black bars,

n=9) (*ANOVA, *P*<0.01) on day 14 p.i.. **D**, Expression of *S1p1* (*Edg1*), *Akt2*, *Cxcr4* and *Cd38* was assessed in FTY720 (open bars, n=9) compared to vehicle- treated controls (black bars, n=9) by quantitative PCR. FTY720 treatment lead to downregulation of *S1p1* and *Akt2* as well as *Cd38* but not *Cxcr4* (*ANOVA for all except *Cxcr4* P< 0.0001) on day 14 p.i. from cells derived from lymph nodes. Results are expressed as 2-ΔΔ CT values. Numbers are mean +/- SEM.

Gene Symbol	Gene Title	Affymetrix Probe Set ID	MOG 91-108 vs naive percentage	MOG 91-108 vs naive average SLR	MOG 91-108 vs CFA percentage	MOG 91-108 vs CFA average SLR	MOG 91-108 vs MOG73-90 percentage	MOG 91-108 vs MOG73-90 average SLR	Function (putative)
Hmgcs2	3-hydroxy-3-methylglutaryl-CoA synthase 2	M33648_g_at	100	3.27	100	1.39	100	2.15	metabolism
Ptpn16	protein tyrosine phosphatase non-r. type 16	U02553cds_s_at	100	3.22	80	1.87	100	3.00	intracellular signaling
Cxcr4	Chemokine receptor (LCR1)	rc_AA945737_at	100	1.94	80	1.48	100	1.67	chemokine receptor
Ccl3	chemokine (C-C motif) ligand 3	U22414_at	90	2.68	70	1.86	60	0.72	chemokine
Jun	v-jun sarcoma virus 17 oncogene hom.	rc_AI175959_at	80	2.19	100	2.95	60	1.12	intracellular signaling
Cd38	CD38 antigen	rc_AA819187_s_at	60	1.75	100	2.62	60	0.98	ectoenzyme cADPR
Max	Max	D14447_at	50	2.79	60	2.96	100	2.21	cell cycle
Cxcl2	chemokine (C-X-C motif) ligand 2	U45965_at	100	6.63	80	2.21			chemokine
Il1b	interleukin 1 beta	E01884cds_s_at	100	4.64	60	1.32			chemokine
Cebpb	CCAAT/enhancer binding protein (C/EBP), beta	S77528cds_s_at	100	4.55	80	2.68			cell cycle
Ass	arginosuccinate synthetase	X12459_at	100	4.55	60	1.77			metabolism
Arg1	arginase 1	J02720_at	100	4.36	50	1.43			metabolism
Fcgr3	Fc receptor, IgG, low affinity III	M32062_g_at	100	4.26	80	1.86			complement receptor
Ptgs2	prostaglandin-endoperoxide synthase 2	S67722_s_at	100	3.95	60	1.96			inflammation
Spp1	secreted phosphoprotein 1	M14656_at	100	3.49	50	1.13			inflammation
Olr1	oxidised low density lipoprotein receptor 1	rc_AI071531_s_at	100	3.37	80	2.04			metabolism
Nos2	nitric oxide synthase 2, inducible	U03699complete_seq_at	100	3.19	80	2.71			inflammation
Anpep	alanyl (membrane) aminopeptidase	M25073_at	100	3.04	50	1.80			metabolism
Vegf	vascular endothelial growth factor	rc_AA850734_at	100	2.81	50	1.28			growth factor
Igsf6	immunoglobulin superfamily, member 6	AJ223184_at	100	2.73	50	0.85			unknown
F3	coagulation factor 3	U07619_at	100	2.32	70	1.54			coagulation
Edn1	endothelin 1	M64711_at	90	1.85	60	1.06			homeostasis
Wap	whey acidic protein	J00801_at	60	1.27	90	1.65			milk protein
Junb	Jun-B oncogene	X54686cds_at	60	1.24	50	0.94			transcription
Anp32a	acidic nuclear phosphoprotein 32 family A	D32209_at	50	0.84	50	1.11			unknown
LOC2868	tropomyosin isoform 6	rc_AA866465_s_at	50	2.02	50		100	3.24	cell structure
Cdkn1b	cyclin-dependent kinase inhibitor 1B	D83792_at					100	3.20	cell cycle
Hmgb1	high mobility group box 1	rc_AA944177_at					80	1.12	transcription
Smstr28	somatostatin receptor 28	X63574_at					60	1.04	metabolism
Klf9	Kruppel-like factor 9	D12769_at					60	1.01	transcription
Cited2	Cbp/p300-interacting transactivator 2	rc_AA900476_at			90	1.84			transcription
Akt2	murine thymoma (v-akt) oncogene hom. 2	rc_AI105076_s_at			80	1.64			intracellular signaling
Hba1	hemoglobin, alpha 1	X56325mRNA_s_at			60	1.27			metabolism
Zfp36	zinc finger protein 36	X63369cds_at			60	0.93			cell cycle
Sv2b	synaptic vesicle glycoprotein 2 b	L10362_at			50	1.39			metabolism
Mmp12	matrix metalloproteinase 12	X98517_at			50	1.16			proteases
Hmox1	heme oxygenase 1	J02722cds_at			50	1.13			unknown
Klf4	Kruppel-like factor 4 (gut)	L26292_g_at			50	1.05			transcription
Il1a	interleukin 1 alpha	D00403_g_at			50	0.99			chemokine
Slc2a1	solute carrier family 2,member 1	M13979_at			50	0.87			metabolism
Dcn	decorin	Z12298cds_s_at			50	0.85			extracellular matrix
Gadd45a	growth arrest and DNA-dam.-induc.45a	rc_AI070295_g_at			50	0.75			cell cycle

Table 1. Genes with increased expression in lymph node cells of LEW.1AV1 (RT1[av1]) rats

LEW.1AV1 (RT1[av1]) rats were immunized with MOG 91-108 in CFA, MOG 73-90 in CFA and CFA alone. Gene expression in lymph node cells was analysed using Affymetrix gene arrays. Gene arrays from rats immunized with MOG 91-108 in CFA (pooled samples of at least n=3 rats, n=5 gene chips) were compared to gene arrays from naive rats (pooled samples, n=2 chips), CFA immunized rats (pooled samples, n=2 chips) and MOG 73-90 in CFA immunized rats (pooled samples, n=1 chip) resulting in each ten comparisons for CFA and naïve to MOG 91-108-immunized rats and five for MOG 73-90 to MOG 91-108-immunized rats. The number of comparisons in which a given gene had a SLR above 1 were counted. If a gene had a SLR above 1 in 50% of the comparisons it was included in the analysis. ESTs and sequences not corresponding to a gene and duplicates were removed from

the table. Genes which fulfilled the criteria in all of the three comparisons are displayed on the top of the table. Genes which fulfilled the criteria only in naive versus MOG 91-108 in CFA comparisons are not displayed here. Lymph node cells were prepared as described (Weissert et al., 2001).