

CCR2⁺Ly-6C^{hi} monocytes are crucial for the effector phase of autoimmunity in the central nervous system

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The chemokine receptor CCR2 plays a vital role for the induction of autoimmunity in the central nervous system. However, it remains unclear how the pathogenic response is mediated by CCR2-bearing cells. By combining bone marrow chimerism with gene targeting we detected a mild disease-modulating role of CCR2 during experimental autoimmune encephalomyelitis, a model for central nervous system autoimmunity, on radio-resistant cells that was independent from targeted CCR2 expression on endothelia. Interestingly, absence of CCR2 on lymphocytes did not influence autoimmune demyelination. In contrast, engagement of CCR2 on accessory cells was required for experimental autoimmune encephalomyelitis induction. CCR2⁺Ly-6C^{hi} monocytes were rapidly recruited to the inflamed central nervous system and were crucial for the effector phase of disease. Selective depletion of this specific monocyte subpopulation through engagement of CCR2 strongly reduced central nervous system autoimmunity. Collectively, these data indicate a disease-promoting role of CCR2⁺Ly-6C^{hi} monocytes during autoimmune inflammation of the central nervous system.

Keywords: autoimmune encephalitis; chemokines; monocytes; multiple sclerosis

Introduction

Multiple sclerosis is the most common non-traumatic disabling neurological disease of adults in the northern hemisphere (McDonald, 2000). Inflammatory demyelination and axonal degeneration of the central nervous system (CNS) are the cardinal

neuropathological features of multiple sclerosis and inflammation plays a critical role in the pathogenesis (Steinman, 1996). This condition is represented in the well-established animal model for brain inflammation and multiple sclerosis, known as experimental autoimmune encephalomyelitis (EAE), which is a vital tool to study the neuroimmunological events related to the

disease (Owens *et al.*, 2001) and resembles many facets of multiple sclerosis (Gold *et al.*, 2006).

During multiple sclerosis, inflammatory immune cells are repeatedly recruited from the periphery, reinforcing the local inflammatory reaction within the CNS. In the context of inflammation, the recruitment of activated leukocytes into the target tissue is controlled by a heterogeneous family of chemotactic cytokines, the chemokines (Izikson *et al.*, 2002; Charo and Ransohoff, 2006).

Since the initial description in the late 1990s of the immunological phenotype of mice lacking the chemokine receptor CCR2 (Boring *et al.*, 1997; Kuziel *et al.*, 1997), there have been numerous publications examining the effects of CCR2 deficiency in a variety of murine models of infectious and autoimmune diseases. In fact, a number of studies indicated that CCR2 and its ligand CCL2 may play a role in the pathogenesis of multiple sclerosis and experimental autoimmune EAE (Mahad and Ransohoff, 2003). In EAE, it has been shown that CCR2 expression increased during the priming phase and peak of disease (Glabinski *et al.*, 2002) and that expression of CCL2 correlated with disease severity in active EAE (Glabinski *et al.*, 2002). In accordance, CCR2-deficient mice are resistant to experimental autoimmune EAE or develop a milder disease (Fife *et al.*, 2000; Izikson *et al.*, 2002; Gaupp *et al.*, 2003). Mice lacking CCL2 show a delayed onset of experimental autoimmune EAE with reduced clinical signs and are also protected against passive transfer of disease (Huang *et al.*, 2001). Interestingly, administration of antibodies against CCL2 had no effect on disease onset, but on the course of established experimental autoimmune EAE (Kennedy *et al.*, 1998). Considering all these data, the CCR2/CCL2 axis could potentially represent an excellent target for the treatment of multiple sclerosis. However, despite growing evidence for the therapeutic potential of CCR2 blockade in autoimmune disease models, so far clinical trials with CCR2 antagonists in humans have been only moderately encouraging or even disappointing, indicating a need to further elucidate the complex system of CCR2/CCL2 interactions (Hamann *et al.*, 2008). Our incomplete understanding of CCR2 function during CNS autoimmunity partly results from the widespread expression of this molecule. In fact, CCR2 has been detected in numerous haematopoietic cells such as monocytes, dendritic cells, basophils and a subset of T cells as well as many non-haematopoietic cells including brain cells such as neurons, astrocytes, microglia and brain endothelia (Mahad and Ransohoff, 2003). Notably, CCR2 can be used to distinguish two distinct monocyte populations in mice that are thought to have different features: CCR2⁺Ly-6C^{hi}CD62L⁺CX₃CR1^{lo} 'inflammatory' monocytes and CCR2⁻Ly-6C^{lo}CD62L⁻CX₃CR1^{hi} 'resident' monocytes (Geissmann *et al.*, 2003).

To establish the relative contribution of individual cell populations carrying CCR2 to the initiation and progression of autoimmune inflammation in the CNS, we combined bone marrow (BM) chimerism with gene targeting. We found that CCR2 expression on myeloid cells is modulating disease by mobilization and recruitment of pathogenic CCR2⁺CD11b⁺Ly-6C^{hi} monocytes to the inflamed CNS. Thus, our study reveals essential and non-redundant *in vivo* functions of this specific monocyte subset during autoimmune disease, suggesting that specific cell

depletion via chemokine receptors may result in therapeutic benefit.

Material and Methods

Mice

CX₃CR1^{GFP/+} mice were a kind gift of Dan Littman, New York (Jung *et al.*, 2000) and CCR2^{-/-} animals were provided by William A. Kuziel, Texas (Kuziel *et al.*, 1997).

For the generation of Tie-2 CCR2 we cloned cDNA of murine CCR2 (kind gift of I.F. Charo, Gladstone Institute) under the control of the endothelial Tie2 promoter (kind gift of U. Deutsch, Theodor Kocher Institute, Bern). To do so we excised the LacZ gene from the Tie2LacZ vector HHNS (Schlaeger *et al.*, 1997) with Sbf and Mlu I and inserted the full length mCCR2 cDNA fragment. Three independent transgenic lines were obtained and analysed. Data represented in this manuscript are from founder line 9. Experiments with the other two lines revealed similar results.

EAE induction, BM chimeras and adoptive transfer experiments

Female 6- to 10-week-old mice from each group were immunized subcutaneously with 200 µg of MOG_{35–55} peptide emulsified in CFA containing 1 mg of *Mycobacterium tuberculosis* (H37RA; Difco Laboratories, Detroit, MI, USA) as described previously (Prinz *et al.*, 2006, 2008). The mice received intraperitoneal injections with 250 ng pertussis toxin (Sigma–Aldrich, Deisenhofen, Germany) at the time of immunization and 48 h later. Mice were scored daily as follows: 0, no detectable signs of EAE; 0.5, distal limb tail; 1.0, complete limb tail; 1.5, limb tail and hind limb weakness; 2, unilateral partial hind limb paralysis; 2.5, bilateral partial hind limb paralysis; 3, complete bilateral hind limb paralysis; 3.5, complete hind limb paralysis and unilateral forelimb paralysis; 4, total paralysis of fore and hindlimbs; and 5, death.

For adoptive transfer experiments mice received 5×10^5 GFP⁺ BM cells from CX₃CR1^{GFP/+} mice *i.v.* and 24 h later CNS cells were isolated and prepared for FACS analysis.

For cell depletion experiments, antibodies were injected three times every third day for high antibody concentration (250 µg) or daily for low concentrations (20 µg). Specific depletion is described elsewhere (Bruhl *et al.*, 2007; Mildner *et al.*, 2007, 2008). Rat IgG2b served as control antibody. All animal experiments have been approved by the ethics review board for animal studies at the Universities of Göttingen and Freiburg.

Tissue and cell preparation and immunohistochemistry

Mice were sacrificed using CO₂. Histology was performed as described recently (Prinz *et al.*, 2003; van Loo *et al.*, 2006). Spinal cords were removed and fixed in 4% buffered formalin. Then, spinal cords were dissected and embedded in paraffin before staining with luxol fast blue (LFB) to assess the degree of demyelination, MAC-3 (BD Pharmingen) for macrophages/microglia, CD3 for T cells (Serotec, Düsseldorf, Germany), B220 for B cells (BD Pharmingen) and amyloid precursor protein (APP, Chemicon, Temecula, CA, USA).

Immunofluorescence was performed on 20 μm cryosections from the diseased CNS. Primary antibodies were added overnight at a dilution of 1:50 for Iba-1 (WACO), 1:500 for iNOS (assay designs), 1:100 for CD3 (eBioscience) and 1:500 for MHC class II (BD Bioscience). Cy3-conjugated secondary antibodies (Dianova) were added at a dilution of 1:100 for Iba-1 and 1:600 for the other antibodies for 1h. Primary endothelial cells were isolated from heart tissue of 3- to 4-week-old tg mice. For this, the organ was removed and digested with collagenase and dispase I (Sigma–Aldrich, Germany). Fc receptors were blocked with CD16/32 antibodies (1 $\mu\text{g}/\text{ml}$, eBioscience). Biotinylated antibodies against CD105 and CD31 (1 $\mu\text{g}/\text{ml}$, eBioscience) were added and afterwards labelled for MACS separation by α -biotin microbeads (Miltenyi Biotec). After 4 days in culture, cells were analysed for cell purity by fluorescence immunohistochemistry. Therefore antibodies against CD105, 1:200 (eBioscience) and CD31, 1:200 (eBioscience) were added overnight. Nuclei were counterstained with 4,6-diamidino-2-phenylindole (DAPI).

RNA isolation and real-time PCR analysis

RNA was extracted from isolated spinal cords or from endothelial cells after 4 days in culture by using RNeasy Mini Kits (QIAGEN) following the manufacturer's instructions. The tissue was flushed with ice cold HBSS and RNA was isolated using RNeasy Mini Kits (Qiagen, Hilden, Germany) following the manufacturer's instructions. The samples were treated with DNaseI (Roche, Mannheim, Germany) and 1 μg of RNA was transcribed into cDNA using oligo-dT primers and the SuperScript II RT kit (Invitrogen, Carlsbad, CA, USA). cDNA (2.5 μl) was transferred into a 96-well Multiply[®] PCR-plate (Sarstedt, Germany) and 12.5 μl Absolute[™] QPCR[®] SYBR Green Master Mix (ABgene, Surrey, UK) plus 9.6 μl ddH₂O was added. The PCR reaction was performed as described recently (Prinz *et al.*, 2006).

Flow cytometry

Blood and CNS cell samples were prepared at 4°C in buffer solution (PBS containing 2% FCS and 0.2% NaN₃) and stained with CD11b, Gr-1, B220, CD45 (all eBioscience), Ly-6C, MHC class II, CD11c, Ly-6G (all BD Pharmingen) or CCR2 (Mack *et al.* 2001). After lysis of erythrocytes with FACS lysis solution for blood samples (Becton Dickinson, San Jose, California), cell suspensions were analysed on a FACS Calibur (Becton Dickinson). For FACS analysis of CNS infiltrated mononuclear cells mice were transcardial perfused with PBS and spinal cord as well as brainstem were removed and homogenized. Cells were separated by Percoll gradient as described before (van Loo *et al.*, 2006). Intracellular FACS staining was performed with GolgiPlug staining kit (BD Pharmingen) according to the manufacturer's instructions. Anti-TNF α antibody (MP6-XT22) or rat IgG1 isotype control was purchased from eBioscience. Data were acquired with WinMDI.

Statistical analysis

Statistical differences in clinical scores were evaluated using a non-paired Student's *t*-test. Differences were considered significant when *P*-value < 0.05.

Results

CCR2 engagement in haematopoietic and non-haematopoietic compartments determines the course of EAE

It has previously been shown that the presence of CCR2 establishes the susceptibility and outcome of autoimmune inflammation of the CNS, but the underlying mechanisms remain unclear (Fife *et al.*, 2000; Izikson *et al.*, 2002). Notably, CCR2 is broadly expressed on large proportions of T and B cells, monocytes, basophils, as well as endothelia and even CNS cells, such as neurons and astrocytes (Horuk, 2001).

In order to determine whether CCR2 engagement on haematopoietic or stromal cells, such as CNS resident cells or endothelia, contributes to the development and progression of EAE, BM chimeric mice were generated, in which CCR2 expression was either limited to the radio-resistant or the radio-sensitive compartment of the host. To do so, we combined BM chimerism with gene targeting as described earlier (Prinz *et al.*, 2006; Mildner *et al.*, 2007). Two months after reconstitution, BM chimeras were immunized with MOG_{35–55} in CFA and the course of disease was assessed over time (Fig. 1A, Supplementary Table 1). The lack of CCR2 expression in the radio-sensitive haematopoietic compartment (CCR2^{-/-} → CCR2^{+/+}) caused a significant delay in disease onset and reduced clinical severity compared with chimeric mice that expressed CCR2 in all compartments (CCR2^{+/+} → CCR2^{+/+}). Interestingly, the lack of CCR2 from the radio-resistant compartment, e.g. endothelia and brain, also had a significant impact on the effector phase of disease (CCR2^{+/+} → CCR2^{-/-}). BM chimeras lacking CCR2 in both the radioresistant and non-radioresistant compartment (CCR2^{-/-} → CCR2^{-/-}, filled squares) have slightly less disease compared to those chimeras that lack CCR2 in the periphery only (CCR2^{-/-} → CCR2^{+/+}) without reaching statistical significance.

In addition, detailed immunohistological analysis of immunized chimeras revealed a distinct lesion pattern (Fig. 1B). The number of infiltrating Mac-3⁺ macrophages and CD3⁺ lymphocytes was fairly high in general as known from irradiated chimeras (Prinz *et al.*, 2006) but strongly decreased when CCR2 was absent from the haematopoietic compartment. This decrease of mononuclear infiltrates correlated with less myelin loss, thus indicating that the presence of CCR2 also regulates demyelination during brain disease. Notably, there was no significant change of histopathology when CCR2 was specifically absent from the radio-resistant compartment alone (CCR2^{+/+} → CCR2^{-/-}).

Endothelial CCR2 expression does not support CNS autoimmunity

CCR2 expression by brain microvascular endothelial cells has been shown to be critical for macrophage transendothelial migration in response to CCL2 (Dzenko *et al.*, 2001, 2005). Also the transportation of CCL2 across an *in vitro* blood–brain barrier model was recently shown to be dependent on CCR2 expressing

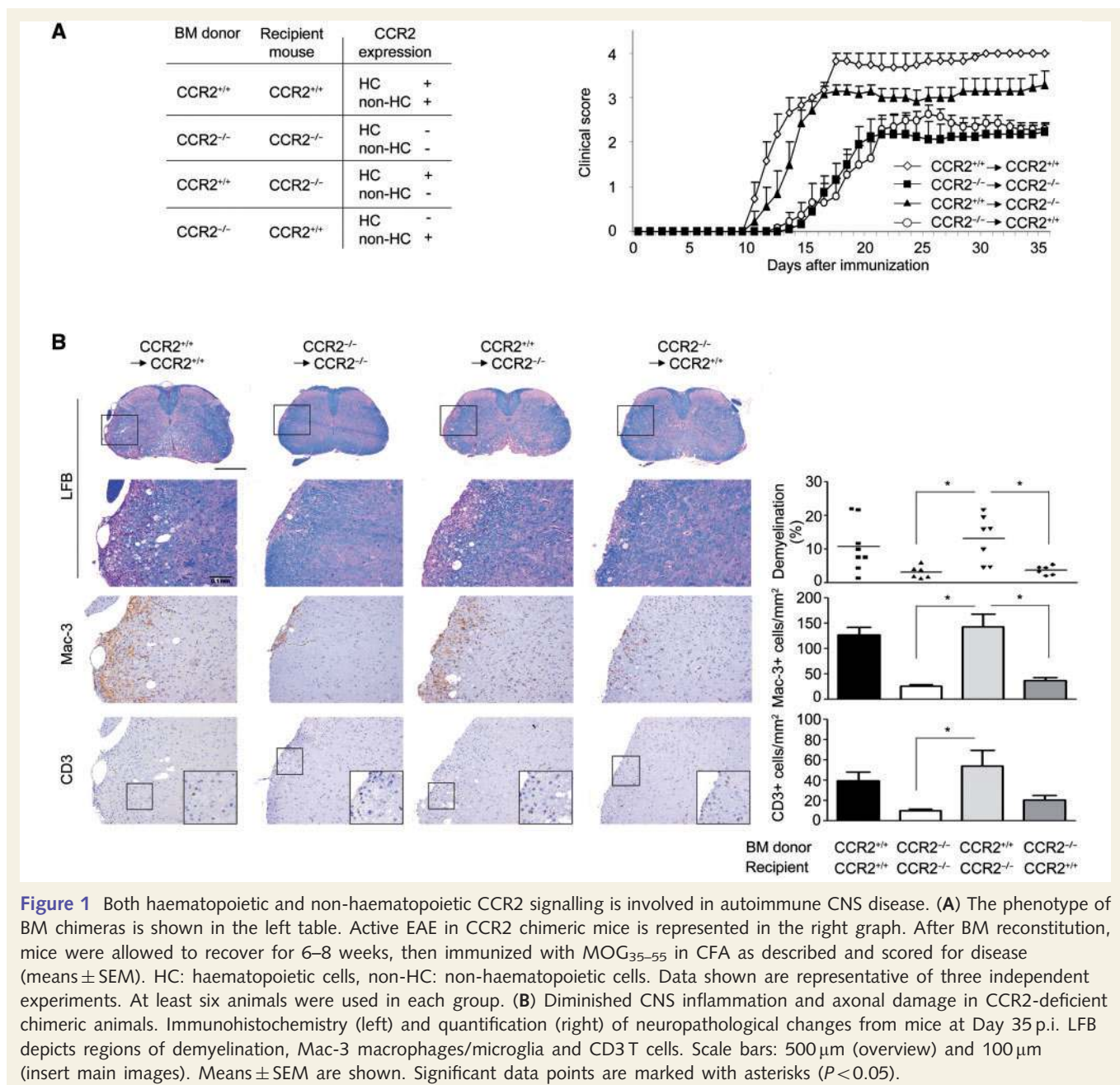


Figure 1 Both haematopoietic and non-haematopoietic CCR2 signalling is involved in autoimmune CNS disease. (A) The phenotype of BM chimeras is shown in the left table. Active EAE in CCR2 chimeric mice is represented in the right graph. After BM reconstitution, mice were allowed to recover for 6–8 weeks, then immunized with MOG_{35–55} in CFA as described and scored for disease (means ± SEM). HC: haematopoietic cells, non-HC: non-haematopoietic cells. Data shown are representative of three independent experiments. At least six animals were used in each group. (B) Diminished CNS inflammation and axonal damage in CCR2-deficient chimeric animals. Immunohistochemistry (left) and quantification (right) of neuropathological changes from mice at Day 35 p.i. LFB depicts regions of demyelination, Mac-3 macrophages/microglia and CD3 T cells. Scale bars: 500 μm (overview) and 100 μm (insert main images). Means ± SEM are shown. Significant data points are marked with asterisks ($P < 0.05$).

endothelial cells (Ge *et al.*, 2008). This transcytosis could have been an important function for the attraction of leukocytes to inflamed brain regions. In addition, our data in BM chimeric mice pointed to a disease-modulating role of the non-haematopoietic compartment (Fig. 1A, CCR2^{+/+} → CCR2^{-/-}). Therefore, we hypothesized that targeted expression of CCR2 on vessels might modulate experimental autoimmune EAE and could restore disease susceptibility in CCR2^{-/-} animals.

To drive the expression of CCR2 in vessels, we generated a DNA construct in which the entire coding region of the murine CCR2 gene was placed under the transcriptional control of a murine Tie2 promoter fragment. The construct, consisting of the 2.1-kb promoter region of the mouse Tie2 gene and one Tie2 intronic enhancer that flanks the 1.2-kb CCR2 open reading

frame region (Fig. 2A), was excised from its prokaryotic backbone and injected into the pronuclei of C57Bl/6 zygotes.

Transgenic founders were identified by transgene-specific PCR analysis (Fig. 2B). All subsequent analyses were performed with heterozygous offsprings of one line (designated tg9) that showed sustained transcription of the transgene and was compatible with two other founder lines obtained (tg14) and tg10-2 (not shown). Transgene expression was subsequently assessed in primary endothelial cell cultures isolated from 3–4-week-old transgenic animals by MACS (Fig. 2C). Cell cultures contained almost exclusively endothelia as indicated by high expression of the specific marker endoglin (CD105) and PECAM-1 (CD31). Total RNA was extracted from cells and after retrotranscription, CCR2 mRNA levels were determined by quantitative real time-PCR.

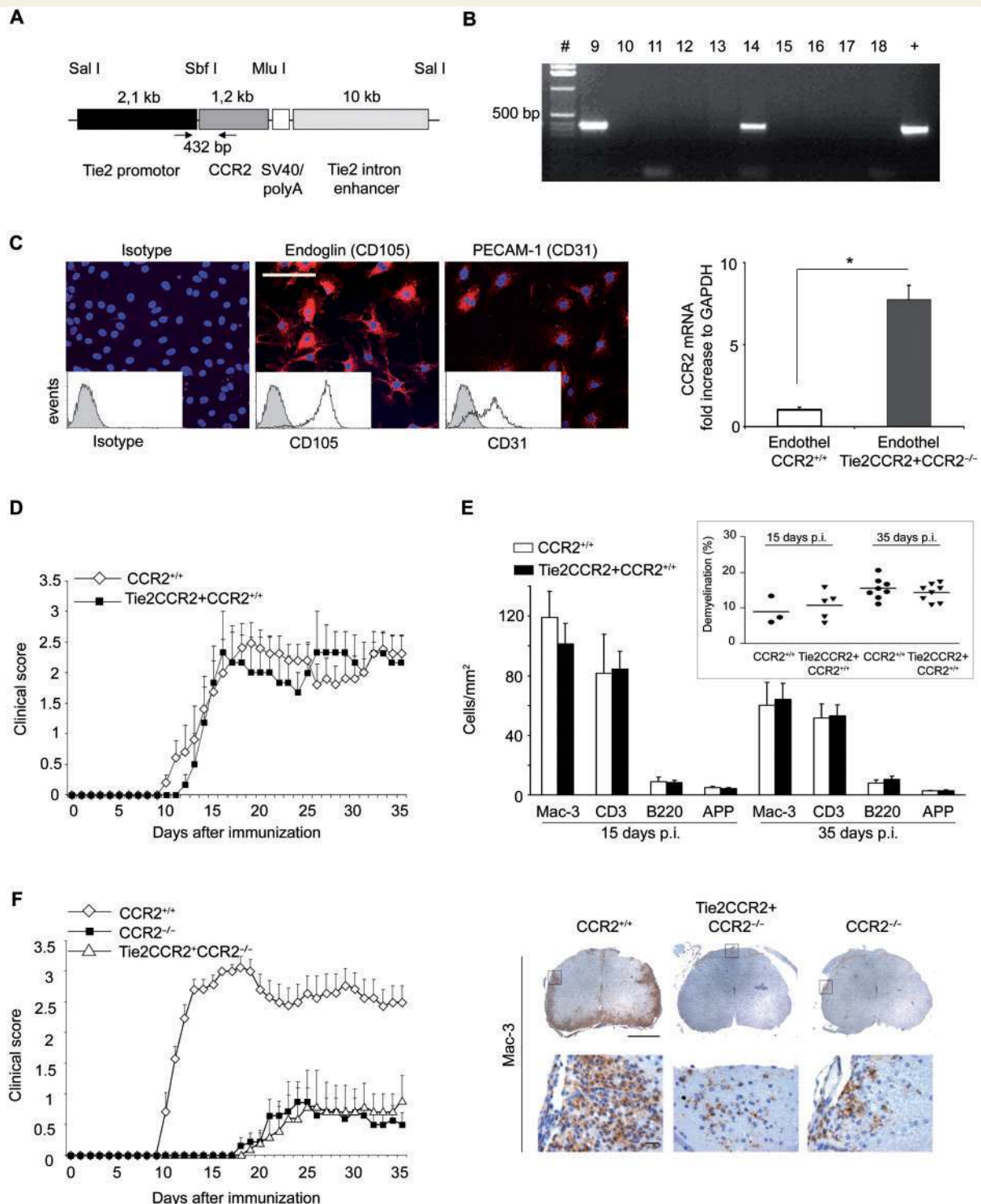


Figure 2 Endothelial targeted CCR2 does not support EAE. (A) Schematic drawing of the Tie2CCR2 construct used for the generation of transgenic mice. Arrows and parenthetical numbers indicate the respective restriction sites. (B) Transgene-specific PCR is shown in several tg lines. (C) Immunofluorescence of primary endothelia from adult Tie2CCR2 mice (left). Isolated cells express specific endothelial markers endoglin (CD 105, red), PECAM (CD31, red). Nuclei are shown by DAPI staining (blue). Scale bar represents 200 μ m. Inserts: FACS analysis on the respective marker. Quantitative real-time PCR analysis (right) of CCR2 expression in endothelia from WT (CCR2^{+/+}) or tg animals on CCR2^{-/-} background. (D) Endothelial CCR2 does not modulate course of MOG-EAE. Disease was induced by active immunization of WT (diamonds) and Tie2CCR2 tg animals on WT background (squares). At least six animals were used in each experimental group. (E) Similar CNS invasion of mononuclear cells (Mac-3, CD3, B220) in WT (white bars) and Tie2CCR2 tg animals on WT background (black bars) at peak of disease (15 d.p.i.) and in the chronic phase (35 d.p.i.). Same amount of axonal damage (amyloid precursor protein, APP) and demyelination (LFB, insert). (F) Endothelia-restricted CCR2 expression does not render CCR2-deficient mice more susceptible to disease. Course of EAE in WT (CCR2^{+/+}, open diamonds), CCR2^{-/-} (closed squares) and Tie2CCR2 mice (open triangle) on CCR2-deficient background (left). The group size was $n \geq 6$. Spinal cord histology reveals diminished inflammation (Mac-3) in CCR2^{-/-} and Tie2CCR2 animals 35 d.p.i. (right). Scale bars: 500 μ m and 20 μ m (insert).

The CCR2 mRNA content in *tg* endothelia was 7.7-fold increased compared to WT cells.

To test whether overexpression of CCR2 in the vessels might modulate EAE, 6- to 8-week-old *tg* animals were immunized (Fig. 2D). No differences were observed between wild-type (WT) mice and Tie2CCR2 *tg* mice regarding their clinical parameters. As expected, concomitant CNS pathology was similar in terms of the distribution as well as the amount of infiltrating macrophages, T and B lymphocytes, axonal damage and demyelination at 35 d.p.i. (Fig. 2E). Even during early disease time points, the entry of mononuclear cells was independent of the expression of CCR2 in endothelia. To check whether endothelia-restricted CCR2 suffices to render CCR2^{-/-} mice more susceptible to EAE, *tg* mice were crossed to the CCR2^{-/-} background and challenged with MOG_{35–55} peptide in CFA (Fig. 2F). However, immunization of *tg* mice did not result in more severe clinical parameters or earlier disease onset compared to the CCR2^{-/-} mice. Spinal cord sections revealed a comparable amount and distribution of macrophages and microglia in diseased Tie2CCR2 animals.

Taken together, these results clearly show that CCR2 signalling on endothelia has no impact on the initiation and progression of CNS inflammation.

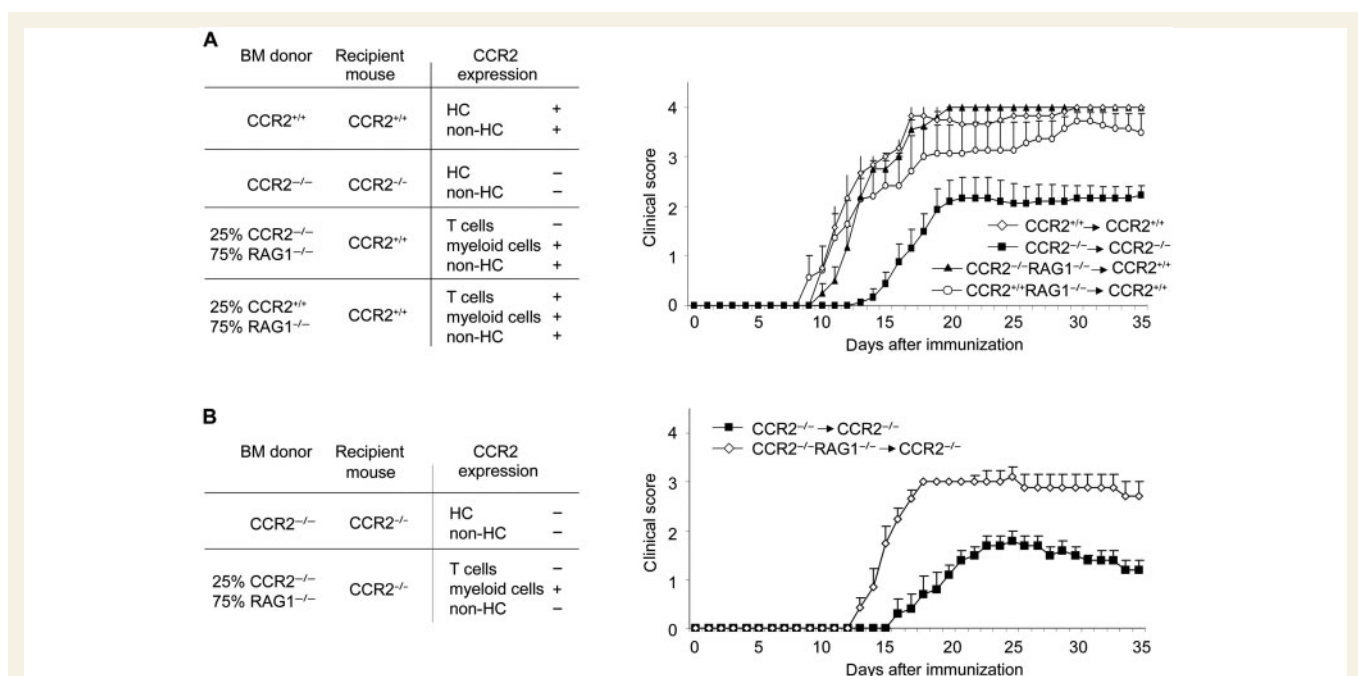
CCR2 expression in myeloid cells is essential for EAE

The results thus far have shown that CCR2-deficiency in haematopoietic cells diminishes development of experimental

autoimmune EAE, whereas CCR2 engagement on vessels seems to be irrelevant for disease. The cell type on which CCR2 exerts its main effects remains unknown. This is mainly due to the fact that CCR2 is expressed in various haematopoietic cell types (Mack *et al.*, 2001). To unequivocally identify the CCR2-expressing haematopoietic cell type involved in EAE induction, we selectively expressed CCR2 on different leukocyte subsets. We generated BM chimeras by transferring either a 4:1 ratio of recombination-activating gene 1-deficient (RAG1^{-/-}) and CCR2-deficient or competent BM into CCR2⁺ recipients (Fig. 3A). Because RAG1^{-/-} mice have defects in lymphocyte development, all lymphocytes in the RAG1^{-/-} + CCR2^{-/-} chimera were CCR2-deficient, whereas most other non-lymphocytes such as monocytes, dendritic cells, basophils, etc. expressed CCR2. Importantly, lymphoid reconstitution levels were comparable in RAG1^{-/-} CCR2^{-/-} and RAG1^{-/-} CCR2^{+/+} chimeras (Supplementary Fig. 1A).

As anticipated from the data above, CCR2-deficient chimeric mice were less susceptible to disease (Fig. 3A). This clearly indicates that the development of encephalitogenic T cells is not affected by the absence of CCR2 expression and that CCR2-deficient lymphocytes show normal *in vivo* activation and proliferation capacity in the wt background. Accordingly, quantification of histopathological changes within the CNS, including infiltration by macrophages and T cells as well as myelin damage, was independent of the expression of CCR2 on lymphocytes (Supplementary Fig. 1B).

Because CCR2 was still present on the radioresistant non-haematopoietic compartment in these mixed chimeras, we



wanted to rule out the possibility that CCR2 expression on host CNS cells might modulate disease as shown in Fig. 1A. Therefore, we transferred a mixture of CCR2^{-/-}RAG1^{-/-} BM cells into CCR2^{-/-} recipients (Fig. 3B). In these mice, only myeloid cells expressed CCR2. In comparison to the EAE-resistant CCR2^{-/-} control chimeras, CCR2^{-/-} RAG1^{-/-} → CCR2^{-/-} chimeras showed a significantly earlier disease onset (18.0 (±0.7) versus 14.0 (±0.5), *P* < 0.01) and a more severe clinical manifestation (mean maximal score 1.8 (±0.2) versus 3.3 (±0.2), *P* < 0.01). Hence, the expression of CCR2 on myeloid cells is essential for the development of experimental autoimmune EAE.

Decreased expansion of Ly-6C^{hi} monocytes in the absence of CCR2 during EAE

Previous reports suggested that CCR2 is required for the egress of CD11b⁺Ly-6C^{hi} monocytes from the BM, resulting in a significant reduction of this specific monocyte subset in the peripheral blood of CCR2^{-/-} mice (Serbina and Pamer, 2006; Tsou *et al.*, 2007). This could potentially cause disease resistance in CCR2^{-/-} animals. To test the hypothesis of reduced expansion of CD11b⁺Ly-6C^{hi} monocytes during EAE, CCR2-deficient mice were immunized and the amount of Ly-6C^{hi} cells in the peripheral blood was determined over time. As expected, CCR2^{-/-} animals developed EAE with strongly delayed disease onset and reduced clinical severity (Fig. 4A). Importantly, the amount of CD11b⁺Ly-6C^{hi} monocytes was substantially reduced in the absence of CCR2 compared to the wild-type situation (Fig. 4B). To ensure that only this leukocyte subtype was affected, we examined the amount of CD4⁺ T lymphocytes in the peripheral blood, which did not differ between CCR2^{-/-} and wild-type mice during disease (Fig. 4B).

We next determined the frequency and kinetics of Ly-6C^{hi} cells in the CNS of CCR2^{-/-} and WT mice at different time points of disease (Fig. 4C, upper panel). As shown before (Mildner *et al.*, 2007), we found similar amounts of Ly-6C^{hi} monocytes as well as Ly-6C^{low} (Ly-6G⁺, data not shown) granulocytes in the CNS of healthy, perfused CCR2^{-/-} mice compared to WT controls, indicating the presence of a meninges-specific myeloid cell population. The amount of CD45^{hi}CD11b⁺Ly-6C^{hi} cells in the inflamed CNS peaked in wild-type mice 10 d.p.i. with a slight decrease in the following days. Further analysis of CNS Ly-6C^{hi} cells revealed that a proportion of these cells express MHC class II (~65%) and additionally CD11c (25%). Intracellular FACS analysis of CD45^{hi}Ly-6G⁻Ly-6C^{hi} cells indicates that these cells produce moderate levels of TNF- α .

In contrast to wild-type mice, CCR2^{-/-} mice revealed significantly reduced numbers of CD45^{hi}CD11b⁺Ly-6C^{hi} cells in the CNS that heightened with delay 15 d.p.i. and returned to normal at 20 d.p.i. The appearance of substantial numbers of CD45^{hi}CD4⁺ T lymphocytes was strongly delayed in CCR2^{-/-} mice compared to the wild-type situation (Fig. 4C, lower panel), indicating that Ly-6C^{hi} monocytes might support the recruitment of lymphocytes during EAE. In summary, our data indicate that the absence of

CCR2 leads to reduced numbers of circulating and expanding CD45^{hi}CD11b⁺Ly-6C^{hi} cells during EAE.

CX₃CR1^{GFP/+} BM cells are recruited to the diseased CNS and participate in local immune regulation

To assess the ability of BM derived myeloid cells to be recruited to the inflamed CNS during EAE, we next performed adoptive transfer experiments. CX₃CR1^{GFP/+} mice were used that allow the distinction of monocyte fractions on the basis of GFP expression (Geissmann *et al.*, 2003). Respective GFP BM cells were adoptively transferred into sick animals and CNS infiltrating cells were examined 24 h later by flow cytometry (Fig. 5A). Importantly, GFP⁺ cells were clearly visible in the diseased, but not in the healthy brain (Fig. 5B). These donor-derived recruited cells were positive for CD11b, GFP and Gr-1, indicating the inflammatory nature of these myeloid cells. We were not able to detect 'resident' monocytes in the inflamed CNS characterized by high GFP expression and absence of the Gr-1 epitope.

Despite higher numbers of recruited GFP⁺ myeloid cells during EAE, it remained unclear whether these monocytes were functional and took part in the process of inflammation and local immune response. In order to test this hypothesis, we examined the induction of immune molecules by donor cells in the host CNS. iNOS expression in engrafted cells as a marker for their inflammatory capacity was clearly visible in CX₃CR1^{GFP/+} transferred cells (Fig. 5C). We furthermore observed an upregulation of MHC class II molecules on a subset of transferred cells as signs of activation. MHC class II expression was only detectable on parenchymal GFP⁺ cells and not on round-shaped cells in the meninges, which is in line with the FACS analysis represented in Fig. 4C. As expected, recruited donor BM cells were devoid of the lymphoid marker CD3. The transferred cells were exclusively found at the sites of inflammation.

These data imply that BM derived inflammatory GFP⁺CD11b⁺Ly-6C^{hi} monocytes are attracted to the site of autoimmune inflammation in the CNS, where they contribute to the local inflammatory response by upregulation of specific immune molecules.

CCR2⁺CD11b⁺Ly-6C^{hi} monocytes are pathogenic during the effector phase of EAE

To exclude the possibility that CCR2-knockout mice have effects from the genetic manipulation that are independent of CCR2 deficiency and do not only affect monocyte subsets, we chose alternative strategies. We took advantage of the α -CCR2 antibody MC-21, which selectively depletes CCR2⁺Ly-6C^{hi}, but not CCR2⁻Ly-6C^{lo} monocytes (Supplementary Fig. 2). In order to unravel the mechanisms by which the α -CCR2 treatment depletes specific monocyte subsets, co-injection experiments using α -CD16/32 antibodies were performed (Fig. 6A). 2 h before α -CCR2 treatment, mice received a single injection

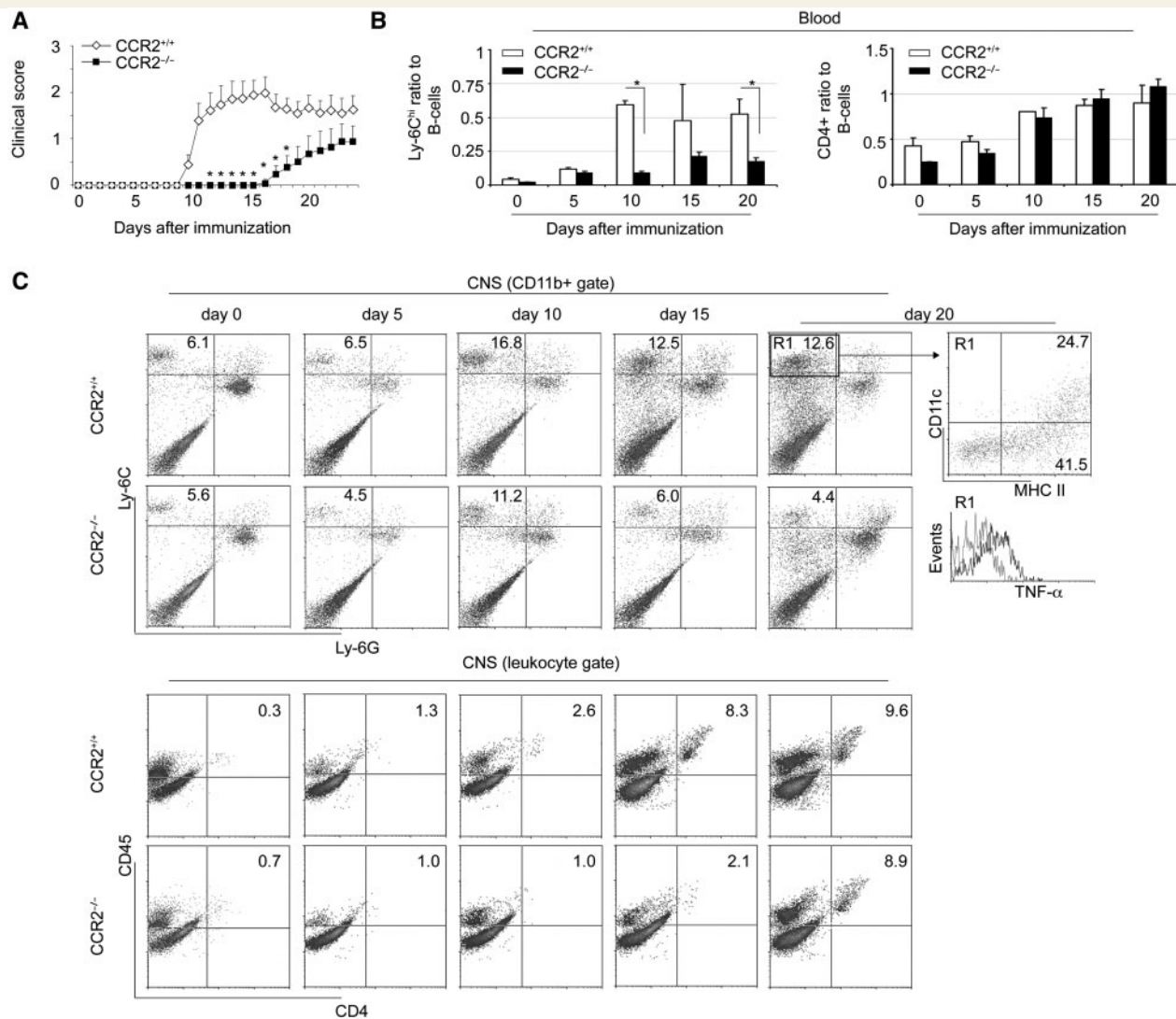


Figure 4 Decrease of CD11b⁺Ly-6C^{hi} monocytes in blood and CNS of CCR2-deficient animals during EAE. (A) CCR2^{-/-} animals (closed squares) display attenuated autoimmune CNS disease compared to WT mice (open diamonds). Data shown are representative of three independent experiments. Statistically significant data points are marked with asterisks ($P < 0.05$). At least eight mice were used in each experimental group. (B) Reduced numbers of blood CD11b⁺Ly-6G⁻Ly-6C^{hi} monocytes in CCR2^{-/-} mice (black bars) compared to the WT situation (white bars) during different time points of disease (left). Increase of circulating CD4⁺ T cell during EAE was not dependent on the presence of CCR2 (right). FACS analysis was normalized to 20 000 B cells. Data are means \pm SEM of at least three mice per group. Asterisks indicate statistically significant differences ($P < 0.05$). (C) Characterization of CNS-infiltrating myeloid cells (above, gated on CD11b⁺ cells only) and lymphocytes (below) in the inflamed CNS during course of disease. The grey line in the TNF- α histogram shows the isotype control staining, whereas the black line represents the TNF- α specific antibody. Three to four mice were used for each FACS time point.

of α -CD16/32 antibody against the Fc γ R II/III. Depletion of Gr-1^{int}CD11b⁺ myeloid cells was blocked by this procedure. Thus, the ablation of CD11b⁺Ly-6C^{hi} monocytes by α -CCR2 treatment is mediated by an antibody-dependent cellular cytotoxicity-mediated mechanism (ADCC), most likely via CCR2-mediated engagement on monocytes and concomitant activation of CD16/32 positive effector cells.

MOG-immunized mice were challenged with α -CCR2 or isotype control antibodies either at high (250 μ g every third day) or low (20 μ g daily) dosages during the preclinical priming or the effector phase of disease (Fig. 6B, videos 1 and 2). When circulating

CCR2⁺Ly-6C^{hi} monocytes were depleted at some point in the priming phase prior to the onset of clinical symptoms, we did not observe any changes in disease development. α -CCR2 and isotype-treated animals developed EAE with a comparable incidence and had similar mean disease onset and severity (Supplementary Table 2). In sharp contrast, when CCR2⁺Ly-6C^{hi} monocytes were removed at peak of disease, mice developed EAE with significantly milder clinical manifestations compared to isotype-treated mice. Even daily injection of low dose α -CCR2 was highly effective, indicating that CCR2⁺ myeloid cells are critical for the effector phase of EAE during a distinct time window.

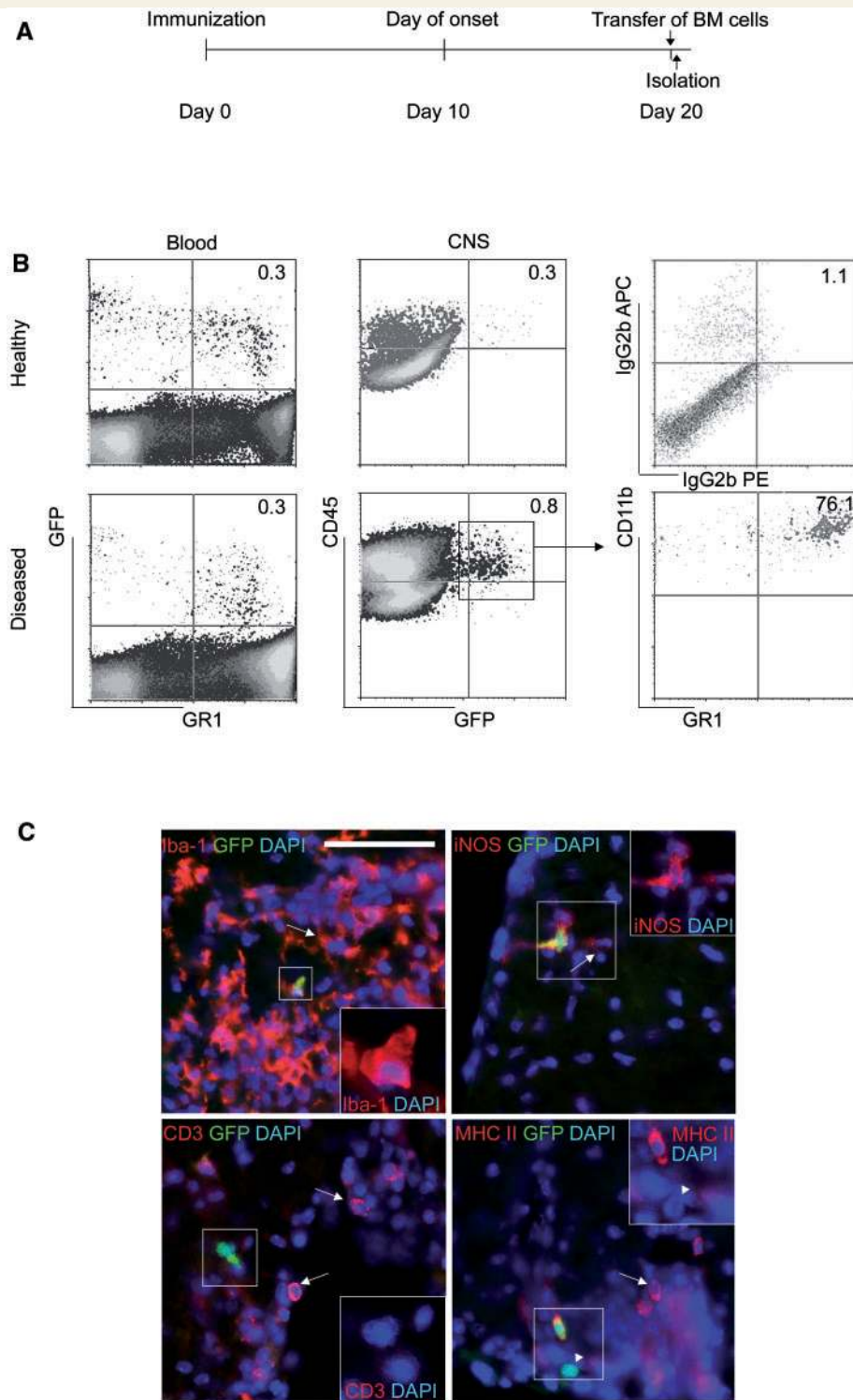


Figure 5 Adoptively transferred $CX_3CR1^{GFP/+}$ BM derived myeloid cells migrate to the inflamed CNS and become an integral part of the local immune response. **(A)** Time scale of adoptive transfer of $CX_3CR1^{GFP/+}$ BM cells during EAE. $CX_3CR1^{GFP/+}$ cells were injected and CNS cells were recovered 24 h later. **(B)** Transferred cells were detectable in the CNS of diseased but not healthy control mice. GFP^+ cells express the surface markers CD45, CD11b and Gr-1. Rat IgG2b isotype controls are depicted for CD11b and Gr-1. **(C)** Recruited GFP^+ cells express the macrophage marker Iba-1 (red) and the immune-related molecules iNOS and MHC class II (red) but no CD3 (red) during CNS inflammation. Arrows point to single positive cells which are not GFP-positive. Arrow head shows a round, non-activated GFP^+ mononuclear cell within the meninges which does not express MHC class II. Nuclei were counterstained with 4,6-diamidino-2-phenylindole (DAPI, blue). Scale bar 20 μm .

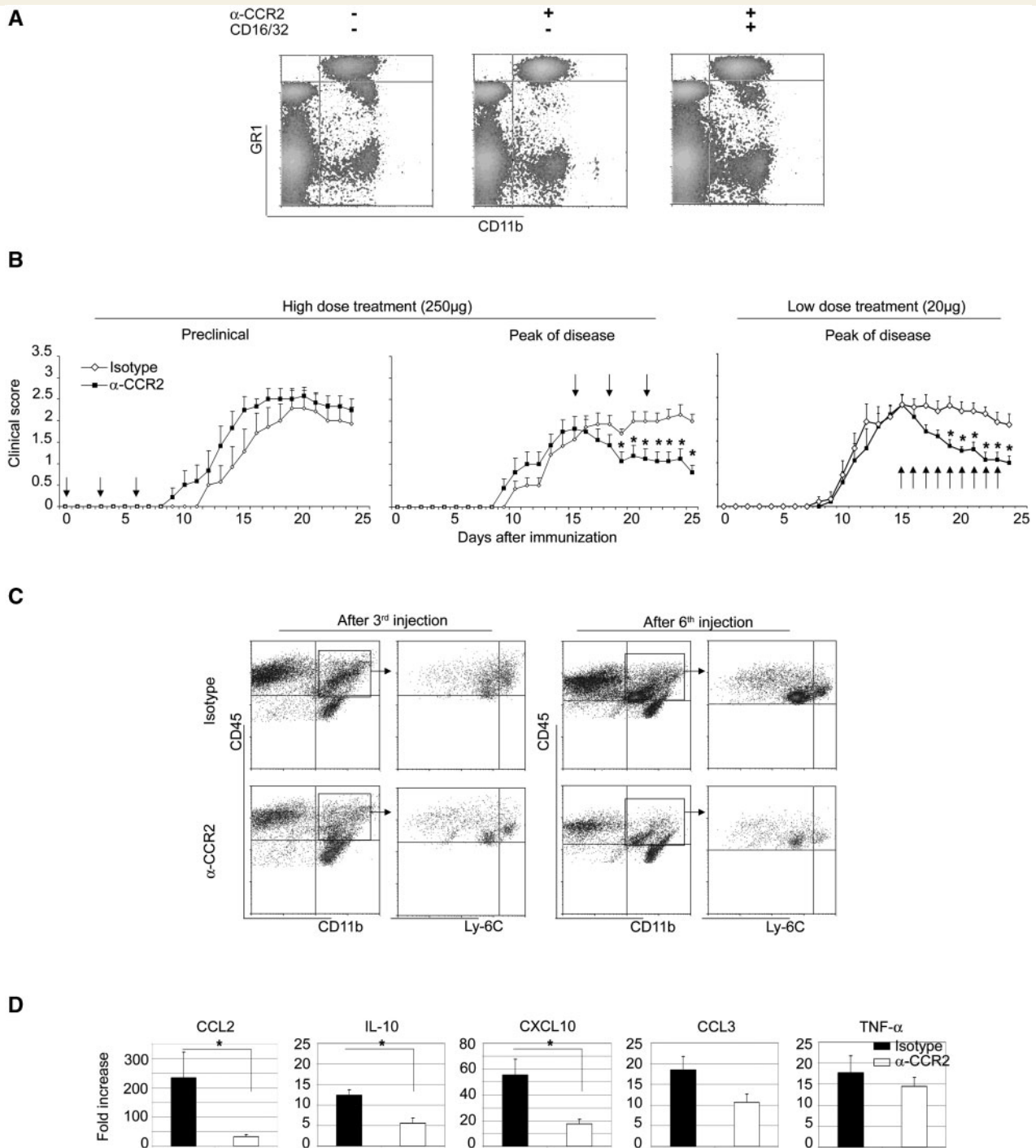


Figure 6 CD11b⁺Ly-6C^{hi} monocytes cells are vital for the effector phase of autoimmune EAE. **(A)** FACS analysis of antibody (α -CCR2) treated animals revealed the absence of Gr-1^{int}CD11b⁺ inflammatory monocytes after usage of the α -CCR2 antibody that was prevented in the presence of α -CD16/32 co-application. One representative animal out of five is shown. **(B)** Depletion of CD11b⁺Ly-6C^{hi} monocytes by high dose (left) or low dose (right) antibody treatment (closed squares) but not isotype control (open diamonds) at preclinical or peak clinical stages. Eight mice per group were used. * P <0.05. Arrows indicate injections days. **(C)** Spinal cord FACS analysis of α -CCR2 treated mice showed reduced cell infiltration after three (left) and even more pronounced after six injections (right) in CNS during disease. **(D)** Quantification of cytokine and chemokine mRNA induction in CD11b⁺Ly-6C^{hi} depleted (α -CCR2, white bars) CNS and isotype-treated (black bars) animals 25 d.p.i. Three mice per group were used. Data indicate means \pm SEM.

Injection of low dosages of α -CCR2 or isotype control antibody did not result in increased serum cytokine levels or depletion of regulatory T cells (data not shown). The depletion efficiency during the α -CCR2 treatment period in the peripheral blood was about 70–90% (Supplementary Fig. 3).

In order to determine both the extent and the composition of CNS-infiltrating cells and subsequent pathological changes in clinically improved animals, spinal cords were examined at different time points after the first antibody injection (Fig. 6C and Supplementary Fig. 4). We found that already after three injections the amount of myeloid cell infiltrates was reduced up to ~70% in the α -CCR2 treated group. However, a small proportion of Ly-6C^{hi} monocytes was still present within the CNS, indicating that the depletion occurs mainly in the peripheral immune system and not directly in the target tissue. After three further injections the overall cell infiltration was strongly decreased, which was much more pronounced in a long-term treatment experiment (Supplementary Fig. 4). Thus, improved clinical disease course was associated with less neuropathological changes in the absence of CCR2⁺Ly-6C^{hi} monocytes.

We next used quantitative real-time PCR analysis to determine the expression of proinflammatory mediators in the CNS of mice lacking CCR2⁺Ly-6C^{hi} cells (Fig. 6D). Among the factors tested, IL-10, CCL 2 and CXCL 10 were significantly reduced in α -CCR2-treated animals, whereas TNF- α and CCL 3 only showed a trend towards lower expression levels.

Taken together, our data indicate that CD11b⁺Ly-6C^{hi} monocytes expressing CCR2 are essential for autoimmune EAE in modulating the effector, but not the priming phase of disease by shaping the local innate immune response in the damaged CNS through immune molecules, e.g. cytokines, oxygen radicals like nitric oxide and surface molecules required for antigen presentation. These data clearly indicate that despite the widespread expression of CCR2 on several stromal and haematopoietic cell populations, engagement of CCR2 on the specific myeloid subtype of CCR2⁺CD11b⁺Ly-6C^{hi} monocytes defines susceptibility to autoimmunity in the CNS.

Discussion

Although CCR2 has a well-established role in CNS autoimmunity, the precise mechanisms and the pathogenic CCR2-dependent cell type are largely unknown. This study shows that disease susceptibility is mainly dependent on a selective monocyte subset, namely CCR2⁺CD11b⁺Ly-6C^{hi} cells, which crucially contribute to local CNS pathology.

A number of CCR2-expressing cells have been considered as potential modulators of the immune-mediated demyelination during experimental autoimmune EAE. Despite its broad expression in blood cells, CCR2 has also been identified in non-haematopoietic cells (Schechter *et al.*, 2004). By using double fluorescent immunohistochemistry, CCR2 expression was demonstrated on neuroectodermal cells, such as neurons and astrocytes (Banisadr *et al.*, 2005). After challenge with lipopolysaccharide, CCR2 expression was upregulated on neurons, astrocytes and microglia in rodents (Banisadr *et al.*, 2002).

To address the possibility that brain-specific CCR2 might contribute to EAE pathogenesis, we created chimeric mice in which only the radio-resistant CNS cells were lacking this chemokine receptor, whereas the peripheral immune compartment was CCR2-competent. Interestingly, chimeras devoid of CCR2 in the brain had moderately reduced clinical disease compared to wild-type control animals, but there was no tendency towards more preserved myelin sheaths and axons in these animals. In addition, the amount and distribution of macrophages and lymphocytes was also not changed, indicating that the recruitment of haematopoietic cells was apparently not altered under these conditions. Under the assumption that CNS-resident cells were not significantly replaced by BM elements during this limited disease period as shown by us and others before (Hickey *et al.*, 1992; Prinz *et al.*, 2006; Mildner *et al.*, 2007), the question remains open which radio-resistant cell type, within the CNS, is responsible for the protective effect upon CCR2 deletion. One convincing possibility is that endothelial CCR2 plays a role in the process of leukocyte extravasation and transmigration. CCR2 is detectable on cultured brain microvascular endothelial cells and mediates binding, internalization and transcytosis of CCL2 (Andjelkovic *et al.*, 1999; Dzenko *et al.*, 2001) which has also been reported to cause diminished expression of the tight-junction-associated proteins occluding (Ge *et al.*, 2008). Furthermore, engagement of CCR2 on endothelial cells seems to be critical for macrophage transendothelial migration, even when macrophages expressed CCR2 (Dzenko *et al.*, 2005). The *in vivo* significance of these data, however, still has to be defined. To determine whether endothelial-specific CCR2 expression might modulate cell recruitment during EAE, we generated transgenic mice in which CCR2 expression is driven by the Tie2 promoter. Despite high expression of the transgene, we did detect a role for this CCR2-expressing compartment for EAE pathogenesis. Our data, therefore, do not support the hypothesis of leukocyte transmigration via CCR2 on endothelial cells.

What other CCR2-expressing CNS cells may be involved in the pathology of EAE? As mentioned above, astrocytes and microglia were suggested to express CCR2, at least under certain conditions (Banisadr *et al.*, 2002). A recent publication demonstrated that CCR2 expression regulates the clinical course of murine Alzheimer-like disease (El Khoury *et al.*, 2007). In this report, amyloid precursor protein transgenic, CCR2-deficient mice died soon after birth, which was accompanied by increased A β protein deposits. The authors concluded from this finding that early microglial recruitment to sites of A β accumulation depends on CCR2. In contrast to Alzheimer-like disease, a defective recruitment of CCR2-deficient microglia to sites of inflammation could be beneficial for the pathology of EAE. Former studies revealed a disease-supporting function of microglia in CNS autoimmune disease (Heppner *et al.* 2005). However, further studies with brain-specific CCR2 knockout mice are needed to clarify the precise role of CCR2 in the radio-resistant compartment.

CCR2 is also evident on a subpopulation of CD4⁺ T lymphocytes. Detailed cell surface analysis defined this T cell subset as regulatory T lymphocytes (T regs) and CCR2 expression was even more strongly induced in activated, CD44⁺ T regs (Bruhl *et al.*, 2004). Nevertheless, it seems unlikely that the milder EAE course

observed in CCR2^{-/-} mice is based on a recruitment defect of T regs since this would result in increased clinical EAE symptoms due to lost T reg suppression activity within the CNS (Matsumoto *et al.*, 2007). The main pathogenic effector T cell subset in EAE are IL-17-producing T cells (Th17 cells) (Langrish *et al.*, 2005). Previous studies have shown that human Th17 cells, as well as *in vitro* generated murine Th17 cells, express CCR2 (Sato *et al.*, 2007; Webb *et al.*, 2008). However, in experiments with mixed BMs chimeras, we could clearly demonstrate that CCR2 expression on lymphocytes is not required for EAE development. Therefore, a crucial role of CCR2 for Th17 cell recruitment is not very likely. In contrast, our results indicate that the loss of CCR2 expressing myeloid cells is responsible for the resistance to disease.

Monocytes and macrophages are highly heterogeneous populations of cells with distinct functional features. Monocytes are blood mononuclear cells with bean-shaped nuclei. They express CD11b, CD11c, CD14 and CD16 in humans and CD11b and F4/80 in mice, and they lack B, T and NK cell markers. Nowadays, monocytes are subdivided into at least two main subsets: a short lived 'inflammatory' subset (CCR2⁺Ly-6C^{hi}CD62L⁺CX₃CR1^{lo}) that homes to inflamed tissue, where it can trigger immune responses, and a 'resident' subset (CCR2⁻Ly-6C^{lo}CD62L⁻CX₃CR1^{hi}) with a longer half life, which homes to non-inflamed tissues (Geissmann *et al.*, 2003). Tissue macrophages like postnatal microglia in the brain (Mildner *et al.*, 2007) and Langerhans cells in the skin (Ginhoux *et al.*, 2006) were recently shown to arise from the CCR2⁺Ly-6C^{hi} monocyte population under certain conditions, indicating a broad role of this myeloid subpopulation for tissue homeostasis during health and disease.

By virtue of their mobility, expression of immune-related receptors, and their ability to produce antibacterial substances such as IL-1β TNF-α and others, CCR2⁺Ly-6C^{hi} monocytes present a plausible candidate for the cell type driving inflammation in the CNS (Serbina *et al.*, 2008). Further, CCR2⁺ cells play a key role in defense against infection by *Listeria monocytogenes*, a Gram-positive intracellular bacterium, via production of TNF-α and iNOS (Serbina *et al.*, 2003). In contrast, CCR2⁺Ly-6C^{hi} monocytes are efficiently recruited to the inflamed CNS during bacterial *Streptococcus pneumoniae* infection, but they are not essential for bacterial containment and killing during infection (Mildner *et al.*, 2008). We found that CCR2⁺Ly-6C^{hi} monocytes were likewise attracted to autoimmune CNS disease, suggestive of a pivotal role of this cell type also during sterile inflammation. In contrast, Ly-6C^{lo} monocytes were not involved in this process.

Egression of Ly-6C^{hi} monocytes from the BM is driven, at least in part, by CCR2-mediated signals (Serbina and Pamer, 2006; Tsou *et al.*, 2007). The number of circulating Ly-6C^{hi} monocytes in CCR2-deficient mice under homeostatic conditions is therefore markedly diminished. Indeed, in our experiments, expansion of Ly-6C^{hi} monocytes in peripheral blood was also strongly reduced during EAE. These findings raise the questions of whether the protection of CCR2^{-/-} animals to EAE, as described before (Fife *et al.*, 2000; Izikson *et al.*, 2002) can solely be attributed to the reduced number of CCR2⁺ monocytes in the blood, rather than to a failure of monocytes to move from the blood into inflamed tissues. Serbina and Pamer (2006) presented experimental

evidence for the former scenario and argued that, at least in the setting of bacterial infection, CCR2-deficient and competent monocytes are attracted equally well from blood to inflamed tissues. To circumvent these difficulties, we designed experiments to explore the role of monocytes at defined disease stages in CCR2^{+/+} animals. Depletion of CCR2⁺CD11b⁺Ly-6C^{hi} monocytes in animals with physiological levels of circulating leukocytes during the effector phase of disease resulted in a significant improvement of clinical disease course and went along with reduced neuropathological damage. Notably, decline of EAE severity was accompanied by reduced levels of all immune mediators examined, including CCL2, CCL3, TNFα and also the prototypical Th2 cytokine IL-10, suggestive of a generally dampened local immune response rather than an altered Th1/Th2/Th17 balance.

How might CD11b⁺Ly-6C^{hi} cells execute their damaging program? In addition to the production of interleukins, expression of proteolytic enzymes and the ability to phagocytose are instrumental for the pathogenic features of these cells. Indeed, previous studies revealed that BM-derived myeloid cells are involved in the process of myelin destruction and neuronal damage (Takahashi *et al.*, 2007). However, it will be interesting to investigate, in the future, whether CCR2⁺CD11b⁺Ly-6C^{hi} monocytes also mediate nervous tissue clearance and facilitate tissue repair and functional recovery during EAE.

Interestingly, there are several potential ligands of CCR2 that might promote autoimmune inflammation. It is generally known that chemokine receptors such as CCR2 are highly redundant and usually engaged by several ligands (Mahad and Ransohoff, 2003). Thus, despite the fact that CCL2 is considered nowadays to be the most relevant ligand for CCR2 during EAE pathogenesis, we cannot exclude that the alternative CCR2 ligands, CCL7 and CCL8, can also shape the immune response in the CNS.

Despite the strength of our conclusions, it is notable that our data, on the impact of CCR2⁺CD11b⁺Ly-6C^{hi} monocytes on autoimmune demyelination are not fully consistent with a report in the literature on this topic which has described rather differing outcomes. Zhu *et al.* (2007) reported that CD11b⁺Ly-6C^{hi} monocytes might have suppressive rather than pathogenic functions. These differences might be due to the fact that the authors focussed mainly on the *in vitro* suppressive function of monocytes on polyclonal CD4⁺ and CD8⁺ T cell proliferation, but did not assess their effect on the clinical course or their ability of trafficking to the diseased CNS *in vivo*.

In summary, our study revealed that CCR2⁺CD11b⁺Ly-6C^{hi} monocytes express MHC class II molecules, produce inflammatory cytokines and participate in the local immune response in EAE. We therefore conclude that this CCR2⁺ cell type is indispensable for disease pathogenesis. Since earlier studies targeting the CCR2/CCL2 axis by blocking ligand/receptor interaction met with limited success in both mice and humans (Mahad and Ransohoff, 2003), we propose that physical depletion of CCR2⁺Ly-6C^{hi} monocytes might be a more promising strategy for the treatment of inflammatory autoimmune disorders. Comparing these distinct strategies in terms of clinical and histopathological benefit might prove useful for the treatment of multiple sclerosis in the future.

Supplementary material

Supplementary material is available at *Brain* online.

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