RESEARCH ARTICLE

Differential Expression of the Chemokine Receptors CX_3CR1 and CCR1 by Microglia and Macrophages in Myelin-Oligodendrocyte-Glycoprotein-Induced Experimental Autoimmune Encephalomyelitis

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Chemokines are important for the recruitment of immune cells into sites of inflammation. To better understand their functional roles during inflammation we have here studied the in vivo expression of receptors for the chemokines CCL3/CCL5/CCL7 (MIP-1α/RANTES/MCP-3) and CX_3CL1 (fractalkine), CCR1 and CX_3CR1, respectively, in rat myelin oligodendrocyte glycoprotein–induced experimental autoimmune encephalomyelitis. Combined in situ hybridization and immunohistochemistry demonstrated intensely upregulated CCR1 mRNA expression in early, actively demyelinating plaques, whereas CX_3CR1 displayed a more generalized expression pattern. CX_3CR1 mRNA expressing cells were identified as microglia on the basis of their cellular morphology and positive GSA/B4 lectin staining. In contrast, CCR1 mRNA was preferentially expressed by $ED1^+GSA/B4^+$ macrophages. The notion of differential chemokine receptor expression in microglia and monocyte-derived macrophages was corroborated at the protein level by extraction and flow cytometric sorting of cells infiltrating the spinal cord using gating for the surface markers CD45, ED-2 and CD11b. These observations suggest a differential receptor expression between microglia and monocyte-derived macrophages and that mainly the latter cell type is responsible for active demyelination. This has great relevance for the possibility of therapeutic intervention in demyelinating diseases such as multiple sclerosis, for example by targeting signaling events leading to monocyte recruitment.

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Introduction

Chemokines are key controllers/mediators of immune cell infiltration into the central nervous system (CNS) in neuroinflammatory disorders. In particular this refers to multiple sclerosis (MS), which is a chronic neuroinflammatory disease of presumed autoimmune origin with debilitating consequences for affected individuals (31, 55). MS is in its common form characterized by a fluctuating relapsing-remitting disease course with infiltration of peripheral blood leukocytes and subsequent plaque formation in various locations of the CNS white matter. Whereas demyelination traditionally has been held as the cause of symptoms during relapses, recent evidence demonstrates that widespread damage to axons is present as well (9, 12, 32, 33). In fact, permanent neurological handicap, especially during chronic progressive stages of the disease, may best be correlated with the degree of nerve fiber damage (10, 59). Considerable efforts are now made to develop new MS treatments based on the interference with any of the steps leading to CNS immune cell accumulation, where chemokines are at the center of interest since they serve crucial roles in attracting immune cells to the target organ (18, 27, 29, 30, 60). Macrophages may be important both for demyelination and secondary axonal damage in MS (61). One of the key candidate signaling pathways for macrophage recruitment to the CNS are the chemokines CCL3/CCL5/CCL7 (MIP-1α/RANTES/MCP-3) exerting their activity via the common receptor CCR1. Prior studies have demonstrated that CCL3 and CCL5 are highly up regulated in plaques during mouse experimental autoimmune encephalomyelitis (EAE) (18) and that CCR1 receptor expressing cells are present in inflammatory lesions both in rodent EAE models and human MS (2, 3, 18, 29, 30, 41, 52, 54, 61). In vivo immunoneutralization of CCL3 in mouse EAE reduces the number of CNS infiltrating mononuclear cells and

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ameliorates clinical disease (28). In addition, Ransohoff and co-workers recently reported that CCR1 is abundantly expressed by infiltrating monocytes in perivascular cell cuffs and at the demyelinating edges of evolving lesions in MS plaques, arguing for a direct role of CCR1 mediated signaling in attracting monocytes/macrophages into the brain compartment (61).

Apart from blood-derived monocytes, microglia residing within the CNS compartment is another progenitor for CNS phagocytic cells. Microglia, sharing a common mesodermal origin with monocytes and peripheral macrophages, normally reside in the CNS in a resting state, but are rapidly activated by virtually all types of disturbances to the CNS homeostasis (45). In their final stage of activation they have become phagocytic cells with very high phenotypical resemblance to peripherally-derived macrophages. One exception is the level of CD45 expression, which can be used to distinguish between these 2 cell populations (22). It has been hypothesized that monocyte-derived macrophages are more actively involved in damaging consequences of MS than microglia-derived phagocytes (6, 24, 33, 63), as indicated, for example, by the preferential appearance of plaques near and around blood vessels. However, phagocytic microglia are also abundant in inflammatory lesions and may exert important effector functions, especially in early stages of MS disease (17, 36). The design of therapeutic strategies aimed at attenuating CNS accumulation of phagocytic cells, therefore, require close examination of both the functional roles of microglia and blood-derived monocytes/macrophages and the mechanisms that guide their accumulation within brain lesions.

In order to examine these issues, we have characterized the expression of 2 chemokine receptors, CCR1 and CX_3CR1, in rat myelin oligodendrocyte glycoprotein (MOG)–induced EAE, a model that shares several important features with MS (33, 38, 56). The rationale to study CCR1 has been described above. CX_3CR1 is one of the most recently discovered members of the chemokine receptor family (23). Together with its corresponding ligand, CX_3CL1 (fractalkine), it forms a chemokine ligand-receptor pair with unique properties, which is very distinct from previously characterized macrophage or monocyte-related chemokines. The functional role of fractalkine in the brain is still enigmatic, but the fact that CX_3CL1 is normally expressed by neurons (39, 43, 50) and CX_3CR1 by brain microglia (4, 19, 43, 50) suggests that it may be involved in neuron-microglia communication. In mice with EAE, levels of both CX_3CR1 mRNA and protein are elevated in activated microglia (25, 44). However, more detailed data on the identity of CCR1 and CX_3CR1 expressing cells during the course of autoimmune neuroinflammatory disease has been lacking.

We have here studied receptor distribution with in situ hybridization combined with histochemistry/immunohistochemistry during different phases of MOG-induced EAE disease and by flow cytometric analysis of leukocytes extracted from the inflamed CNS. Our results demonstrate distinctly non-overlapping expression of CCR1 and CX_3CR1 in peripherally-derived macrophages and microglia, respectively. Furthermore, the intimate association between CCR1 expressing, phagocytic cells and the actively demyelinating edges of lesions strongly argues for a pathogenic role for blood-derived macrophages and supports the feasibility of developing MS therapies based on specific monocyte chemoattractant inhibitors.

Material and Methods

Animals. Female DA rats at 10 to 14 weeks of age (150-200 g) were obtained from B&K Universal AB (Sollentuna, Sweden). A total of 42 animals (6 for each time point) were used for histological analyses and 26 animals for the flow cytometry study. All rats were housed under specific pathogen-free conditions and 12 hour/12 hour light-dark cycle in order to minimize the influence of environmental factors. All animal experiments were approved and performed in accordance with Swedish national guidelines on animal experimentation.

Preparation of MOG. Recombinant rat MOG corresponding to the N-terminus of the protein (amino acids 1-125) was expressed in *E.coli* and purified to homogeneity by chelate chromatography as previously described (1). The purified protein dissolved in 6 M urea was dialyzed against PBS to obtain a preparation that was stored at -20°C until use.

Immunization. Rats were anaesthetized with isoflurane (Baxter Medical AB, Kista, Sweden) and injected intradermally at the base of the tail with 0.2 mL inoculum, containing 20 μg recombinant rat MOG in saline, emulsified (1:1) with incomplete Freund's adjuvant (IFA) (Difco, Detroit, Mich) (56). The rats were scored and weighed daily from day 7 after immunization until sacrifice by 2 alternating investigators. The following score was used: Grade 1, flaccid tail or tail paralysis; Grade 2, hind leg paresis; Grade 3, hind leg paralysis, and Grade 4, tetraplegy, moribund state, or death. A disease

remission was defined as an improvement in disease grade from 3-4 to 1, or from 2-4 to 0 that was maintained for at least 2 consecutive days. A relapse was defined as an increase in the clinical grade of at least 2 points that lasted for 2 or more days. Healthy rats and Freund's complete adjuvant (FCA) or IFA immunized animals served as controls except for the flow cytometry study in which pools of 5 healthy animals were used. At various time points (day 12-60) after immunization and according to score rats were killed with CO_2 and perfused via the ascending aorta with sterile PBS and ice-cold 4% paraformaldehyde. Animals used for flow cytometry analysis were perfused only with sterile PBS in order to minimize contamination of non-extravasated leukocytes. Brains and spinal cords were quickly dissected out and routinely embedded in paraffin wax alternatively homogenized in PBS followed by Ficoll separation of infiltrating leukocytes.

Histopathology and immunohistochemistry. Histopathological evaluation was performed on paraformaldehyde-fixed, paraffin-embedded sections of the spinal cord and brain sampled at day zero, 13, 18, 21, 24, 29 and day 40 after immunization. The CNS tissue was divided into multiple parts from cervical to sacral spinal cord and 3 segments of the brain (cerebrum, cerebellum and brainstem). Individual parts were embedded in paraffin. Serial 4-μm thick sections were stained with haematoxylin and eosin (H&E), Klüver-PAS stain (Luxol fast blue [LFB] combined with periodic acid Schiff [PAS]) or with Bielschowsky silver impregnation to assess inflammation, demyelination and axonal damage, respectively. Similar sections processed for immunhistochemistry (IHC) were pretreated using an antigen retrieval technique consisting of 5×5 minutes of boiling in 10 mM Na-citrate buffer (pH 6.0) at 97°C in a microwave oven followed by blocking in 5% normal sheep serum diluted in PBS and finally incubation in 1% H_2O_2 in PBS. The following primary monoclonal antibodies were used; W3/13 (T-cells; Harlan Sera Lab), ED-1 (monocytes and macrophages; Serotec) and G-A-5 (glial fibrillary acidic protein specific (GFAP) of astrocytes; Boehringer, Mannheim, Germany). Primary antibodies were diluted 1/30 (W3/13), 1/500 (ED-1) and 1/20 (G-A-5) in PBS/0.5% BSA/0.6%Triton. Subsequent incubation with a biotinylated sheep anti-mouse IgG antibody (Life Sciences) and processing with the avidin biotin peroxidase (ABC) detection system (ABC Elite, Vector, Burlingame, Calif) was used to detect binding of primary antibody. Biotinylated lectin (GSA/B4, Vector) combined with the ABC detection system was used for the detection of microglia in various stages of activation. Control sections lacking the primary antibody step or incubated with irrelevant isotyped matched control antibodies were processed in parallel. As an additional control, labeling was examined also in sections from peripheral lymphoid organs tissue and in normal appearing white matter (NAWM).

In situ hybridization histochemistry. Paraffin embedded sections were mounted onto Superfrost slides (Super Frost Plus, Pittsburgh, Pa) and pretreated as for IHC. The in situ hybridization histochemical (ISH) protocol was essentially that of Swanson and Simmons (58) although post-fixation and treatment with acetic anhydride and proteinase K were replaced with an antigen retrieval technique. Briefly, deparaffinized sections from the brain, spinal cord and peripheral lymphoid organs were subjected to a 5×5 minutes of boiling in 10 mM sodium-citrate buffer (pH 6.0) at 97°C in a microwave oven. Sections were then hybridized with ^{35}S-uridine triphosphate (New England Nuclear, Mass) -labeled antisense cRNA probes encoding rat CCR1 (a 1280 base pair cDNA fragment of rat CCR1; GenBank acc. U92803) and CX_3CR1 (a 882 base pair cDNA fragment of rat CX_3CR1; GenBank acc. RN04808). Sense transcribed probes hybridized to slides processed in parallel served as controls of labeling specificity. Post-hybridization washes were performed in a series of washing solutions with subsequently lower salt content. A final high stringency wash was performed in $0.1 \times$ SSC with one mM DTT at 75°C for 30 minutes. IHC on selected slides was performed immediately after this step as described above. The hybridization signal was visualized with a phosphorimager screen (Fujifilm, Sweden), followed by exposure of X-ray film (Beta max, Kodak) and finally photographic emulsion dipping (NTB2, Kodak). Emulsion dipped slides were developed after 7 to 28 days and counterstained with haematoxylin (HTX).

Imaging. Digital images were captured with a Kappa DX 30 camera and imported into Adobe Photoshop (v. 6.0 Adobe Systems Inc.) and optimized for of brightness, contrast, sharpness and balance. Individual files were exported to Canvas (v. 8.0 Deneba Systems Inc.) for assembly into plates, which were rendered at initial resolution of 300 DPI.

Lesional staging. Areas representing NAWM, periplaque white matter (PPWM), active demyelinated plaques, inactive demyelinated plaques, and remyeli-

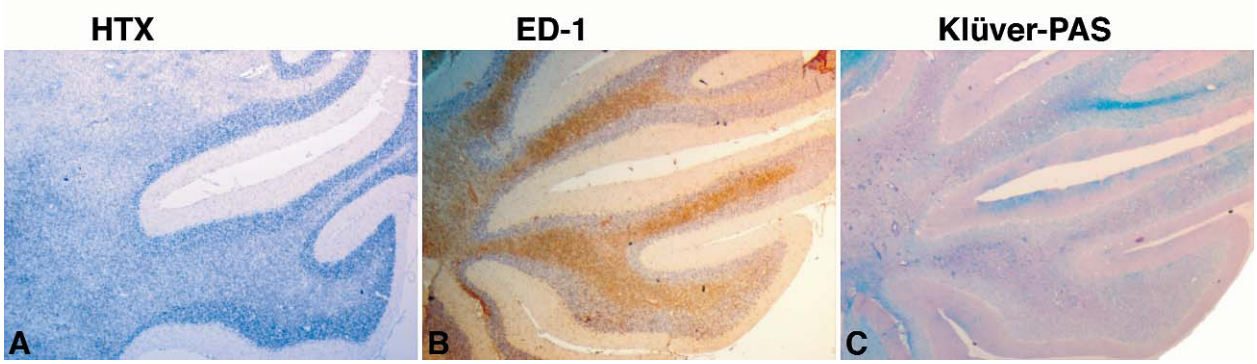

Figure 1. Photomicrographs of near adjacent sections from a large cerebellar plaque 40 days post-immunization. **A.** HTX staining demonstrates dense infiltration of inflammatory cells seen as a blue coloring beneath the granular cell layer also stained in blue. **B.** Numerous ED1-positive macrophages are present within the lesion overlapping with the area of inflammatory cell infiltration. **C.** LFB /PAS (Klüver-PAS) staining demonstrate different areas of active/inactive complete/incomplete demyelination. The major part of the plaque consists of a completely demyelinated inactive zone, easily identified by the complete lack of myelin staining. There are numerous macrophages containing PAS reactive degradation products, seen as a reddish coloring, indicative of active myelin breakdown. Note the relative lack of ED-1 and HTX stained macrophages and inflammatory cells, respectively, in the upper right corner where a LFB-positive myelinated fiber tract can be seen.

nated shadow plaques were identified and selected independently by three examiners (D.S., A.E.D. and H.L.) in H&E and Klüver-PAS stained slides. Actively demyelinating plaques were further carefully mapped into subareas using camera lucida drawings for the correlation of chemokine receptor distribution with histopathology. These were classified as follows: Early active lesions (EA; pink label in Figure 3) with dense infiltrates of macrophages, lymphocytes and microglia, disintegrating myelin sheets and macrophages containing LFB stained myelin degradation products; Late active lesions (LA; green label in Figure 3) with myelin-stripped axons and numerous macrophages containing PAS positive myelin degradation products; Inactive complete demyelinated lesions (DM) with no evidence of ongoing tissue destruction at plaque borders, but continued presence of infiltrating inflammatory cells, albeit at lower density than at LA and EA stages, and macrophages without LFB or PAS staining, suggesting remaining empty vacuoles after complete destruction of internalized myelin; Remyelinated shadow plaques (RM) with pale LFB myelin staining due to abnormally thin remyelinated sheaths and the presence of low numbers of inflammatory cells.

Flow cytometry analysis of leukocytes and microglia. Spinal cords were dissected out and placed in 10 mL 50% Percoll (= 1.070 g/mL; Pharmacia Biotech, Uppsala, Sweden) diluted in Hanks' balanced salt solution (HBSS, Sigma-Aldrich CO, Irvine, United Kingdom) containing 0.1% bovine serum albumin (BSA, Vector), 4.5 g/L glucose (Life Technologies, Paisley, United Kingdom) and 500 units DNAse type I (Boehringer). Homogenization was performed using a B pistil and 15 mL holder (Kontes, Vineland, NJ) after which samples were transferred to 50 mL tubes. A discontinuous centrifugation gradient was obtained by first adding 10 ml 50% Percoll and then by adding 7 mL 63% Percoll (= 1.087 g/mL) below and 20 mL 30% Percoll (= 1.045 g/mL) above. The gradient was centrifuged for 30 minutes at 1000g at 4°C. Myelin was discarded from fraction I (top), and the cells were collected from the interfaces between the remaining gradient densities. The cells were washed twice in Hanks' balanced salt solution (HBSS)/0.1% BSA/1% glucose. Cell counts and viability determined by Trypan blue (Sigma-Aldrich) dye exclusion were performed. For FACS analysis the samples were transferred to 4 mL tubes, centrifuged at 600g for 5 minutes at 4°C. The supernatant was discarded and the cells were resuspended in cold blocking buffer (HBSS/0.1% BSA/4% normal donkey serum [Research Diagnostics Inc. (RDI), Flanders, NJ]/ 0.02% NaN3/4.5 g/L glucose) and incubated for 10 minutes at 4°C. The first antibody incubation was performed for 30 minutes at 4°C with rabbit anti-rat CX_3CR1 (Torrey Pines Biolabs Inc., San Diego, Calif), goat anti-rat CCR1 (RDI) and mouse anti-rat CD45 (Pharmingen, San Diego, Calif) or ED-2 (Serotec). Unspecific binding was assessed by incubating control samples with unrelated isotype-matched mouse antibodies and rabbit or goat IgG. After two steps of washing and centrifugation, cell pellets were resuspended and incubated for 30 minutes at

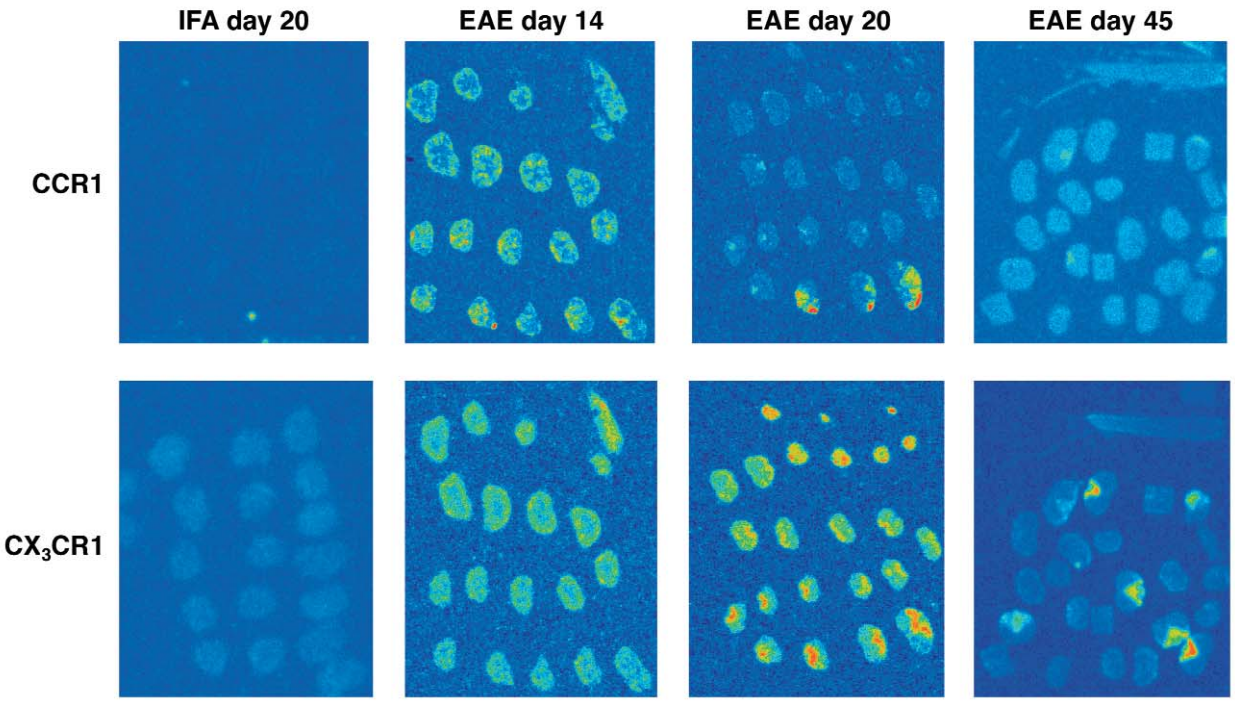

Figure 2. Phosphorimages of representative adjacent sections during the different phases of MOG-EAE illustrating the kinetic evolution of the expression patterns for CCR1 and CX_3CR1 in the inflamed spinal cord. The typical appearance of the expression pattern of CCR1 and CX_3CR1 mRNAs at 14, 21, 45 days post-immunization as well as in normal controls and IFA immunized animals is depicted with 17-23 parts of the cord from one individual rat in each picture. The sacral part of the cord has been cut longitudinally. Notably, no mRNA for CCR1 was present in any tissue derived from control animals or in areas of CNS tissue lacking histopathological signs of inflammation. In contrast, CX_3CR1 mRNA expressing cells were distributed diffusely throughout the CNS parenchyma and were present also in non-EAE immunized animals.

4°C with secondary step antibodies; phycoerythrin labeled donkey anti-goat IgG (RDI), fluorescein labeled donkey anti-rabbit IgG (RDI) or allophycocyanin labeled streptavidin (Becton Dickinson, San Jose, Calif) diluted 1:100 in blocking buffer. The incubation was followed by two steps of washing and centrifugation. Finally, cell pellets were resuspended in 300 μL 1% paraformaldehyde (Merck, Darmstadt, Germany) in 1 × PBS (Sigma-Aldrich) and analyzed with a FACSCalibur (Becton Dickinson) equipped with the CellQuest 3.01 software. Data is presented as mean fluorescence intensity of the whole populations.

Results

Clinical and histopathological disease in MOG-induced EAE of the DA rat. Clinical signs of disease became evident from day 9 to 13 post-immunization (p.i.) and were corroborated by histopathological demonstration of active demyelination, signs of acute axonal injury and dense infiltration of peripheral blood derived monocytes and neutrophils into the CNS from day 13 p.i. Histopathology at onset of clinical symptoms revealed diffuse infiltration of leukocytes into the CNS, particularly in the spinal cord, while focal infiltration of immune cells with plaque formation was regularly seen at later relapse stages. In most cases the number of infiltrating cells and signs of histopathological damage closely mirrored clinical symptoms. ED1 immunohistochemical labeling was used as a marker of phagocytic cells (microglia/monocyte-derived macrophages), and the distribution of labeled cells displayed a large degree of overlap with HE stained cell infiltrates (Figure 1). LFB/PAS staining of myelin products in adjacent sections enabled lesional stage mapping with regard to active/inactive complete/incomplete demyelination, as well as remyelination (Figures 1, 3). Numerous T-cells were present during the acute phase, whereas T-cells during chronic or relapse phases were sparse. Microglia and macrophages were differentiated by morphological criteria essentially as described by Storch et al, (57) except that GSA/B4 lectin was used instead of AIF-1.

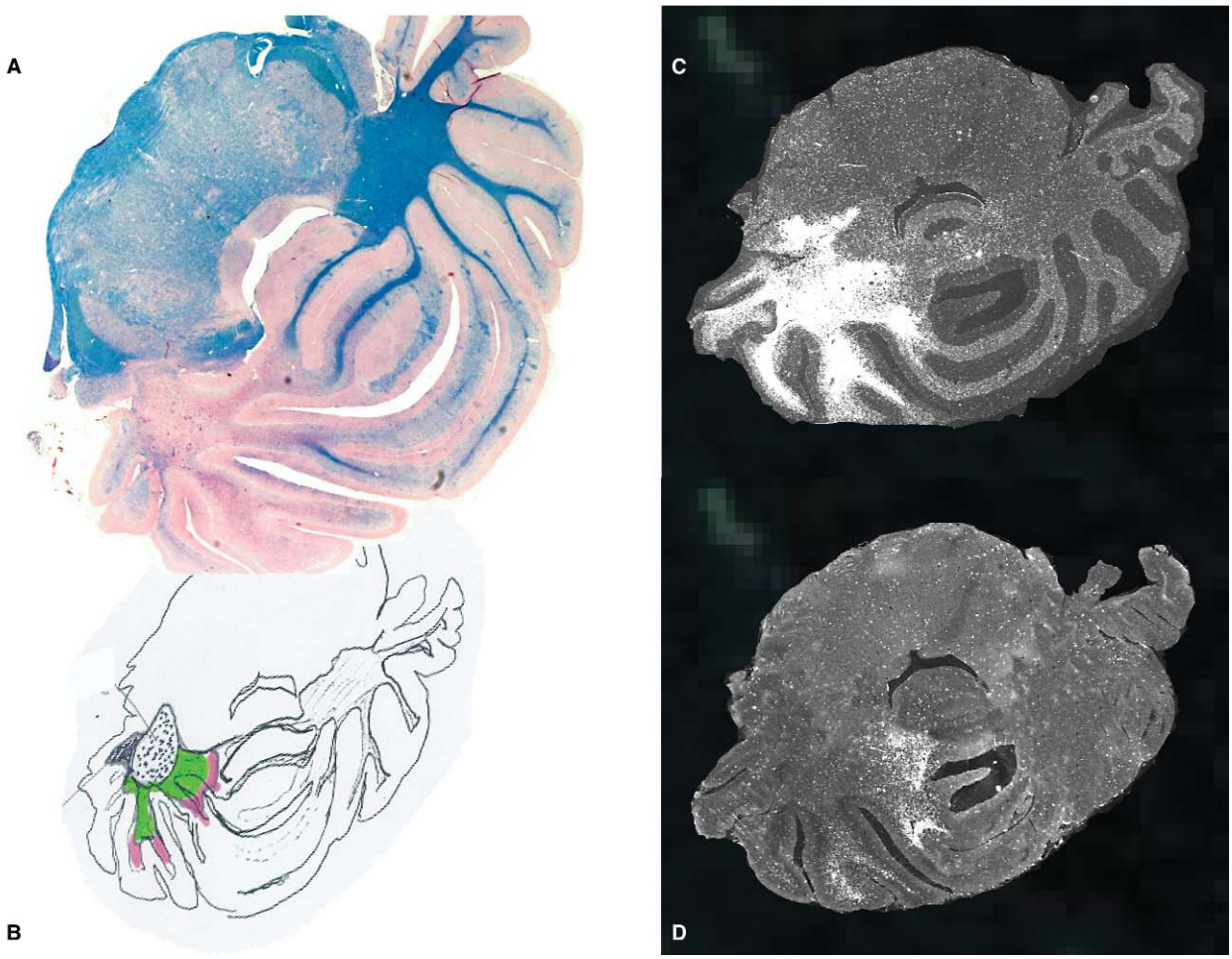

Figure 3. Photomicrographs and camera lucida drawing of a cerebellar plaque identical to that shown in Figure 1. **A.** LFB/PAS (Klüver-PAS) staining of a section through the brain stem and cerebellum visualizes a large plaque that demonstrate different areas of active/inactive complete/incomplete demyelination, as well as a smaller zone of remyelination. **B.** Camera lucida drawing of the section with stage mapping (see Materials and methods) of the lesion. **C.** Dark-field image of the in situ hybridization signal for CX_3CR1 mRNA. **D.** Dark-field image of the in situ hybridization signal for CCR1 mRNA. Note the generalized expression of CX_3CR1 mRNA and the focal expression of CCR1 mRNA in the active edges of the lesion. Early active lesions (EA; pink label), Late active lesions (LA; green label) Inactive complete demyelinated lesions (DM, grey dotted label) Remyelinated shadow plaques (RM, Filled grey label).

GSA/B4 labeling of microglia and perivascular, meningeal and blood-derived macrophages rendered a similar labeling pattern as ED-1 in lesions. In addition, there were GSA/B4$^+$ED-1$^-$ activated microglia in the periplaque region, most of which displayed a morphology typical of initial stages of microglia activation (Figure 4). Occasional animals in chronic stages of EAE displayed a more widespread activation of microglia throughout the parenchyma (Figures 1, 3).

Kinetic evolution and cellular localization of CCR1 and CX3CR1 expressing cells during MOG-EAE. No positive CCR1 mRNA hybridization signal was present in brain sections from normal and CFA immunized animals. In contrast, CX_3CR1 mRNA expression was readily evident also in resting microglia of control animals (Figures 2, 4). Both transcripts displayed dramatically increased hybridization signals already from day 14 p.i. (Figure 2). CCR1 and CX_3CR1 mRNA expressing cells were detected throughout all brain lesions, which, at the acute stage, were restricted mainly to the spinal cord as focal aggregates of infiltrating leukocytes. This contrasted sharply with a much more differentiated distribution of CCR1 and CX_3CR1 expressing cells within the inflammatory lesions during later stages. At day 21, when large, non-uniform lesions were emerging within the spinal cord and, to a lesser extent, the cerebellum and

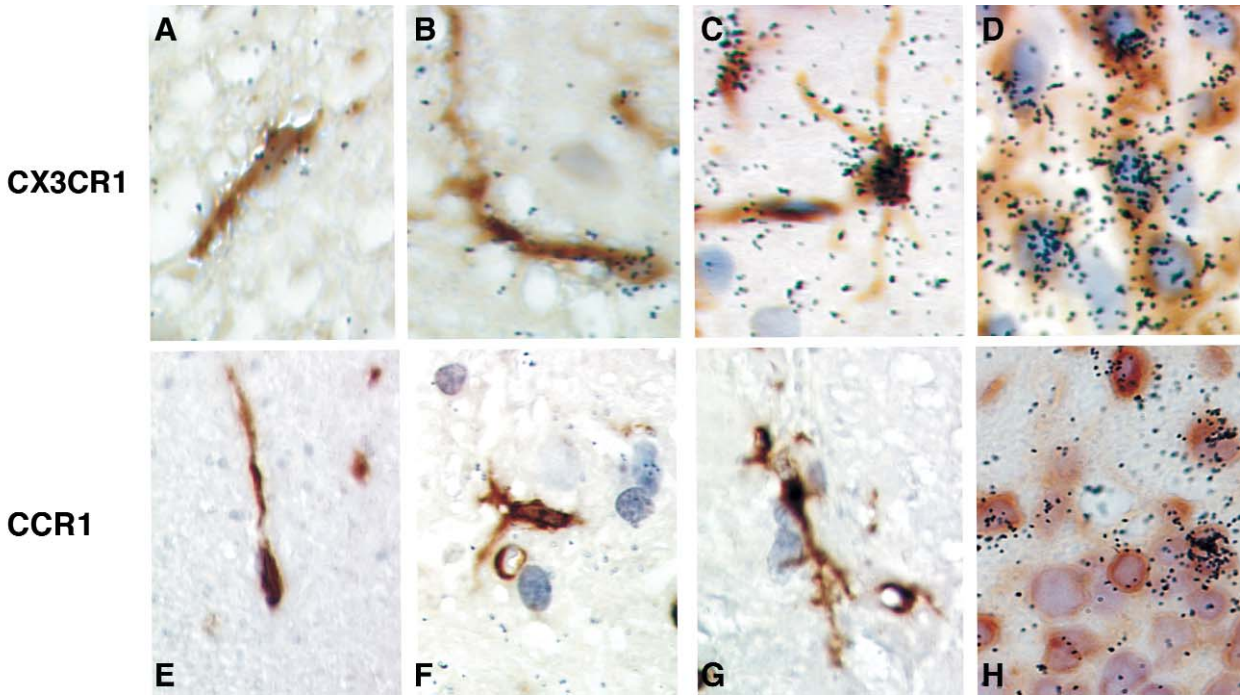

Figure 4. Photomicrographs of sections processed for combined GSA/B4 immunohistochemistry and CX_3CR1/CCR1 mRNA in situ hybridization in control tissue and from various locations in EAE animals. **A, E.** Resting microglia cell in normal appearing white matter (NAWM) of a control animal displays a weakly positive CX_3CR1 mRNA labeling and no labeling for CCR1. **B, F.** Microglia cell in the NAWM of an EAE animal displaying morphology typical of initial stages of activation as well as slightly stronger CX_3CR1 mRNA labeling and negative for mRNA expressing CCR1. **C, G.** Activated microglia in the lesion border zone of an EAE animal with dense CX_3CR1 mRNA labeling mirrored by negative labeling for CCR1. **D, H.** Large amoeboid GSA/B4 positive phagocytes with intense CX_3CR1 and CCR1 mRNA labeling located in the core zone of an EAE plaque. Scale bar = 25 μm.

optic nerve, CCR1 mRNA expressing cells were aggregating focally within lesions, whereas CX_3CR1 mRNA expressing cells were distributed throughout the entire lesions and adjacent periplaque regions. In addition, an increased CX_3CR1 mRNA signal was evident also in the remaining CNS parenchyma. This discrepancy, with a focal versus generalized expression, was further accentuated as rats developed a chronic progressive stage of the disease. The kinetic evolution of the expression patterns for CCR1 and CX_3CR1 in the inflamed spinal cord is visualized by phosphorimage profiles in Figure 2.

A detailed analysis consisting of time-staging of the lesions using various kinetics markers that reflect the degree of myelin phagocytosis and intracellular degradation revealed that CCR1 mRNA expressing cells were confined to lesions with intense inflammation and signs of early-active demyelination, ie, entire lesions of the acute phase and active edges of older lesions. In contrast, increased CX_3CR1 mRNA signals were present in multiple areas within and outside of plaques, in particular areas representing late active and inactive, complete demyelination, but also the periplaque region and early active plaque zones. In the so called shadow plaques, demyelinated brain lesions with signs of ongoing remyelination, the expression of CX_3CR1 was lower, however, not completely reaching the levels seen in the NAWM of non-immunized control rats. CCR1 and CX_3CR1 expression and adjacent histopathological stains in a well differentiated cerebellar brain lesion with subregions representing all of the above-mentioned kinetic stages is depicted in Figure 3.

Cellular identity of CCR1/CX3CR1 expressing cells. The observations described above prompted further investigations into the cellular identity of the CCR1 and CX_3CR1 expressing cells. This analysis was performed using combined in situ hybridization for CCR1 and CX_3CR1 mRNA and immunohistochemical staining for GFAP (astrocytes), W3/13 (T-cells and plasma cells), ED-1 (macrophages and final stage activated microglia) and GSA/B4 (microglia, perivascular and menigeal macrophages). Expression of CCR1 mRNA was detected exclusively in a subpopulation of

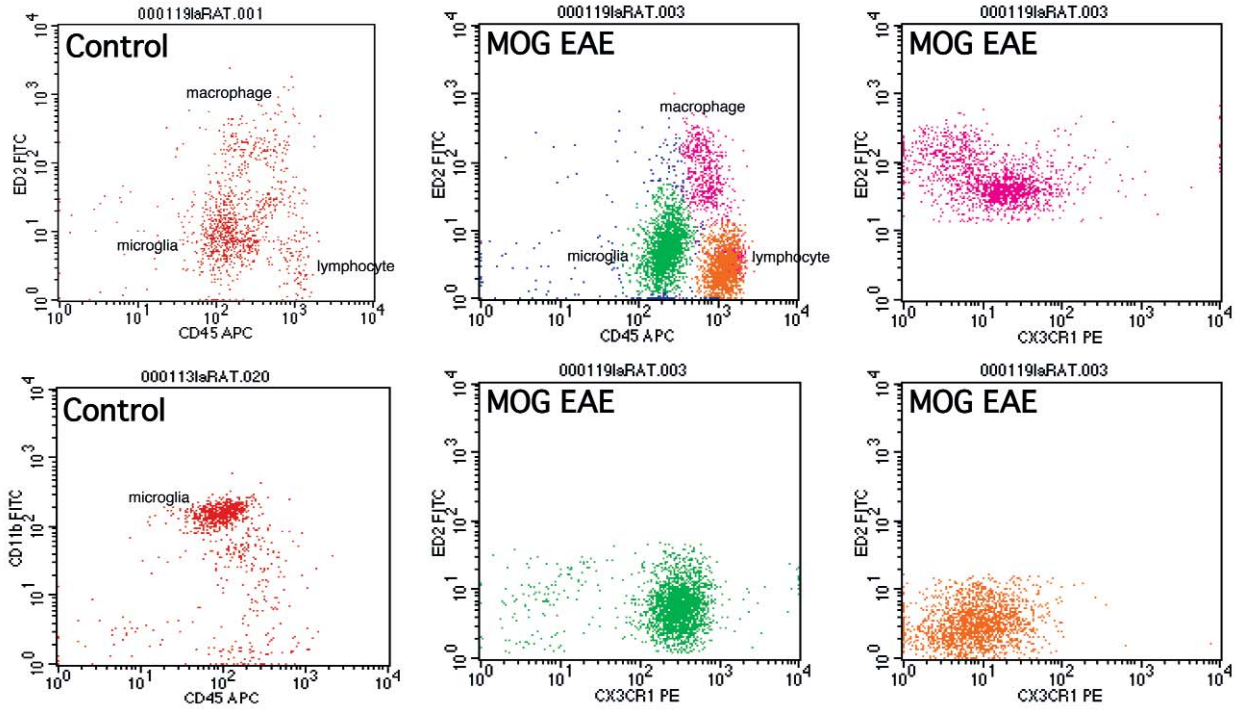

Figure 5. Flow cytometry plot depicting the distribution of microglia, macrophage and lymphocyte populations extracted from the spinal cord of control (left panel) and EAE (right panel) animals using CD11b/ED-2 and CD45 gating. Microglia (ED-2^{Low}CD11b^{High}-CD45^{Low}), monocytes/macrophages (ED-2^{High}CD11b^{High}-CD45^{High}) and lymphocytes (ED-2^{Low}CD45^{High}-Cd11b^{Low}) are represented as distinct populations. Note that virtually no lymphocytes or macrophages were present in spinal cord tissue from control animals.

the ED-1$^+$ cells and in the amoeboid form of the GSA/B4$^+$ cells, but not in T-cells or astrocytes. CX_3CR1 mRNA was detected abundantly outside the inflammatory lesions in GSA/B4$^+$ cells morphologically resembling microglia in various stages of activation, as well as in amoeboid cells in the plaque regions. The ED1$^+$ cells could further be subdivided in 2 different populations, one with and one without detectable expression of CX_3CR1 mRNA (Figure 4). Thus, GSA/B4$^+$ cells in the meninges, ie, meningeal macrophages, were CCR1 mRNA-positive but devoid of CX_3CR1 mRNA, while GSA/B4$^+$ amoeboid cells morphologically identified as microglia in plaque regions displayed an inverse pattern. Combined with the fact that the expression of CCR1 and CX_3CR1 mRNAs is partly non-overlapping in late stage lesions (Figure 3), this suggests that CCR1 expression is confined to the infiltrating macrophage population, while CX_3CR1 mRNA is expressed by microglia.

Differential expression of CCR1 and CX_3CR1 in FACS sorted microglia and macrophage populations. Surface protein expression of CCR1 and CX_3CR1 were studied in microglia and CNS infiltrating leukocytes extracted from the spinal cord of EAE animals and controls. Cell populations of interest were identified by the differential expression of the cell surface markers CD45 and CD11b, using the following gating; CD11b^{High}-CD45^{Low} (microglia), CD11b^{High}-CD45^{High} (monocytes, macrophages), CD45^{High}-Cd11b^{Low} (lymphocytes). In some cases CD11b was replaced with ED-2 in order to distinguish between perivascular macrophages/pericytes (ED-2$^+$) and microglia and monocytes (ED-2$^-$). Thus, triple stainings were performed for ED-2 or CD11b, as well as for CD45 and CCR1 or CX_3CR1, respectively. This confirmed that CCR1 and CX_3CR1 are differentially expressed in microglia/monocyte populations also at the protein level (Figure 5). CCR1 expression was detected both in the lymphocyte and macrophage populations, but not in microglia. The expression was highest in animals with more severe clinical symptoms, probably as a reflection both of higher numbers of infiltrating cells and higher relative CCR1 expression. Conversely, rats with less clinical symptoms had relatively lower CCR1 expression judged by the mean fluorescence intensity (Figure 6, Table 1). No lymphocytes or macrophages were present

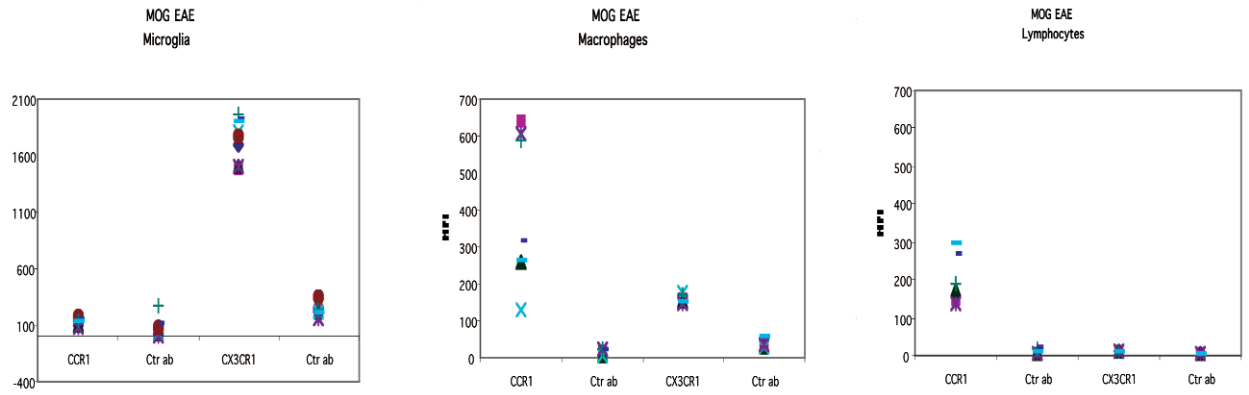

Figure 6. Box plots of mean fluorescence signal for CCR1 and CX_3CR1 and their respective control antibodies in microglia, macrophage and lymphocyte populations extracted from spinal cord tissue of EAE animals. Each data point represents a single animal.

		CX_3CR1 Median (range)	**Control ab** Median (range)	**CCR1** Median (range)	**Control ab** Median (range)
EAE animal					
n = 5-7	Microglia	1803 (1479-1803)	215 (163-231)	134 (89-159)	38 (10-266)
n = 5-7	Macrophage	155 (149-174)	36 (29-60)	319 (131-641)	20 (5-25)
n = 5-7	Lymphocyte	10 (9-13)	4 (4-5)	175 (136-297)	8 (7-22)
Control animal					
(pool of five) x 2	Microglia	1727 (1690-1764)	299 (253-344)	131 (92-169)	51 (20-82)

Table 1. *Mean fluorescent intensity (MFI). Day 18+24.* Mean fluorescence signal readings for CCR1 and CX3CR1 and their respective control antibodies in microglia, macrophage and lymphocyte populations extracted from spinal cord tissue of EAE animals.

in control animals and, as in EAE animals, the microglia population was devoid of CCR1 protein. In contrast, CX_3CR1 was expressed to a high extent and in a similar manner in the microglia population of all EAE animals studied. CX_3CR1 immunoreactivity was also detected in microglia derived from control animals (pooled cells from 5 animals) demonstrating the constitutive nature of CX_3CR1 in resident microglia. The macrophage population from EAE animals displayed much lower CX_3CR1 expression in relation to microglia from the same animals, whereas the lymphocyte population was completely devoid of positive cells.

Discussion

We have here examined the expression of receptors for the chemokines CCL3/CCL5/CCL7 and CX_3CL1, CCR1 and CX_3CR1, respectively, in MOG-induced EAE in the DA rat. This model shares several important features with human MS disease, including a chronic relapsing-remitting disease course and development of focal MS-like plaques with demyelination and axonal damage. The disease pattern and histopathological changes documented in this study are in line with previous findings reported for this disease model (33, 56). Microglia and macrophages constitute the dominating cell populations in inflammatory lesions of MOG-EAE rats, especially at later stages of the disease. In contrast, only sparse T-cells were present during the relapse phase, confirming previously reported results (33). Interference with monocyte recruitment constitutes a promising therapeutical strategy for the treatment of neuroinflammatory diseases, in particular MS. However, the usefulness of this strategy is difficult to predict due to the lack of knowledge about functional distinctions between phagocytes derived from peripheral blood and those whose progenitors are already present within the CNS compartment. Our results demonstrate very distinct expression profiles of CCR1 and CX_3CR1. CCR1 mRNA was heavily upregulated only in areas of early active demyelination, whereas mRNA for CX_3CR1 was

more generally expressed and demonstrated highest expression levels in areas of late active and complete inactive demyelination. Furthermore, CX_3CR1 mRNA expression was restricted to GSA/B4$^+$ cells with microglia morphology, whereas CCR1 mRNA was detected exclusively in ED1$^+$GSA/B4$^+$ cells with amoeboid morphology. The notion of a differential receptor expression was further corroborated by FACS analysis of infiltrating leukocytes and microglia extracted from the spinal cord, demonstrating high levels of CCR1 and low levels of CX_3CR1 in the macrophage population and an inverse relation in microglia. These observations suggest that CCR1$^+$ cells under in vivo conditions originate from the non-microglia pool of phagocyte progenitors, ie, blood-derived monocytes. Our results also include the novel observation that macrophages infiltrating the brain and spinal cord parenchyma successively down regulate CCR1 during plaque evolution, so that older lesions are completely devoid of CCR1 expressing cells. This is consistent with the early zone of the plaque being an area in which active recruitment from the periphery of CCR1$^+$ monocytes, and possibly also myelin-reactive CCR1-expressing T-cells (13, 33), takes place. Numerous phagocytic cells are still present at later stages of plaque evolution. However, they express relatively less CCR1 and are thus likely to be less responsive to migratory stimuli in the form of locally produced CCR1 ligands.

In contrast to CCR1, expression of CX_3CR1 mRNA was detected also in microglia of healthy control rats. Expression was increased in inflammatory lesions, which is in line with the results of a previous study using RNAse protection (25). However, widespread expression throughout the brain parenchyma associated with a generalized activation of microglia also in NAWM was present during initial stages of EAE (Figures 2, 4B, F). This may bear some resemblance to the situation in MS, in which some clinical cases display histopathological signs of axonal damage without signs of demyelination, possibly due to the propagation of a residual inflammatory process by macrophages and activated microglia (34). Discrepancies in the expression pattern between rat and human make it difficult to extrapolate data from rodent EAE models with regard to the role of CX_3CL1 signaling in MS (8, 14, 15, 23). However, we have recently found that CX_3CR1 is upregulated also in a marmoset EAE model, with a similar expression profile as reported here (unpublished observations, Sunnemark-Lassmann). A direct effect of CX_3CL1 on rat T-cells is unlikely since we found no CX_3CR1 in CNS infiltrating lymphocytes and very low levels in circulating blood cells, a finding previously reported also for murine lymphocytes (26). In addition, TH1 polarized T-cells display even lower levels of CX_3CR1 (16). Our data also demonstrate much lower relative levels of CX_3CR1 in infiltrating macrophages, including a subpopulation totally devoid of receptor expression, compared to microglia. Perhaps this may reflect a shift from CX_3CR1 to CCR1 in the receptor expression by differentiation of cells of monocyte-lineage. The role of CX_3CR1 in neuroinflammation, thus, remains elusive. In fact, CX_3CR1 knock-out mice do not demonstrate any discernible differences with regard to disease susceptibility or severity compared to controls (20). Still, the fact that CX_3CR1 mRNA induction was only detected within areas of the plaque with inflammatory features and not in remyelinated zones argues for a role of CX_3CL1 signaling in the pathogenesis of inflammation. Alternatively, degeneration of neurons could be the cause of increased CX_3CR1 expression due to priming of microglia to fractalkine shedded from injured neurons by a yet undefined neuronally-derived signal. Interestingly, an anti-inflammatory and neuroprotective effect of CX_3CL1 was recently proposed (64, 65). CX_3CL1 has also been proposed as a survival factor for microglial cell in the CNS, since in vitro treatment with fractalkine inhibits Fas-mediated cell death of brain microglia (5). Contrasting to this, CX_3CR1 has been implicated in the propagation of inflammation in other organs, eg, cardiac allograft rejection (20, 46), cardiovascular disease (35, 40, 42) and glomerulonephritis (7, 11).

Several inhibitors of specific chemokine signaling pathways are currently being investigated as potential drug candidates for the treatment of neuroinflammatory disease. Ideally one would like to find a single critical step accessible for blocking that would provide significant clinical benefit. However, this task is made difficult by the increasing complexity of the chemokine-signaling network. The failures of prior studies of specific inhibitors of cytokines in the treatment of MS also warrant caution of unrealistic optimism (51, 53, 62). To date, several exploratory studies of inhibitors of chemokine receptors have been published (37, 48, 49). In addition to these chemokine pathways, the results presented herein support CCR1 as a potential drug target due to its role in the recruitment of phagocytic cells to early active zone of demyelination. In fact, data from our laboratory provide strong experimental support for this hypothesis. Thus, administration of 5-[4-(4-

chlorophenyl)-4-hydroxypiperidin-1-yl]-2,2-diphenylpentanenitrile, a low molecular weight compound which specifically bind and blocks CCR1 (21), results in greatly suppressed disease activity both with regard to clinical symptoms and histopathological signs of inflammation in the same rat EAE model used here (10a). Early treatment results in a complete block of all symptoms, whereas treatment from the first bout leads to an ameliorated disease. A synchronized bout/relapse in the animals upon stopping drug administration suggests an effect down stream of the regulatory events that initiate and propagate the systemic inflammatory activation. Nevertheless, this encourages further attempts at developing drugs intervening with monocyte recruitment for the treatment of neuroinflammatory conditions such as MS. However, it is important to consider the fact that MS is a heterogeneous condition and further studies are needed to evaluate not only the therapeutic potential in various stages of the disease, but also possible side effects of chemokine receptor antagonists.

In conclusion, the present study demonstrates that both CX_3CR1 and CCR1 are expressed and extensively up regulated during rat MOG-EAE. Whereas CX_3CR1 is primarily expressed by parenchymal microglia, CCR1 is exclusively expressed by infiltrating leukocytes. CCR1 expressing cells were present only in active zones of demyelination, while CX_3CR1 mRNA expression was present not only at high levels in plaque areas but also to a lower expression level throughout the nervous tissue outside of lesions. These observations provide strong evidence for a pathogenic role of monocyte-derived macrophages in neuroinflammatory diseases and strengthen the notion of monocyte recruitment as a target for disease modifying therapy in MS.

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