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Regulation of Adaptive Immunity by the Fractalkine Receptor during Autoimmune Inflammation

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Fractalkine, a chemokine anchored to neurons or peripheral endothelial cells, serves as an adhesion molecule or as a soluble chemotactant. Fractalkine binds CX3CR1 on microglia and circulating monocytes, dendritic cells, and NK cells. The aim of this study is to determine the role of CX3CR1 in the trafficking and function of myeloid cells to the CNS during experimental autoimmune encephalomyelitis (EAE). Our results show that, in models of active EAE, Cx3cr1−/− mice exhibited more severe neurologic deficiencies. Bone marrow chimeric mice confirmed that CX3CR1 deficiency in bone marrow enhanced EAE severity. Notably, CX3CR1 deficiency was associated with an increased accumulation of CD115+Ly6C+ monocytes and increased IL-17 levels in lymphoid tissues of CX3CR1-deficient mice. Analyses of peripheral responses during disease initiation revealed a higher frequency of IFN-γ– and IL-17-producing T cells in lymphoid tissues of CX3CR1-deficient mice as well as enhanced T cell proliferation induced by CX3CR1-deficient dendritic cells. In addition, adoptive transfer of myelin oligodendrocyte glycoprotein-reactive wild-type T cells induced substantially more severe EAE in CX3CR1-deficient recipients when compared with wild-type recipients. Collectively, the data demonstrate that besides its role in chemoattraction, CX3CR1 is a key regulator of myeloid cell activation contributing to the establishment of adaptive immune responses. The Journal of Immunology, 2013, 191: 1063–1072.

Multiple sclerosis (MS) is a chronic, inflammatory demyelinating disease of the CNS causing significant neurologic disability in young adults. Supported by experimental evidence largely collected from its major model, experimental autoimmunne encephalomyelitis (EAE), MS is considered for many in the field a predominantly T cell–mediated disease. Although the exact cause of MS remains unsolved, CNS inflammation is a key component of the pathophysiology of MS. Whereas chemokines are known to promote tissue inflammation via recruitment of immune cells to these sites, recently it was shown that chemokine–chemokine receptors also participate in cellular activation and modulate effector functions of certain immune cell subsets (1, 2).

The discovery of the chemokine receptor CX3CR1 and its unique ligand fractalkine (CX3CL1) in the mid 1990s represented a major advancement in the understanding of myeloid cell function (3–5). Fractalkine is a distinct chemokine, expressed as a membrane-bound glycoprotein on neurons and peripheral endothelial cells (3, 6, 7). The fractalkine receptor (CX3CR1) is present on microglia and circulating monocytes, dendritic cells, and NK cells. Fractalkine, first known as neurotactin due to its abundant expression in the brain, plays different roles in different tissue compartments and disease states. We discovered that CX3CL1 signaling promotes microglial survival and controls microglial neurotoxicity through its unique receptor CX3CR1 under certain neurodegenerative and inflammatory conditions. Importantly, in humans, two single-nucleotide polymorphisms give rise to four allelic receptor variants. The most studied forms are CX3CR1V249T/C280 (also considered as the reference receptor) and the variant alleles CX3CR1V249T/C280 and CX3CR1V249T/C280 present in 20–30% of the population. These changes decrease fractalkine affinity and correlate with enhanced susceptibility to age-related macular degeneration (8, 9) and protection from atherosclerosis (10–12). Notably, a genetic study in a Serbian population (397 MS patients) found a significantly lower frequency of the CX3CR1V249T/C280 haplotype in secondary progressive compared with relapsing–remitting patients. Therefore, understanding the function of CX3CR1 during disease initiation will be valuable to understand the immune pathology of MS.

Monocytes and dendritic cells (13–15) are critical mediators of innate and adaptive immune responses. Notably, CX3CR1 distinguished a monocyte subset in peripheral blood so-called resident and phenotypically recognized as LFA-1+/CD11b+/Ly6C−/CCR2−/CX3CR1I249/T280, whereas the MCP-1 (or CCL2) receptor CCR2, marked inflammatory monocytes, distinguished as LFA-1+/CD11b+/Ly6C+/CCR2+/CX3CR1I249/M280 (14, 16, 17). The recent characterization of red fluorescent protein (RFP)–CCR2 knock-in mice and the generation of mouse models carrying CX3CR1-GFP/CX3CR1-GFP...
CXC3R1 AND MYELOID CELLS DURING AUTOIMMUNE INFLAMMATION

CCR2 reporter proteins provided additional support for a distinct molecular signature of CXC3R1 and CCR2 during embryonic development (18) and brain inflammation. CCR2 is critical for efficient accumulation of Ly6Chigh/CCR2low monocytes to the CNS. Furthermore, microglia in the adult naive and EAE-inflamed CNS were found CXC3CR1high/CCR2low (19), and, similar to the populations described in peripheral blood, the EAE bone marrow monocytes also appeared as CCR2highCX3CR1low or CCR2low CX3CR1high (19).

Due to the contrasting expression of CX3CR1 on the two major monocyte populations, we sought to investigate the role of CX3CR1 in accumulation of myeloid cells to the CNS during EAE and its effects on brain pathology. For this, we used Cx3cr1GFP/GFP (or Cx3cr1+/−) mice and Cx3cr1GFP/GFP.Ccr2+/RFP and controls (Cx3cr1+/+ and Cx3cr1GFP/GFP.Ccr2+/RFP) to characterize myeloid cells in brain and lymphoid tissues at peak of EAE disease and investigate the role of these cells in promoting T cell activation and proliferation. Our results show that Cx3cr1+/− mice exhibited more severe neurologic signs. Radiation bone marrow chimeric mice confirmed that CX3CR1 deficiency in bone marrow enhanced EAE severity. Notably, CX3CR1 deficiency was associated with an increased accumulation of CD11b+/Ly6C−/CCR2− CD11c+ dendritic cells into EAE-affected brains that correlated with enhanced demyelination and neuronal damage. Wild-type (WT; Cx3cr1+/+) infiltrating T cells showed an increased expression of IL-10 when compared with CX3CR1-deficient cells. Analyses of peripheral responses during disease initiation revealed a higher frequency of IFN-γ and IL-17–producing T cells in lymphoid tissues of CX3CR1-deficient mice. Analyses of peripheral responses during disease initiation revealed a higher frequency of IFN-γ and IL-17–producing T cells in lymphoid tissues of CX3CR1-deficient mice. Analyses of peripheral responses during disease initiation revealed a higher frequency of IFN-γ and IL-17–producing T cells in lymphoid tissues of CX3CR1-deficient mice.

Active EAE induction
Active EAE was induced in mice 8–10 wk old by s.c. immunization with 100 μg MOG35–55 peptide in CFA, as previously described (18). Mice were weighed and examined daily for EAE signs and scored as follows: 0, no signs of neurologic disease; 1, lack of tail tone; 2, abnormal gait, hind limb weakness; 2.5, partial hindlimb paralysis; 3, complete hindlimb paralysis; 3.5, ascending paralysis; 4, tetraplegia; and 5, death. Mice were sacrificed when they reached a score of 2.5–3.0 (1). Mice were sacrificed at 11 d postimmunization (p.i.), at peak of EAE disease (16–21 d p.i.), or 60 d p.i. (chronic phase).

Passive EAE induction
Mice were immunized with 300 μg MOG35–55 peptide in CFA. Spleen and lymph nodes were harvested, and single mononuclear cell suspensions were prepared 10 d p.i. and cultured in the presence of 20 μg/ml MOG35–55, 20 ng/ml murine rIL-23 (R&D Systems), and 10 μg/ml anti-IFN-γ (R4-6A2; BioXcell) in complete media (20–22). After 3-d incubation, cells were collected and washed in DMEM containing 50 μg/ml pristamin, and 20–50 × 10⁷ cells were i.p. injected into WT or Cx3cr1+/− recipient mice. A separate aliquot of the isolated cells was subjected to IFN-γ and IL-17 cytokine ELISPOT assays. This was performed on primed T cell from WT and CX3CR1-knockout (KO) mice, to normalize the number of cells injected per recipient and compare similar number of effector cells per experiment. Prior to injection, recipients were injected with 200 ng per-tussis toxin i.p. on the day of cell transfer and 48 h after transfer. Mice were weighed and EAE scored daily.

Generation of bone marrow chimeric mice
Recipient mice (5–6 wk old) were irradiated with a dose of 9 Gy and allowed to recover overnight before bone marrow reconstitution. Bone marrow cells were isolated from femur and tibia, as previously described (1). Briefly, mice were sacrificed by CO2 asphyxiation, followed by cervical dislocation, and flushed bone marrow cells were resuspended in Iscove’s media without FBS at 15 × 10⁶ cells/ml. Recipient mice were anesthetized 1–2 min (or until animals’ loss of righting reflexes) in an induction chamber with oxygen flow rate of 1 L/min and isoflurane delivery to 3–4%, and 15–20 × 10⁶ cells were injected via the retro-orbital sinus in a volume of 100–150 μl. Mice were placed in a clean cage and monitored until righting reflex was gained. Six weeks after reconstitution, EAE was induced. Efficiency of engraftment was confirmed by flow cytometry 4 wk after bone marrow reconstitution by virtue of CD45.1 and CD45.2 congenic markers in donor and recipients, respectively.

Isolation of mononuclear cells and flow cytometry
Perfused brains and spinal cord tissues were dissected from mice at peak of EAE disease; mononuclear cells were separated over discontinuous 70/30% Percoll gradients, as previously described (23); and cellular pellets were resuspended in cell-staining buffer (BioLegend, San Diego, CA). Blood for single-stained controls was collected from the submandibular vein, and RBCs were depleted by hypotonic lysis and washed in staining buffer. Isolated cells were incubated on ice for 5 min with anti-mouse CD16/CD32 (clone 2.4G2; BD Pharmingen) to block FcRs and then incubated on ice for 30 min with a mix of fluorochrome-conjugated anti-mouse Abs, as follows: CD45-PerCP, allophycocyanin-Cy7, or allophycocyanin (clone 30-F11; BD Pharmingen); CD115-PE (clone AF598; Bioсиescience); CD11b-PE (clone M1/70); CD11c-PerC57 (clone N418; Bioсиescience); Ly6C-Alexa 647 (clone ER-MP20; AbD Serotec); CD80-allophycocyanin (clone 16-10A1); CD86-PerCP (clone GL-1); and I-A/I-E–Pacific Blue (mouse MHC-II clone M5/114.15.2). After washing, cells were resuspended in 2% paraformaldehyde and analyzed in a LSR-II (BD Biosciences, Franklin Lakes, NJ). Similar analyses were carried out in cell suspensions from lymph node and spleens at 11 d p.i. Lymphoid tissues were passed through a 70-μm nylon mesh, and spleen RBCs were depleted by hypotonic lysis prior to flow cytometry.

To quantify the proportion of resident and infiltrating myeloid cells undergoing cell proliferation, CNS mononuclear cells were stained with a mix of CD45-allophycocyanin (clone 30-F11) and CD11b-PerCP (clone M1/70) Abs for 30 min on ice, fixed with 4% paraformaldehyde (PFA) for 30 min, and then incubated at room temperature for 10 min in permeabilization buffer (eBioсience). Cells were then stained with Ki67-V450 (clone B56) in permeabilization buffer for 20 min, washed, resuspended in 2% PFA, and acquired on an LSRII. Flow cytometry data are presented as number or percentage of cells, or as mean fluorescence intensity for MHC-II, CD80, and CD86 markers.

Immunohistochemistry staining of brain sections
Following buffer perfusion, mice were perfused with 4% PFA. Dissected tissues were postfixed 24–36 h in 4% PFA and cryoprotected in 20% glycerol in 80 mM phosphate buffer (pH 7.6) for 48 h at 4°C. Free-floating 30-μm sections were prepared using a freezing microtome and stored at −20°C until use (2). After blocking, tissues were stained overnight at 4°C with anti-mouse CD45 Abs (clone IBL-3/16; AbD Serotec, Raleigh, NC), anti-mouse NeuN (clone A60; Millipore, Billerica, MA), anti-calbindin mAb (clone C26D12; Millipore), or anti–myelin basic protein (MBP) Ab (Invitrogen). Tissues were then incubated with biotin-labeled secondary Ab and developed with avidin-biotin peroxidase system (Vector Laboratories, Burlingame, CA) with diaminobenzidine as a substrate. Tissues were then incubated with biotin-labeled secondary Ab and developed with avidin-biotin peroxidase system (Vector Laboratories, Burlingame, CA) with diaminobenzidine as a substrate. Tissues were then incubated with biotin-labeled secondary Ab and developed with avidin-biotin peroxidase system (Vector Laboratories, Burlingame, CA) with diaminobenzidine as a substrate.
ethanol solutions (100, 95, and 70%) and stained for 3 min in 0.5% solution of cresyl violet, followed by dehydration (70, 95, and 100% ethanol). To assess differences, myelin content, three tissue sections per mouse (n = 4 per group), was imaged, and the area of myelin immunoreactivity in eight random images per section was obtained using Image-Pro Plus 6.2.1 (Media Cybernetics) in a blinded manner. Similarly, calbindin-positive cells were counted in 10 randomly acquired images per section in 3 different tissues per mouse. Individual cells were counted using the count analysis tool in Adobe Photoshop extended CS4 v11 (Adobe).

T cell proliferation assay
Mice immunized with 300 μg MOG35–55 and sacrificed 11 d p.i. T cells were enriched from spleens by magnetic negative selection (Stemcell Technologies). For dendritic cell isolation, spleens were extracted and dissociated in spleen dissociation media (Stemcell Technologies). For dendritic cell isolation, spleens were extracted and dissociated in spleen dissociation media (Stemcell Technologies) and enriched for CD11c+ cells (Stemcell Technologies). T cells were labeled with the proliferation dye CFSE (eBioscience) and plated in complete media with 20 μg MOG35–55 at 1 × 10^5 cells/well in a 96-well plate. Either Cx3cr1 WT or KO CD11c+ cells were added to the T cells at an equal ratio, and then the cells were incubated at 37°C for 3 d. Cells were labeled with viability dye eFluor780 (eBioscience), and then blocked with CD16/CD32 (BD Pharmlingen) and labeled with a mixture of Abs for flow cytometric analysis using LSR II.

Cytokine ELISPOT assay
Mice were immunized with 100 μg MOG35–55 and sacrificed 11 d p.i. Spleen and lymph nodes were harvested and cells were plated at 1 × 10^6 per well with 10 μg/ml MOG35–55 in complete HL-1 serum-free media in a 96-well plate filter plate that was previously sensitized with purified capture anti-IL-17 (clone 17CK15A; eBioscience) or anti–IFN-γ (clone AN-18; eBioscience) and blocked with 1% PBS/1% BSA. After 24 h of incubation at 37°C/5% CO2, the cells were washed with 0.5% Tween 20 in PBS, then labeled with biotin anti-IL-17 detection Ab (clone EBio1B7B7) or biotin anti–IFN-γ detection Ab (clone R4-6A2), and incubated at 4°C overnight.

The cells were then incubated for 2 h at room temperature with streptavidin alkaline phosphatase (Invitrogen) diluted in PBS containing 1% BSA/0.5% Tween 20 and developed with 5-bromo-4-chloro-3-indoly phosphate/NBT phosphate substrate (KPL). Plates were read, and spots were counted using ImmunoSPOT software. Similarly, IFN-γ- and IL-17-producing cells were assayed in total brain leukocyte populations (isolated via Percoll gradients, as described above) from WT and Cx3cr1-KO mice at peak of EAE disease. Number of spots in nonstimulated control wells was subtracted from the stimulated well, and data are presented as spot-forming cells per total million of cells added in the assay.

Quantitative RT-PCR
Tissues were dissected from perfused mice at the peak of EAE disease. Cerebellum was carefully detached using a scalpel and stored separately from forebrain and spinal cord tissues. Total RNA was isolated using TRIzol reagent, according to the manufacturer’s instructions. Quantity was assessed with a Nanodrop 1000, and RNA quality was confirmed over 1% accuracy from forebrain and spinal cord tissues. Total RNA was isolated using TRIzol reagent, according to the manufacturer’s instructions. Quantity was assessed with a Nanodrop 1000, and RNA quality was confirmed over 1% transcribed using TagMan reverse-transcription reagents. Quantitative real-time PCR was performed with TaqMan Master mix and gene expression assay. Samples were analyzed on an Applied Biosystems 7900HT thermal cycler. All TaqMan reagents were from Applied Biosystems. Reactions were run in triplicates, and expression levels were normalized to β-actin.

Statistical analysis
Data are presented as mean ± SEM. Transcript data for IL-17 were analyzed using ANOVA. For all other experiments, differences between groups were analyzed using an unpaired t test with GraphPad Prism software (San Diego, CA). The p values are shown in the data, as follows: *p < 0.05, **p > 0.01, ***p < 0.01.

Results
Peripheral CX3CR1 deficiency confers more severe EAE
Two mouse models of CX3CR1 deficiency were reported in the earlier 2000s (24, 25). In mixed 50:50 hybrids of the C57BL/6 and 129/Sv strains, Cx3cr1−/− mice upon active immunization with MOG35–55 peptides revealed a slightly earlier disease onset (24, 26, 27). A different study using mutant mice engineered by disrupting the CX3CR1 locus by insertion of the GFP reporter protein showed that CX3CR1-deficient mice on the CD1dR+/− and CD1d−/− background developed EAE disease with earlier onset and correlated with a selective deficiency of NK cells in the CNS (28). Due to the expression of CX3CR1 by distinct monocyte/macrophage populations (18, 19), we sought to extend these studies and characterize the myeloid compartment in the CNS during EAE in the absence of CX3CR1 signaling. To begin to address this issue, active EAE was induced in Cx3cr1−/−/GFP (Cx3cr1−/−, KO) mice backcrossed to the C57BL/6J background for >14 generations and WT littermates via s.c. injection of 100 μg MOG35–55 peptide, and the mice were observed for EAE disease. All mice developed signs of EAE (Fig. 1A). However, CX3CR1-deficient mice showed EAE signs at an earlier time point (Fig. 1B). The peak of disease was also manifested earlier in Cx3cr1−/− mice (Fig. 1B), and the EAE signs were significantly more severe in the absence of CX3CR1, as shown by the comparison of maximum EAE scores between the groups (Fig. 1A, 1C). To delineate the contribution of peripheral versus CNS-resident cells to disease progression, we generated radiation bone marrow chimera models. Cx3cr1−/− bone marrow and active EAE was induced and monitored, as described earlier. Reconstitution of WT or Cx3cr1−/− recipient mice with Cx3cr1−/−−/− bone marrow (KO→WT and KO→KO) showed a severe and nonremitting form of EAE, with a high proportion of mice exhibiting ascending paralysis that was sustained up to 45–50 d p.i. (Fig. 1D, 1E). Peak EAE was comparable in WT→KO and KO→WT chimera mice (Fig. 1D); however, recipients of KO bone marrow failed to recover from EAE, whereas recipients of WT bone marrow (WT→KO) exhibited a progressive recovery, and motor function was regained in both hind limbs. Control bone marrow chimera mice (WT→WT and KO→KO; Fig. 1E) showed a similar EAE phenotype in which the genotype of the circulating bone marrow–derived cells correlates with neurologic disease, as observed in the mixed chimera mice (Fig. 1D). Overall, the results show that EAE severity observed in CX3CR1-deficient mice was due to absence of fractalkine signaling on peripheral bone marrow–derived cells.

Increased severity of CNS pathology in CX3CR1-deficient mice correlates with the increased accumulation of dendritic cells in the CNS
To investigate the mechanism underlying the increased severity of EAE in the absence of CX3CR1 signaling, we examined CNS inflammation at the time of peak disease in CX3CR1-KO mice also carrying the CCR2-RFP reporter protein. Heterozygous mice with normal receptor function (Cx3cr1+/GFP Ccr2+/RFP) and CX3CR1-deficient mice carrying the CCR2-RFP reporter (Cx3cr1−/−/GFP CCR2−/RFP) were investigated for the accumulation of monocyte subsets upon active EAE induction (Fig. 2A, 2B). Myeloid subsets in the CNS were distinguished by flow cytometry using Abs against CD45 to distinguish CD45high infiltrating leukocytes from CD45low microglial cells. CD45high cells were further analyzed for the monocyte marker CD115 expression and CD115+ cells for Ly6C and CD11c expression. The results show that, in heterozygous mice, >85% of CD115+ monocytelineage cells were Ly6C(high) (also CCR2(high) by virtue of RFP expression), indicating a predominant infiltration of this population to the CNS during EAE, demonstrating the importance of CCR2 for recruitment of this subset into the CNS. In contrast, accumulation of the Ly6Clow population was dramatically increased in the absence of CX3CR1 (Fig. 2B), and 60% of these cells also expressed the CD11c marker characteristic of dendritic cells. The population of Ly6C−CD11c+ cells expressed CX3CR1 as revealed by comparison of CD11c marker expression in the various myeloid populations. Ly6C(low) cells expressed higher...
levels of CX3CR1 than Ly6C<sup>high</sup> cells (Fig. 2C, *p* = 0.008 for comparison of CX3CR1-GFP expression between Ly6C<sup>CD11c</sup> and Ly6C<sup>−</sup>CD11c<sup>−</sup> cells; *p* = 0.016 for comparison of CCR2-RFP between Ly6C<sup>CD11c</sup> and Ly6C<sup>−</sup>CD11c<sup>−</sup> groups; *p* = 0.046 for comparison of CX3CR1-GFP and CCR2-RFP expression within Ly6C<sup>−</sup>CD11c<sup>−</sup> population; and *p* = 0.008 for comparison of CX3CR1-GFP and CCR2-RFP expression within Ly6C<sup>CD11c</sup> population). These results suggest that Ly6<sup>CD11c</sup>/CCR2<sup>+</sup> and Ly6C<sup>low</sup>/CX3CR1<sup>+</sup> subsets represent distinct populations with specialized functions within the CNS.

To establish a relationship between the infiltration of myeloid cells and CNS pathology, brains were sectioned and analyzed at the peak of EAE by immunohistochemistry. Tissues were stained with Abs against CD45, as a generalized marker of inflammation (Fig. 2D–F), MBP to assess demyelination (Fig. 2G–I), and calbindin as a marker for Purkinje cells in cerebellar regions (Fig. 2J–L). When compared with naive CX3CR1-KO tissues (Fig. 2D), infiltration of peripheral cells was evident in diseased WT mice (Fig. 1E) and CX3CR1-KO tissues. However, CD45 immunoreactivity was found most dramatic in forebrain and cerebellar white matter of CX3CR1-KO mice (Fig. 2F, Supplemental Figs. 1, 2). Myelin staining revealed a defined pattern in naive (Fig. 2G) and diseased WT mice (Fig. 2H), with myelin fibers darkly stained and axons easily visualized. In WT tissues affected by EAE, strong myelin immunoreactivity is observed in areas close to inflammatory cuffs (Fig. 2H). In contrast, CX3CR1-KO tissues revealed a decreased intensity in the myelin staining; axons appeared thinner and shorter, and less defined axons were visualized in the cerebellar region (Fig. 2I). Purkinje cells were detected along the granular cell layer of naive mice (Fig. 2J) and diseased WT mice (Fig. 2K). In contrast, in CX3CR1-KO mice, calbindin-positive neurons appeared with an altered morphology. Deteriorated/degenerating cells were evident by the presence of a disrupted lining of the granular cell layer, and ovoids at the ends of the axons were clearly visualized (Fig. 2L, Supplemental Fig. 1). Quantification of myelin immunoreactive area shows that naive CX3CR1-KO mice do not differ from naive WT mice (Fig. 2M). Similar results were found when assessing the number of calbindin-positive neurons (Fig. 2N). However, upon EAE induction, a reduction in both myelin (Fig. 2M, *p* = 0.03 between WT naive and WT-EAE groups, *p* = 0.0002 between KO naive and KO-EAE groups, and *p* < 0.0001 between WT-EAE and KO-EAE groups) and neuronal counts was detected with CX3CR1-KO mice revealing a more dramatic reduction when compared with diseased WT mice (Fig. 2N, *p* = 0.04 between WT-EAE and KO-EAE, and *p* = 0.021 between KO naive and KO-EAE groups). Therefore, the results indicate that an increased inflammatory reaction, dominated by a myeloid subset with a CD115<sup>+</sup>Ly6C<sup>low</sup>CD11c<sup>−</sup> phenotype, correlated with enhanced demyelination and neuronal damage in CX3CR1-KO mice.

**Differential cytokine expression in CX3CR1-KO mice is indicative of an increased proinflammatory environment in the CNS**

To further examine mechanisms by which this myeloid subset conferred CNS pathology, we sought to investigate the frequency of IFN-γ and IL-17 MOG<sub>35-55</sub>-specific producing T cells in the periphery. For this, we used lymph nodes and spleen tissues from actively immunized WT and CX3CR1-KO mice in ELISPOT assays performed at 11 and 60 d p.i. The results show that during disease initiation IFN-γ Ag-specific producing T cells were detected in higher frequency in lymph node tissues (Fig. 3A). IL-17-producing T cells were significantly abundant in both lymph nodes (Fig. 3A) and spleen tissues of CX3CR1-KO mice (Fig. 3B) when compared with WT mice at 11 d p.i. By day 60 p.i., the frequency of Ag-specific T cells producing IFN-γ or IL-17 was undetectable in lymph nodes (data not shown). However, in the spleen, both WT and KO mice revealed IFN-γ–producing cells in comparable frequencies. In addition, the frequency of IL-17–producing T cells was higher in CX3CR1-deficient mice when compared with WT mice, with a notable decrease at 60 d p.i. (Fig. 3B). The results suggest that CX3CR1-KO mice exhibited increased numbers of peripheral IL-17–secreting Ag-specific T cells. Due to the differential inflammatory response visualized...
by tissue immunohistochemistry, cytokine expression was also assessed in the CNS by quantitative RT-PCR and analyzed separately in forebrain, cerebellum, and spinal cord regions. Compared with WT mice, CX3CR1-KO mice exhibited increased mRNA transcript expression of IFN-\(\gamma\) in cerebellar and spinal cord tissues (Fig. 3C), and increase in IL-17 was revealed in forebrain and cerebellar regions (Fig. 3D, \(\ast p = 0.005\)). The anti-inflammatory cytokine IL-10 was detected at significantly higher levels in spinal cord tissues of WT mice (Supplemental Fig. 3A, \(\ast\ast p = 0.0008\)), whereas in all CNS tissues of CX3CR1-deficient mice, TNF-\(\alpha\) levels were higher when compared with diseased WT mice (Supplemental Fig. 3B). Cytokine ELISPOT assays (Fig. 3E) using total brain leukocyte populations at peak of EAE disease revealed a significant increase in the number of IL-17–producing cells from CX3CR1-KO mice and a higher ratio of IL-17/IFN-\(\gamma\) spot-forming cells when compared with WT mice (Fig. 3F, \(\ast p < 0.05\)). Overall, our results suggest an important role for CX3CR1/CX3CL1 in the regulation of APC effector function and in turn in the modulation of the development of a proinflammatory environment.
CX3CR1 and myeloid cells during autoimmune inflammation

Effect of CX3CR1 deficiency on APC effector function

To address the role of CX3CR1 on dendritic cells and their effects to modulate T cell function, we performed in vitro proliferation assays using MOG35-55-prime myeloid cells isolated from actively immunized WT mice at day 11 p.i. T cells were then mixed with WT or Cx3cr1−/− APCs (CD11b+CD11c+) isolated from spleens and lymph nodes of WT and KO recipients (Fig. 7). Donor CD4+ T cells were analyzed (Fig. 4B). Cultures containing WT dendritic cells (Fig. 4C, 4D) revealed less proliferating T cells when compared with cultures containing CX3CR1-KO peripheral dendritic cells (Fig. 4C, 4D). A similar result was obtained when using CNS-derived myeloid cells (Supplemental Fig. 4). To further address the role of CX3CR1 on adaptive immunity via modulation of myeloid cell function, we examined expression of class II histocompatibility Ags I-A^d and the costimulatory molecules CD80 and CD86 in spleen and lymph node myeloid cells (Fig. 5A, 5C) from actively immunized mice at 11 days p.i. and from brain leukocyte populations at peak of EAE disease (Fig. 5B, 5D). The proportions of myeloid subsets CD11b+Ly6C^hi CD11c^+ and CD11b+Ly6C^lo CD11c^+ in spleen and lymph nodes of actively immunized mice were comparable between WT and CX3CR1-KO mice. However, CX3CR1-KO cells exhibited increased surface expression of MHC-II and IL-17 when compared with WT mice (Fig. 6A, 6B). Cultures containing WT or Cx3cr1−/− T cells (Fig. 7A, 7B) or Cx3cr1−/− T cells (Fig. 7A, 7C, 7D) induced a similar EAE phenotype as shown by the maximum EAE score in the four experimental groups analyzed. From four different experiments analyzed, the incidence of EAE was higher in the KO recipients with ~80% of mice displaying neurologic signs. EAE manifestations were more severe in Cx3cr1−/− recipient regardless of the genotype of the T cells used for the passive transfer of EAE.

Discussion

The diversity and polarization capacity of the myeloid cells, including microglia, monocytes, macrophages, and dendritic cells, have introduced a challenge to understand the function of the myeloid lineage during conditions that involve microbial pathogens, autoimmune inflammation, or neurodegeneration (29). In general, the mononuclear phagocyte system plays an important role in development, scavenging, inflammation, and antimicrobial defenses. However, cell signaling pathways elicited in response to extracellular signals guide the development of distinct population of myeloid cells (30). Main subsets of blood monocytes that have been studied in mice can be distinguished on the basis of Ly6C/CX3CR1 expression. Inflammatory Ly6C^hi/CX3CR1^lo murine monocytes are believed to be recruited from blood to tissues following infection, where they undergo activation and their response appears to be pathogen dependent. In contrast, Ly6C^lo/CX3CR1^hi monocytes appear to populate normal tissues and exhibit long-range crawling over the endothelium of capillaries, small veins, and arteries, a process that may be involved in surveillance. Ly6C^− monocytes are suggested to be involved in tissue repair, but a role in regulating T cell function is not yet described. It has also been defined that a fraction of murine monocytes exhibits suppressor functions and can be found in the spleen or liver of mice and inhibits T cell proliferation. In humans, CD14^+ monocytes that consist of CD16^+ and CD16^− cells re-
semble Ly6C$^+$ murine monocytes. In contrast, murine patrolling Ly6C$^2$ monocytes are more similar to the human CD14$^{\text{low}}$ subset (13). Although the heterogeneity of monocytes is complex, current data support the notion that Ly6C$^+/\text{CCR2}^{\text{high}}/\text{CX3CR1}^{\text{low}}$ and Ly6C$^2/\text{CCR2}^{\text{low}}/\text{CX3CR1}^{\text{high}}$ commit to differentiate more readily into M1-like and M2-like inflammatory monocytes or dendritic cells, respectively (30).

The fractalkine receptor, CX3CR1, plays neuroprotective roles in selected CNS pathologies, including the CX3CR1-SODG93A transgenic mouse model of amyotrophic lateral sclerosis. Also, the absence of CX3CR1 led to increased levels of IL-1$\beta$ and enhanced neuronal damage after peripheral LPS administration in a model of low-level endotoxemia (2). In addition, CX3CR1-deficient mice exhibited an increased loss of tyrosine-hydroxylase–positive neurons in the substantia nigra pars compacta after acute 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) intoxication, which correlated with robust microglial activation. However, no effect on striatal dopaminergic contenta was observed when Cx3cr1$^{2/2}$ mice were treated with methamphetamine (31). Also during EAE, CX3CR1 appears to be protective (28) by affecting

**FIGURE 4.** Effect of CX3CR1 on APCs for T cell proliferation. MOG35–55-primed T cells were enriched from WT mice 11 d p.i., labeled with CFSE, and incubated with CD11b$^+$ CD11c$^+$ dendritic cells isolated from WT or Cx3cr1$^{2/2}$ mice in presence of 20 $\mu$g/ml MOG35–55 Ag. Proliferation was assessed by flow cytometry on live (A) and CD4$^+$ T cells (B). A representative experiment is shown using primed WT T cells cultured with WT (C, blue line) or Cx3cr1$^{2/2}$ (C, red line) dendritic cells and compared in overlapping histograms (C); faint gray line represents the positive control. Data are presented as percentage of WT T cell proliferation in three to five different experiments in the presence of WT (D, open bar) or CX3CR1-KO dendritic cells (D, gray bar), *$p < 0.05$.

**FIGURE 5.** Effect of CX3CR1 deficiency on APC-related molecules. Lymph nodes and brain mononuclear cells were isolated at 11 d p.i. and at peak of EAE disease, respectively, and analyzed for the myeloid subsets CD45, CD11b, Ly6C, and CD11c by flow cytometry. Mean fluorescence intensity for MHC-II in lymph node (A) and brain leukocytes (B) and CD80 and CD86 (C, D) was compared between WT (open bars) and KO mice (gray bars). Each dot represents data from pooled inguinal lymph nodes from one mouse and from one brain at peak EAE disease. *$p < 0.05$.
CX3CR1 and Myeloid Cells During Autoimmune Inflammation

Clinical and experimental studies indicate that CX3CR1 plays a role in the development of autoimmune diseases, such as multiple sclerosis (MS), rheumatoid arthritis, and experimental autoimmune encephalomyelitis (EAE). CX3CR1 is expressed on several cell types, including monocytes, dendritic cells, and microglial cells. The function of CX3CR1 in these cells is not fully understood, but it is thought to be involved in the regulation of inflammation and immune responses.

In a recent study, the authors investigated the role of CX3CR1 in myeloid cell trafficking and function in mouse models of MS. They found that CX3CR1-deficient myeloid cells proliferate more readily in the CNS, which suggests that CX3CR1 is important for the regulation of myeloid cell accumulation and function in the CNS.

Moreover, the authors observed that CX3CR1-deficient myeloid cells exhibit increased expression of markers of inflammation, such as CD11b and Ly6C. This population is associated with smaller infarct size and enhanced recovery in a model of focal cerebral ischemia, suggesting that CX3CR1-deficient myeloid cells may play a protective role in this setting.

Additionally, the authors found that absence of CX3CR1 correlates with enhanced brain inflammation and more severe EAE neurologic signs. Reconstitution of WT recipients with CX3CR1-deficient bone marrow revealed that peripheral CX3CR1 also myeloid cell lineage and potentially microglial cells, and additional human studies should be directed to identify possible associations of these polymorphic variants with CNS inflammation.

Overall, the findings of this study are of great relevance due to the implication of the human polymorphic variants (V249I and T280M) in altering fractalkine-receptor binding affinity (12) as well as expression level of CX3CR1 (9). The association of the variant receptor with chronic inflammatory diseases, including Crohn’s disease (36), atherosclerosis, and coronary artery disease (10, 12, 37), has been demonstrated. Importantly, protective roles in atherosclerosis and acute coronary events have been suggested (38, 39), contrasting an association to increased susceptibility to age-reacted macular degeneration. The decreased binding of the variant receptors to the ligand CX3CL1 on peripheral endothelial cells may explain the protective effects in settings of peripheral inflammation. However, little is known about the association of human CX3CR1 variants in CNS conditions. From our data in experimental mice with defective CX3CR1 signaling, we argue that abnormal CX3CR1–CX3CL1 interactions will be deleterious due to increased influx of highly activated myeloid populations that might potentially enhance and sustain T cell responses within the CNS. A genetic analysis of MS patients revealed significantly lower frequency of the CX3CR1I249/T280 haplotype in secondary-progressive patients when compared with relapsing–remitting patients (40). Therefore, there is a possible protective effect of the reference I249 allele on secondary-progressive MS when linked with T280. Findings in MS patients added to our results show that CX3CR1 deficiency alters not only the NK cell compartment, but also myeloid cell lineage and potentially microglial cells, and additional human studies should be directed to identify possible associations of these polymorphic variants with CNS inflammation.

The results presented showed that absence of CX3CR1 correlated with enhanced brain inflammation and more severe EAE neurologic signs. Reconstitution of WT recipients with CX3CR1-deficient bone marrow revealed that peripheral CX3CR1 has a profound effect in CNS inflammation, as a more severe neurologic disease developed in these mice. The pathology in the CX3CR1-deficient mice showed that the inflammatory reaction correlated with more demyelination and neuronal damage. To further investigate mechanisms that lead to differences in myelin density between WT and CX3CR1-deficient mice, we compared Olig2 and ciliary neurotrophic factor (CNTF) expression by immunohistochemistry in brain sections from WT and KO mice at peak disease (our unpublished data). Olig2+ cells appear increased in cerebellar areas of EAE-affected CX3CR1-KO mice when compared with WT mice. Expression of CNTF was abundant in cerebellar regions of naive and EAE WT mice, but was reduced in diseased KO mice. Therefore, we hypothesize that CX3CR1-KO mice exhibit a defect in demyelination. How the inflammatory reaction contributes to modulation of CNTF expression is still under investigation.
Comparison of myeloid cell subsets showed an enrichment of monocyte-derived (CD115+) Ly6C\(^+\)CD11c\(^+\) dendritic cells in the EAE brain. Although Ly6C\(^+\) monocytes migrate to CNS under steady-state conditions, they do not tend to home to sites of inflammation. However, our data showed that, in absence of CX3CR1 signaling, this particular Ly6C\(^+\)CD11c\(^+\) subset more readily infiltrates the inflamed brain, where it undergoes proliferation and may play an important role in sustaining encephalitogenic T cell responses. We focused our studies on investigating the role of CX3CR1 for APC function, including modulation of costimulatory molecules and MHC-II presentation, and found that CX3CR1-deficient APCs showed a higher expression of molecules involved in T cell costimulation. In humans, immunosenescence has been associated with the peripheral expansion of a subgroup of patients, but their exact contribution of CX3CR1 in particular tissues under the pressures of the surrounding environment is yet to be determined.

Our data support the notion that CX3CR1 plays neuroprotective roles during EAE and suggest that peripheral CX3CR1 expression restricts trafficking of Ly6C\(^{low}\)CX3CR1\(^{high}\) cells to the CNS most likely by interaction with the peripherally expressed ligand. As we advance our knowledge in the understanding of myeloid cell subtypes, it is important to clarify whether they arise from a common bone marrow precursor or whether they differentiate within particular tissues under the pressures of the surrounding environment to exert specialized functions. CX3CR1, as an inflammatory and regulatory receptor, poses an intriguing biology, and its role in the human population under various neuroinflammatory conditions is yet to be determined.

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Disclosures
The authors have no financial conflicts of interest.

References


Supplementary Figure 1. **CX3CR1-deficient mice exhibit more severe cerebellar pathology.** Brain tissues were stained with anti-CD45 antibodies (brown staining) as a marker of global inflammation and counterstained with Nissl (A-C), anti-MBP antibodies to assess demyelination (D-F) and anti-calbindin antibodies to visualize cerebellar Purkinje cells (G-L) in Cx3cr1<sup>−/−</sup> naïve (A, D, G and J), diseased WT (B, E, H and L) and Cx3cr1<sup>−/−</sup> (C, F, I, and L) mice.

Supplementary Figure 2. **Severe spinal cord pathology in CX3CR1-deficient mice.** CD45 staining shows accumulation of inflammatory cells in spinal cord tissues from WT (A). Lesions in CX3CR1-KO as previously reported revealed increased infiltration of hematogenous cells (B), which correlates with enhanced demyelination (C and D). CD45 immunoreactive cells developed with DAB (Brown).

Supplementary Figure 3. **Cytokine expression in CNS tissues.** Cytokine expression was measured at peak of EAE disease in forebrain (FB), spinal cord (SC) and cerebellar (Cer) tissues of WT (open bars) and Cx3cr1<sup>−/−</sup> mice (shaded bars) using qRT-PCR in TaqMan assays for IL-10 (A), and TNF-α (B). Dots on graph represent sample from an individual mouse. * P < 0.05, ** P < 0.01.

Supplementary Figure 4. **Effect of CX3CR1 on CNS myeloid cells for T cell proliferation.** MOG<sub>35-55</sub> primed T cells were enriched from WT mice 11 days p.i., labeled with CFSE and incubated with CNS-derived CD11b<sup>+</sup> isolated from passively immunized mice at 45 days p.i. Myeloid cells were isolated from WT (A) or KO (B) CNS tissues and also compared in overlapping histograms (C).
Supplementary Figure 3.