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Production of CCL2 by Central Nervous System Cells Regulates Development of Murine Experimental Autoimmune Encephalomyelitis through the Recruitment of TNF- and iNOS-Expressing Macrophages and Myeloid Dendritic Cells

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Experimental autoimmune encephalomyelitis (EAE) is a T cell-mediated demyelinating disease of the CNS that serves, arguably, as a model for the human disease multiple sclerosis. Disease pathogenesis is thought to result from the activation of autoreactive CD4 (3, 4) or CD8 (5) IL-17-producing (6) T cells in the periphery, the migration of these activated T cells (7) through CD49d- (8, 9) and β-integrin-mediated (10, 11) mechanisms into the CNS, and their reactivation by CNS myeloid dendritic cells (DC) (12). In addition to the requirement for autoreactive T cells, macrophages have been shown to be necessary for EAE development (13–15). Infiltrating T cells are thought to both produce and induce expression of TNF from glial sources (16). Responsiveness to TNF through glial cell-expressed TNF receptor I (17) as well as responsiveness to IFN-γ (18) has been shown to regulate the expression of critical chemokines such as CCL2, which have been hypothesized to direct the accumulation of inflammatory cells in the CNS in a spatial and temporal fashion (19).

Chemokines are small molecular mass proteins that are produced by a wide variety of cell types and induce cell migration through the stimulation of G protein-coupled receptors (20, 21). Many chemokines have been shown to be expressed in the CNS of mice induced to develop EAE (22, 23). Of the many chemokines shown to be expressed in the CNS of animals with EAE, deletion or neutralization of only CCL2 (24–26), CCL3 (26, 27), CCL20 (28), CXCL10 (29), or CXCL13 (30) has been shown to decrease disease severity, suggesting a level of nonredundancy. In comparison to the chemokines shown to functionally regulate EAE pathogenesis, CCL2 (31), CCL3 (32), and CXCL10 (33) have been suggested to be involved in multiple sclerosis.

It is well accepted that chemokines play an important role in the pathogenesis of EAE. In order for activated T cells to adhere to endothelium and subsequently to extravasate, it has been postulated that chemokines are secreted by or across endothelial cells to activate integrin adhesiveness (34). Most chemokine up-regulation has been shown to occur after the onset of inflammation (35). One of the major chemokines shown to be involved in EAE is CCL2 (24), and deletion of this chemokine (25) or its receptor CCR2 (36, 37) has been shown to reduce CNS inflammation and subsequent EAE, prompting development of pharmacologic agents directed against CCR2 for use in inflammatory diseases (38). Despite this information, it remains unknown as to whether endogenous CNS cells (microglial and/or astrocytes) or infiltrating inflammatory cells (T cells and/or macrophages) are required to express CCL2 in order for mice to manifest severe clinical EAE. We addressed this question by creating radiation bone marrow chimeric mice in which the CNS glia or the peripheral leukocytes were deficient for CCL2 expression. Furthermore, we asked what population of CNS-infiltrating cells that either glial or peripheral leukocyte expression of CCL2 regulated to develop severe EAE. We found that optimal EAE developed as a result of glial expression of CCL2 resulting in the CNS accumulation of myeloid DC and macrophages that express TNF and iNOS that lead to demyelination.
Materials and Methods

Mice
Female C57BL/6 mice (H-2b; CD45.2) were purchased from Harlan Sprague Dawley; CCL2−/− mice (H-2b; CD45.2) (39) were provided by Dr. Barrett Rollins (Dana-Farber Cancer Institute, Boston, MA); and C57BL/6 CD45.1 (H-2b; CD45.1) mice were purchased from The Jackson Laboratory. All mouse strains were bred and maintained in the Center for Comparative Medicine at Northwestern University according to institutional and Public Health Service policies.

Antibodies
Fluorochrome-conjugated mAbs to murine CD45.1 (A20), CD45.2 (clone 104), CD3 (clone 500A2), CD4 (clone RM4-5), CD11b (clone M1/70), CD11c (clone HL3), Gr-1 (clone RB6-8C5); INOS (clone 6), TNF (clone MP6-XT22), CD16/32 (clone 2.4G2), and isotype control Abs were purchased from BD Biosciences and used in flow cytometric applications. Purified anti-IFN-γ (clone R4-6A2), anti-IL-17 (clone TC11-18H10.1), and anti-CCL2 (clone 2H5) as well as biotinylated anti-IFN-γ (clone XMG1.2), biotinylated anti-IL-17 (TC11.8H4.1), and biotinylated anti-CCL2 (clone 4E2/MCP) were also purchased from BD Biosciences and used for ELISA, and the mice were healthy at the start of each experiment.

Generation of radiation bone marrow chimera mice
Recipient mice were lethally irradiated with 5 Gy from a 137Cs source in two doses separated by a 4-h time period. Bone marrow cells were collected from the femurs of donor mice, washed in HBSS, and counted. The hematopoietic systems of irradiated recipient mice were reconstituted by i.v. injection of 5 × 10^6 donor bone marrow cells in 0.2 ml HBSS. Irradiated recipient mice were maintained for 5 wk in specific pathogen-free conditions, fed autoclaved mouse chow, and given water containing the antibiotic Baytril (enrofloxacin). The chimeric status of each irradiated recipient mouse was screened by flow cytometric analysis of peripheral blood leukocyte expression of either CD45.1 or CD45.2. All peripheral leukocyte compartments (T, B, and myeloid) were normally reconstituted and the mice were healthy at the start of each experiment.

Flow cytometry
For cell surface staining, cells (0.5–1 × 10^6) were incubated with anti-mouse FcγRII (CD16/CD32) for 15 min at 4°C to block Fc-mediated binding. Cells were washed in PBS containing 1% BSA and 0.05% sodium azide (flow buffer (FB)), followed by incubation with murine-specific Abs at predetermined optimal concentrations for 15 min at 4°C. Cells were then washed and resuspended in 0.5 ml FB. For intracellular staining, 1 × 10^6 cells were stimulated with 20 ng/ml PMA and 1 μg/ml ionomycin in the presence of 10 μg/ml brefeldin A (Sigma-Aldrich) for 3 h. Following culture, the cells were washed in HBSS and surface stained as described above for CD4, CD11b, CD11c, CD45.1, CD45.2, and Gr-1. Cells were washed in HBSS and then fixed using 0.5% paraformaldehyde and permeabilized with 0.5% saponin in FB and incubated with anti-TNF and anti-iNOS for 30 min. Following staining, the cells were washed in FB and data collection was performed on an LSR II flow cytometer (BD Biosciences) using Diva software. Offline analysis of the data was performed using FCS Express software.

Induction of EAE
EAE was induced in mice by immunization with 0.1 ml of an emulsion containing 50 μg myelin oligodendrocyte glycoprotein (MOG35-55) peptide (MEVGWYRSPFSRVHLYRNGK; >98% purity; Peptides International) and 400 μg Mycobacterium tuberculosis strain H37Ra (VWR). Clinical disease was scored as follows: 0, asymptomatic; 1, distal tail atonia; 2, partial hindlimb weakness accompanied by tail atonia; 3, partial hindlimb paralysis accompanied by tail atonia; 4, complete hindlimb paralysis and tail atonia.

Cytokine/chemokine analysis
The presence of CCL2 in tissue samples was performed as previously described (24). Briefly, CNS tissue was homogenized in 1 ml PBS containing 0.05% Tween 20 and then clarified by centrifugation (400 x g) for 10 min. The relative amounts of CCL2 protein in the extracts were quantified by a sandwich ELISA kit (BD Biosciences) according to the manufacturer’s instructions. CCL2 levels were calculated by comparison to standard curves generated from recombinant CCL2. The detection limit of this assay was determined to be 125 pg/ml. Cell-specific production of IFN-γ and IL-17 was determined using ELISPOT assay. Microplates (Whatman) were coated overnight with the capture Abs (purified anti-IFN-γ at 4 μg/ml or IL-17 at 2 μg/ml) in sterile PBS. Following washing with PBS, the plates were blocked for 1 h with PBS containing 1% BSA and washed again with PBS. Cells isolated from experimental mice were plated at a concentration of 1 × 10^5/ml in DMEM containing 5% FCS (HyClone), 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 0.1 M nonessential amino acids, and 5 × 10^–3 M 2-ME (all of the latter from Invitrogen) in the presence of 0.5, 0.5, or 50 μM MOG35-55 and incubated at 37°C and 5% CO2 for 24 h. After incubation, plates were washed with PBS and biotinylated detection Abs (2 μg/ml) were added to the wells overnight at 4°C. Cytokine-producing cells (spots) were visualized by addition of anti-biotin-alkaline phosphatase Abs (DakoCytomation) for 15 min followed by addition of NBT/5-bromo-4-chloro-3-indolyl phosphate substrate (Bio-Rad). Plates were dried overnight and images of the ELISPOT wells were captured with an ImmunoSpot Series 3B Analyzer (Cellular Technology). Image analysis of the ELISPOT results was performed using the ImmunoSpot 3.2 Analysis software (Cellular Technology).

Isolation of CNS mononuclear cells
CNS-infiltrating mononuclear cells were isolated from individual mice by digestion of the isolated spinal cord tissue homogenate with type 3 collagenase (Worthington Biochemical) followed by density centrifugation as previously described (40).

Immunohistochemistry and histology
Mice were anesthetized with sodium pentobarbital (Abbott Laboratories) and perfused intracardially through the left ventricle with ice-cold PBS. Tissues were embedded in OCT before cryostat sectioning. Immunohistochemical staining was performed on 8–10-μm cryostat sections. Frozen sections were blocked with 5% normal serum (depending upon the species of origin for the secondary antibodies) in PBS for 30 min at room temperature and incubated with primary mAbs (anti-CCL2) for 2 h at room temperature. Sections were treated with 3% H2O2 to quench endogenous peroxidase activity and then incubated with either secondary Abs that were directly conjugated to HRP or an avidin-HRP complex (Vectastain ABC kit, Vector Laboratories). Biotin-avidin binding was detected by the use of diaminobenzidine (Sigma-Aldrich). Demyelination was evaluated by Luxol fast blue as previously described (41). Representative photomicrographs were taken using a Nikon E800 microscope (Fryer Company) equipped with a SPOT digital camera (Diagnostic Instruments). Images were created using Metamorph Meta Imaging Series 4.5 software (Universal Imaging).

Statistical analysis
Comparisons of disease incidence were analyzed by the χ2 analysis, using Fisher’s exact probability test. Statistical significance of cytokine levels, disease onset, and disease severity was analyzed using Student’s t test for comparisons of two means. Values of p ≤ 0.05 were considered significantly different.

Results
Generation of radiation bone marrow chimera mice
It has been previously reported that a genetic deletion of CCL2 resulted in significantly attenuated clinical symptoms of EAE, presumably because of decreased macrophage trafficking to the CNS (25). In this earlier work it was unknown whether the endogenous CNS cells or whether CNS-infiltrating leukocytes were the critical cellular source of CCL2 that, in turn, regulated disease development. We decided to ask the questions of what cellular chemokine production was important for disease development and how did that production influence subsets of CNS-accumulating leukocytes. To answer these questions we created radiation bone marrow chimera mice because glial cells in the CNS (astrocytes and microglia) do not turn over with the same kinetics as do cells in blood and peripheral lymphoid tissue after irradiation, thus allowing for differential reconstitution of peripheral (blood and lymphoid tissues) and CNS compartments with genetically modified bone marrow-derived cells (42). Three groups of mice were made: group 1, wild-type donor CD45.1+ bone marrow transferred into lethally irradiated wild-type CD45.2+ recipient mice (referred to as the wild type) that served as an irradiation control whose peripheral leukocytes were CD45.1+ with both peripheral leukocytes...
CCL2 REGULATION OF MACROPHAGES AND mDC IN EAE

FIGURE 1. Radiation bone marrow chimera construction. Three experimental groups were created: 1) lethally irradiated CD45.2 wild-type C57BL/6 mice reconstituted with CD45.1 wild-type C57BL/6 bone marrow (designated as wild type); 2) lethally irradiated CD45.1 wild-type mice reconstituted with CD45.2 wild-type C57BL/6 bone marrow (designated as leukemia CCL2−/−); and 3) lethally irradiated CD45.2 CCL2−/− C57BL/6 mice reconstituted with CD45.1 wild-type C57BL/6 bone marrow (designated as CNS CCL2−/−). At 5 wk postreconstitution, peripheral blood from the recipient mice was phenotyped by flow cytometry for the presence of donor CD45 expression. Chimerism refers to the percentage of circulating donor leukocytes. The chimeric construction shown is representative of six independent experiments.

and CNS glia capable of expressing CCL2; group 2, wild-type donor CD45.1+ bone marrow transferred into lethally irradiated CD45.2−/−CCL2-deficient recipient mice (referred to as CNS CCL2-deficient) whose CNS cells, including astrocytes and microglia, were deficient in CCL2 expression; and group 3, CCL2-deficient donor CD45.2+ bone marrow transferred into lethally irradiated wild-type CD45.1+ recipient mice (referred to as leukemia CCL2-deficient) whose peripheral leukocytes were CCL2-deficient. The results shown in Fig. 1 demonstrate a high level of chimerism attained by the bone marrow reconstitution procedure. In this representative analysis, wild-type irradiation control mice had 87% of their leukocytes derived from donor bone marrow at the time the experiment was begun. CNS CCL2-deficient mice had 90% of their leukocytes derived from donor bone marrow. Leukocyte CCL2-deficient mice had 91% of the leukocytes derived from donor bone marrow. In subsequent replicate experiments (six in total), wild-type chimerism ranged from 87 to 98%, leukocyte CCL2-deficient chimerism ranged from 91 to 92%, and CNS CCL2-deficient chimerism ranged from 90 to 95%.

CCL2 production by resident CNS cells is required for optimal clinical disease development

Once the irradiation bone marrow chimeraic mice were screened and found to have an acceptable level of hematopoietic reconstitution, they were immunized with MOG35-55 in CFA and given pertussis toxin for development of EAE. The experimental groups constructed in Fig. 1 were immunized with MOG35-55 in CFA and observed for the development of clinical disease symptoms. A, Demonstrates a delay in clinical disease onset as well as a decrease in clinical disease severity in the CNS CCL2−/− chimeric group compared with the wild-type and leukemia CCL2−/− chimeric groups (p < 0.05, days 12–21 after disease induction; n = 7 for each group). B, Shows a significant decrease (*, p < 0.05) in cumulative clinical disease score for all the animals in the CNS CCL2−/− chimeric group compared with the other two groups over the duration of the experiment shown in A. The gray boxes indicate the range of cumulative scores, the line in the gray box denotes the mean, and the error bars denote the SD of the mean. The experiment in A and B is representative of four repeats with identical results.

clinical disease. We examined CCL2 expression levels in the CNS following chimera construction and disease induction in an effort to understand to what degree CCL2 expression was affected. The results shown in Fig. 3A indicate that the CNS CCL2-deficient chimeric mice produced significantly less CCL2. We performed an in situ immunohistochemical analysis of CNS tissue for the expression of CCL2 in an effort to understand whether there was a compensatory level of chemokine expression in a particular bone marrow chimeric group. The results shown in Fig. 3B demonstrate extensive CCL2 immunostaining in the CNS of wild-type control mice. Specifically, both glia (stellate and ramified appearing cells) as well as leukocytes (round cells) showed very prominent CCL2 expression, consistent with the ELISA data. The CNS CCL2-deficient chimeric group showed that only the round leukocytes expressed CCL2 while the leukocyte CCL2-deficient chimeric group showed that only the stellate and ramified, but not the round leukocytes, expressed CCL2. Furthermore, analysis of positive staining cells using MetaMorph software indicated that the wild-type and leukocyte CCL2-deficient groups had considerable
The wild-type control group, spinal cords were harvested from 5 to 6 mice and pooled for flow cytometric analysis of leukocyte subpopulation expression of iNOS and TNF. The results shown in Fig. 5 indicate that the CNS-infiltrating leukocytes in wild-type control mice (left column) and leukocyte CCL2-deficient mice (right column) that developed severe EAE consisted of large macrophage

FIGURE 3. Decreased CCL2 in the spinal cords of the CNS CCL2<sup>−/−</sup> chimeric mice. The experimental groups constructed as shown in Fig. 1 were immunized with MOG<sub>35–55</sub> in CFA and observed for the development of clinical disease symptoms. At the peak signs of clinical disease in the wild-type control group, spinal cord tissue was harvested and assessed for CCL2 content by ELISA (A) as previously described (24). The data indicate a significant (*, p < 0.05) decrease in spinal cord CCL2 content compared with both the wild-type control and the leukocyte CCL2<sup>−/−</sup> groups. B, Spinal cords from wild-type, CNS CCL2<sup>−/−</sup>, and leukocyte CCL2<sup>−/−</sup> chimeric groups were stained with anti-CCL2 mAb. The wild-type section showed intense CCL2 expression; the CNS CCL2<sup>−/−</sup> section showed CCL2 expression by the round leukocytes (arrows); and the leukocyte CCL2<sup>−/−</sup> section showed CCL2 expression by the stellate and ramified cells representative of glia (arrows), but not the round (blue) leukocytes. Magnification, ×200. The results are representative of three independent experimental replicates.

CNS CCL2 deficiency results in less accumulation of macrophages and myeloid DC (mDC)

It was clear from the clinical data that CNS glial expression of CCL2 was required to elicit maximum disease. Therefore we asked what cell populations were influenced to migrate to and/or accumulate in the CNS. Three chimeric mouse groups were made: 1) wild-type control, 2) leukocyte CCL2-deficient, and 3) CNS CCL2-deficient. EAE was induced by immunization with MOG<sub>35–55</sub> in CFA. At the peak of clinical disease presentation in the wild-type control group, spinal cords were harvested and analyzed for the presence of macrophages (CD45<sup>high</sup>CD11b<sup>+</sup>CD11c<sup>+</sup>), microglia (CD45<sup>low</sup>CD11b<sup>+</sup>), lymphocytes (CD45<sup>high</sup>CD11b<sup>+</sup>), and mDC (CD45<sup>high</sup>CD11b<sup>+</sup>CD11c<sup>+</sup>). The results in Fig. 4A demonstrate similar CNS percentages of macrophages, microglia, lymphocytes, and mDC in the wild-type and leukocyte CCL2-deficient mice. In contrast, the CNS CCL2-deficient groups showed decreases in the CNS percentages of both the macrophage and mDC subpopulations. Because this was a representative analysis of the proportion of leukocyte subpopulations in the CNS, we decided to quantify these results by enumerating the actual numbers of macrophages, microglia, lymphocytes, and mDC in the CNS of 5 individual mice per group. The results in Fig. 4B demonstrate a significant (*, p < 0.05) reduction in the number of macrophages (Mp) and mDC in the CNS of CNS CCL2-deficient mice compared with both the wild-type and leukocyte CCL2-deficient groups. These results suggested that CNS glial CCL2 production functions as a regulator of selective CNS macrophage and mDC accumulation.

CNS CCL2-deficient mice have less spinal cord infiltration by iNOS- and TNF-expressing macrophages and mDC

To determine the mechanism of CNS glia-derived CCL2-regulated disease development, we investigated inflammatory mediator production by macrophages and mDC. Chimeric mice were constructed and EAE induced. At the peak signs of clinical disease in the wild-type control group, spinal cords were harvested from 5

FIGURE 4. Decreased macrophage and mDC accumulation in spinal cords of CNS CCL2<sup>−/−</sup> chimeric mice. The experimental groups constructed in Fig. 1 were immunized with MOG<sub>35–55</sub> in CFA and observed for the development of clinical disease symptoms. At the peak signs of clinical disease in the wild-type control group, spinal cords were harvested from five individual mice in each group and the infiltrating leukocytes were analyzed by flow cytometry. The cells were stained with mAbs specific for CD45, CD11b, CD11c, CD3, and B220. Leukocytes were selected by CD45 vs side light scatter gating. A, Relative percentages of macrophages (CD45<sup>high</sup>CD11b<sup>+</sup>CD11c<sup>+</sup>), microglia (CD45<sup>low</sup>CD11b<sup>+</sup>), lymphocytes (CD45<sup>high</sup>CD11b<sup>+</sup>), and mDC (CD45<sup>high</sup>CD11b<sup>+</sup>CD11c<sup>+</sup>). The results for the wild-type and leukocyte CCL2<sup>−/−</sup> groups. However, the CNS CCL2<sup>−/−</sup> group showed a lower percentage of macrophages and mDC. B, Quantification of the flow cytometry analysis performed on individual mice. These data demonstrate a significant (*, p < 0.05) decrease in the numbers of macrophages (Mp) and mDC in the CNS of the CNS CCL2<sup>−/−</sup> chimeric group when compared with the wild-type and leukocyte CCL2<sup>−/−</sup> groups. The data are representative of two identical experimental replicates.
and mDC populations that expressed intracellular iNOS and TNF. In contrast, the spinal cords of CNS CCL2-deficient mice contained fewer macrophages and mDC expressing iNOS and TNF (middle column). The numbers of iNOS- and TNF-expressing mDC and macrophages were quantified from each individual mouse in the three chimeric groups shown in Fig. 5. The results in Fig. 6 demonstrate a significant ($p < 0.05$) decrease in macrophage- and mDC-mediated iNOS and TNF expression in the CNS CCL2-deficient chimeric group compared with both the wild-type and leukocyte CCL2-deficient groups. Collectively, these data suggest that CNS glial expression of CCL2 functions to attract and/or retain myeloid cells (macrophages and mDC) in the CNS expressing the effector molecules iNOS and TNF.

Effect of compartment-specific CCL2 expression on peripheral autoimmune responses

In an effort to understand whether limiting CCL2 expression to either the peripheral leukocyte or the CNS glial compartments influenced the Ag-specific autoimmune response, we evaluated the MOG$_{35-55}$-specific recall effector T cell response from each of the three radiation bone marrow chimera groups. Wild-type, leukocyte CCL2-deficient, and CNS CCL2-deficient bone marrow chimeric mice were immunized with MOG$_{35-55}$ in CFA. At the peak signs of clinical disease in the wild-type control group, CNS and splenic lymphocytes were harvested and assessed for the ability to produce IFN-$\gamma$ or IL-17 by ELISPOT assay. The results shown in Fig. 7 indicate that CNS lymphocytes from wild-type control, leukocyte CCL2-deficient, and CNS CCL2-deficient mice had equal frequencies of both IFN-$\gamma$- (Fig. 7A) and IL-17-producing (Fig. 7B) T cells. Moreover, splenic lymphocytes from wild-type control, leukocyte CCL2-deficient, and CNS CCL2-deficient mice had equal frequencies of both IFN-$\gamma$- (Fig. 7C) and IL-17-producing (Fig. 7D) T cells. Furthermore, there was no difference between the three bone marrow chimeric groups in the number of CNS or peripheral FoxP3$^+$ cells. These results suggested that compartment-specific CCL2 production did not alter EAE development through changes in either effector autoimmune (peripheral or CNS) or regulatory T cell responses.
FIGURE 8 demonstrate that the spinal cord from wild-type control mice were cut and stained with Luxol fast blue. The results shown in medium at the termination of the experiment, and cryostat sections demyelination as determined by Luxol fast blue staining (41). Ransohoff and colleagues, who found that CCL2 deletion resulted in dramatically decreased clinical disease (25). Furthermore, CCL2 has been shown to be a critical pathogenic mechanism of CNS autoimmune disease development by using a radiation bone marrow chimeric mouse approach. Because the turnover rate of cells in the CNS is much lower following radiation than that of peripheral leukocytes, CNS cells retain the genotype of the recipient animal despite bone marrow reconstitution with genetically altered hematopoietic cells. This includes the CNS microglial population that eventually will turn over and be replaced by donor bone marrow monocytes. Use of the reconstituted mice within 5 wk of chimeric construction ensured adequate levels of chimerism (Fig. 1). In other words, the peripheral leukocytes were derived from donor bone marrow while the CNS microglia was of host/recipient origin. This type of bone marrow chimeric approach has been useful to probe the roles of IL-23 (43), iNOS (44), TNF (45), and TNFR (17) expression in the pathogenesis of EAE and allowed us to compartmentalize CCL2 gene deficiency. We created three radiation bone marrow chimeric groups: mice that served as wild-type irradiation controls (wild type), mice whose leukocytes (including lymphocytes and macrophages) were deficient for CCL2 (leukocyte CCL2/−/−), and mice whose glia (astrocytes and microglia) were deficient for CCL2 (CNS CCL2/−/−). Our major conclusion was that CNS glial CCL2 expression recruited iNOS- and TNF-producing macrophages and mDC to the CNS, which was necessary for optimum disease development.

The functional significance of CCL2 in the development and progression of chronic-progressive EAE induced by MOG35–55-specific T cell activation was originally shown by Ransohoff and colleagues, who found that CCL2 deletion resulted in dramatically decreased clinical disease (25). Furthermore, CCL2 has been shown to regulate the relapsing phase of EAE induced by proteolipid protein 139–151-specific T cell activation by reducing CNS accumulation of macrophages (24). The question of which cells, CNS-infiltrating macrophages or CNS glia, are needed to produce CCL2 in order for EAE development was addressed in the present

FIGURE 7. Compartmentalization of CCL2 expression does not affect CNS or peripheral effector T cell responses. The experimental groups constructed in Fig. 1 were immunized with MOG35–55 in CFA and observed for the development of clinical disease symptoms. At the peak signs of clinical disease in the wild-type control, spinal cords and spleens were harvested from three individual mice in each group and Ag-specific Th1 and Th17 responses were determined by ELISPOT assay. A, the data indicate similar frequencies of Ag-specific, IFN-γ-producing T cells in the CNS from all three bone marrow chimeric groups. B, the data indicate similar frequencies of Ag-specific, IL-17-producing T cells in the CNS from all three bone marrow chimeric groups. Identical analyses were performed on individual spleens and no differences among the groups were noted for either IFN-γ (C) or IL-17 (D). The data are representative of two identical experimental replicates.

Effect of compartment-specific CCL2 expression on CNS demyelination

The data thus far indicated that lack of CCL2 expression by the glial compartment resulted in accumulation of fewer mDC and macrophages expressing iNOS and TNF in the CNS without an effect on CNS Ag-specific T cell responses. Therefore, we sought to determine whether the CNS CCL2/−/− chimeric group had less demyelination as determined by Luxol fast blue staining (41). Radiation bone marrow chimeric groups were constructed as described in Fig. 2. CNS tissue was snap frozen in OCT mounting medium at the termination of the experiment, and cryostat sections were cut and stained with Luxol fast blue. The results shown in Fig. 8 demonstrate that the spinal cord from wild-type control mice showed extensive demyelination (Fig. 8A, demyelination is demarcated by black line). Additionally, there are numerous inflammatory cells in the tissue. The spinal cord from the leukocyte CCL2/−/− chimeric group also showed extensive demyelination (Fig. 8B, demyelination is demarcated by black line). In contrast, the spinal cord from the CNS CCL2/−/− chimeric group showed little demyelination, if any (Fig. 8C). These data are consistent with the idea that the spinal cords from CNS CCL2/−/− mice have significantly fewer mDC and macrophages producing iNOS and TNF (cf. Figs. 4 and 6). Furthermore, the results suggest that CNS glial CCL2 expression is a regulator of disease through the ability to affect accumulation of end-stage effector cells involved in self tissue damage.

Discussion

In the present report we sought to test the hypothesis that CNS-derived CCL2 production was a critical pathogenic mechanism of CNS autoimmune disease development by using a radiation bone marrow chimeric mouse approach. Because the turnover rate of cells in the CNS is much lower following radiation than that of peripheral leukocytes, CNS cells retain the genotype of the recipient animal despite bone marrow reconstitution with genetically altered hematopoietic cells. This includes the CNS microglial population that eventually will turn over and be replaced by donor bone marrow monocytes. Use of the reconstituted mice within 5 wk of chimeric construction ensured adequate levels of chimerism (Fig. 1). In other words, the peripheral leukocytes were derived from donor bone marrow while the CNS microglia was of host/recipient origin. This type of bone marrow chimeric approach has been useful to probe the roles of IL-23 (43), iNOS (44), TNF (45), and TNFR (17) expression in the pathogenesis of EAE and allowed us to compartmentalize CCL2 gene deficiency. We created three radiation bone marrow chimeric groups: mice that served as wild-type irradiation controls (wild type), mice whose leukocytes (including lymphocytes and macrophages) were deficient for CCL2 (leukocyte CCL2/−/−), and mice whose glia (astrocytes and microglia) were deficient for CCL2 (CNS CCL2/−/−). Our major conclusion was that CNS glial CCL2 expression recruited iNOS- and TNF-producing macrophages and mDC to the CNS, which was necessary for optimum disease development.

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report. Additionally, the present approach was useful for determining a role for CCL2 in CNS mDC accumulation, which to this point has not been addressed. Using radiation bone marrow chimeric mice we found that when the glia could not produce CCL2, the severity of EAE was significantly diminished (Fig. 2, A and B). An evaluation of CNS tissue levels of CCL2 revealed that there was a significant decrease of CNS CCL2 in the CNS CCL2-deficient chimeric mice (Fig. 3A). CCL2 has been shown to be produced by astrocytes (46, 47) and microglia (47) in the CNS during the development of EAE. Endothelial cells (48, 49) and neurons (50) have also been shown to express CCL2. However, these cell types, like astrocytes and microglia, would be incapable of CCL2 expression in the CNS CCL2-deficient chimeric mouse. Therefore, without the ability of these diverse cell populations to express CCL2, there would be less CCL2 in the target tissue, thereby resulting in fewer inflammatory cells that could potentially be recruited and/or retained in the CNS, and ultimately decreased EAE would be seen in the CNS CCL2+/− chimeric group. The remaining CCL2 expression in the CNS CCL2−/− chimeric mice (Fig. 3) was most likely a result of activated macrophages, as CCL2 has been shown to be expressed by CNS-infiltrating macrophages (51). However, this level of CCL2 expression was most likely below threshold levels necessary to result in the induction of a sufficient inflammatory infiltrate and severe EAE. Our immunohistochemical analysis of CCL2 expression in situ also supported this idea (Fig. 3B). Therefore, we think that CNS glial chemokine production is an important pathogenic factor in the development of EAE.

Concomitant with a diminution of CCL2 expression and clinical EAE, we also observed a significantly decreased accumulation of macrophages and mDC in the CNS of the CNS CCL2−/− chimeric mouse group (Fig. 4). This was noted as both a decrease in the proportion of the CNS infiltrate (Fig. 4A) as well as the absolute number of macrophages and mDC in the CNS (Fig. 4B). It is well accepted that macrophages express CCR2 (52), and in the absence of this receptor mice have difficulty generating inflammatory reactions that require macrophages (53), clearing macrophage-dependent infections in a variety of tissue sites (54, 55), and developing EAE (36). These deficiencies in macrophage function are a result of the inability to respond to CCL2. Macrophages have been demonstrated to be important for EAE development (10, 13, 14), presumably producing inflammatory cytokines/molecules such as NO and TNF (56–59) and/or participating in demyelination (60). Moreover, our data demonstrated that there were fewer macrophages and mDC in the CNS expressing the effector molecules iNOS and TNF (Figs. 5 and 6), indicating that glial production of CCL2 is critical for the optimal accumulation of iNOS- and TNF-expressing macrophages that are thought to be the end-stage effector cells in the pathophysiology of EAE.

Similar to macrophages, DC have been shown to express CCR2, and thus to be responsive to CCL2 (61, 62). DC migration to the CNS has recently been shown to be an important functional mechanism of tissue-specific Ag presentation that has been postulated to drive the progression of EAE (12, 63). Therefore, it is reasonable to postulate that our observation of decreased EAE as well as decreased mDC accumulation in the CNS in the absence of glial production of CCL2 is related to less than optimal DC-mediated Ag presentation. However, the CNS Ag-specific T cell responses were identical in all three radiation bone marrow chimeric groups (Fig. 7). Alternatively, the decreased EAE seen in the CNS CCL2−/− chimeric mice could result from decreased CNS-infiltrating TNF- and iNOS-producing DC (Tip-DC). Tip-DC have been shown to be absent from the spleen in Listeria monocytogenes-infected CCR2−/− mice that have an inability to clear infection (64). Therefore, in our experiments it is more likely that the mechanism of reduced clinical EAE in the CNS CCL2−/− chimeric group is the collective reduction of TNF- and iNOS-expressing, CNS-infiltrating macrophages and mDC rather than alterations in the Ag-specific T cell responses.

Despite the inability of glia to produce CCL2 in the CNS CCL2−/− chimeric mice, these animals still develop some clinical EAE (Fig. 2), albeit significantly decreased, with an accompanying leukocyte infiltrate (Fig. 4). A number of additional chemokines such as CCL3, CCL4, CCL5, and CXCL10 have been detected in the CNS CCL2−/− chimeric mouse (data not shown), suggesting some redundancy in the chemokine system. Moreover, macrophages and mDC express CCR1, CCR5, CCR7, and CXCXR3 (21), making them capable of migrating in response to other chemokine ligands. Despite the redundancy of receptor and ligand expression, glial CCL2 production appears to be a major pathogenic factor necessary for severe CNS demyelinating disease induction. Indeed, CXCXR3−/− and CCR5−/− mice show symptoms of clinical EAE similar to wild-type controls, arguing against a major role for these receptors and their ligands in regulating disease pathogenesis (65, 66) through redundant mechanisms. Collectively, our model for EAE pathogenesis suggests the following steps. Peripheral T cells become activated and migrate to the CNS where they induce glia to produce cytokines and chemokines, which results in the CNS accumulation of lymphocytes, macrophages, and dendritic cells. Specifically, glial-specific CCL2 production results in the accumulation of TNF-producing macrophages and Tip-DC whose effector cytokines are thought to contribute to disease pathology through induction of demyelination, rather than through alteration of CNS immune responses. Our data in Figs. 6–8 support this idea. Given the central importance of CCL2 and its receptor CCR2 in disease pathogenesis, these molecules remain an attractive therapeutic target for intervention. Understanding the importance of the chemokine contribution to disease development and progression is important, as new chemokine receptor-specific therapies are being developed for use in inflammatory diseases (67). Small molecular mass antagonist molecules efficacious in the inhibition of CCR2 responses have been developed (68) and show efficacy in EAE (38). Further understanding of the roles of chemokines, the cell and tissue-specific expression patterns, and the types and functions of responding cells in disease pathogenesis will allow for more effective antagonist drug development.

Disclosures

The authors have no financial conflicts of interest.

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