Chronic expression of monocyte chemoattractant protein-1 in the central nervous system causes delayed encephalopathy and impaired microglial function in mice

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ABSTRACT Increased central nervous system (CNS) levels of monocyte chemoattractant protein 1 [CC chemokine ligand 2 (CCL2) in the systematic nomenclature] have been reported in chronic neurological diseases such as human immunodeficiency virus type 1-associated dementia, amyotrophic lateral sclerosis, and multiple sclerosis. However, a pathogenic role for CCL2 has not been confirmed, and there is no established model for the effects of chronic CCL2 expression on resident and recruited CNS cells. We report that aged (>6 months) transgenic (tg) mice expressing CCL2 under the control of the human glial fibrillary acidic protein promoter (huGFAP-CCL2hi tg/H11545) manifested encephalopathy with mild perivascular leukocyte infiltration, impaired blood brain barrier function, and increased CD45-immunoreactive microglia, which had morphologic features of activation. huGFAP-CCL2hi tg/H11545 mice lacking CC chemokine receptor 2 (CCR2) were normal, showing that chemokine action via CCR2 was required. Studies of cortical slice preparations using video confocal microscopy showed that microglia in the CNS of huGFAP-CCL2hi tg/H11545 mice were defective in expressing amoeboid morphology. Treatment with mutant CCL2 peptides, a receptor antagonist and an obligate monomer, also suppressed morphological transformation in this assay, indicating a critical role for CCL2 in microglial activation and suggesting that chronic CCL2 exposure desensitized CCR2 on microglia, which in the CNS of huGFAP-CCL2hi tg/H11545 mice, did not up-regulate cell-surface expression of major histocompatibility complex class II, CD11b, CD11c, or CD40, in contrast to recruited perivascular macrophages that expressed enhanced levels of these markers. These results indicate that huGFAP-CCL2hi tg/H11545 mice provide a useful model to study how chronic CNS expression of CCL2 alters microglial function and CNS physiology.—Huang, D., Wujek, J., Kidd, G., He, T. T., Cardona, A., Sasse, M. E., Stein, E. J., Kish, J., Tani, M., Charo, I. F., Proudfoot, A. E., Rollins, B. J., Handel, T., Ransohoff, R. M. Chronic expression of monocyte chemoattractant protein-1 in the central nervous system causes delayed encephalopathy and impaired microglial function in mice. FASEB J. 19, 761–772 (2005)

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Monocyte chemoattractant protein 1 [MCP-1; CC chemokine ligand 2 (CCL2) in the systematic nomenclature] is expressed by a variety of cells at sites of tissue injury (1–3). In vitro and in vivo, CCL2 mediates migration of monocytes, dendritic cells, and T cells (4–8). CCL2 and its cognate receptor CCR2 are critical, not only for local inflammation but also for expression of T helper cell type 1 (Th1) or Th2-polarized immune responses (9–13). Therefore, CCL2 has been proposed to function at the interface of innate and adaptive immunity.

The central nervous system (CNS) is separated from the peripheral immune/inflammatory apparatus by the blood brain barrier (BBB), which inhibits the invasion of leukocytes. Conversely, CNS contains marrow-derived microglia that function as CNS-specific inflamma-
tory cells and respond to intrinsic and extrinsic noxious stimuli (14, 15). Microglial responses can be protective or neurotoxic (16). Upon the detection of insults to CNS tissue in their territory, the highly branched, ramified resting microglia undergo a stereotyped process of transformation, through a series of predictable, morphologic changes including de-ramification, process shortening and thickening, and acquisition of amoeboid morphology.

Cocultured with granulocyte macrophage-colony stimulating factor (GM-CSF), neonatal microglia can differentiate into antigen-presenting cells (APCs), and M-CSF governs microglial differentiation toward macrophages (17). Activated microglia up-regulate expression of molecules related to antigen presentation including major histocompatibility complex class II (MHC-II), CD80, CD86, CD40, and CD11c (17–19). Microglial activation is associated with high levels of intrathecal CCL2 (20–25) in numerous CNS disorders including human immunodeficiency virus (HIV) -associated dementia (HAD) (refs 16, 26), amyotrophic lateral sclerosis (ALS; refs 27, 28), multiple sclerosis (MS; ref 29), and spongiform encephalopathies (30). The role of CCL2 in the activation of microglia has, however, not been studied. Further, the consequences of elevated levels of CNS CCL2 in patients with chronic diseases such as HAD, Alzheimer’s disease (AD), and MS remain largely unknown.

Astrocytes are the major cellular source of CCL2 in patients with neurological disorders and in their corresponding animal models as well (21, 23, 31–35). Young (<6 months) mice overexpressing CCL2 under the control of an astrocyte-specific promoter of the gene encoding human glial fibrillary acidic protein [huGFAP-CCL2 hi transgenic (tg) + ] exhibit minimal inflammatory infiltration in the CNS. However, upon challenge with intravenous injection of pertussis toxin (PTx), huGFAP-CCL2 hi tg + mice developed reversible encephalopathy. This disease model, PTx-induced reversible encephalopathy dependent on MCP-1 (CCL2) overexpression (PREMO), is a surrogate for the clinical syndrome of transient decompensation of CNS function, frequently observed in patients with HAD, AD, and MS (36) and usually associated with clinical or subclinical, upper respiratory or gastrointestinal infections.

In the current report, we describe studies of unmanipulated huGFAP-CCL2 hi tg + mice from 7 to 15 months of age. Persistently elevated levels of CCL2 in the CNS led to neurological impairment, a syndrome designated as delayed encephalopathy spontaneously occurring in mice with MCP-1 (CCL2) overexpression (DESMO). The pathologic triad in DESMO includes perivascular infiltrates containing activated macrophages, microglia with morphological activation, and impaired BBB. We found minimal evidence of autoimmunity to neural components. There were no consistent findings of demyelination or of reduced neurons, axons, or synapses. Results from an in vitro microglial activation assay suggested that chronic exposure to CCL2 desensitized microglial receptor(s) to this chemokine and suppressed signaling through CCR2. Results from the current studies indicated that chronic elevation of CCL2 in the CNS exerted detrimental effects on CNS physiology. We propose that impaired microglial function, coexisting with mild perivascular inflammation and reduced BBB integrity, underlies the observed neurological impairment. This murine model provides an opportunity to isolate and characterize the effects of chronic exposure of CNS tissues to CCL2 and reveals an unexpected dependence of microglial activation on signaling through CCR2.

**MATERIALS AND METHODS**

**Mice**

The generation of huGFAP-CCL2 hi tg + mice and their littermate controls and huGFAP-CCL2 hi tg − mice lacking CCR2 (huGFAP-CCL2 hi tm − · CCR2 −) and their littermate controls was described previously (36). Replacement of CX3C chemokine receptor 1 (CX3CR1) gene by insertion of a gene fragment coding enhanced green fluorescent protein (GFP) created mice deficient for CX3CR1 (CX3CR1GFP/GFP; ref 37), which were subsequently backcrossed onto C57BL/6J for 10 generations. Mating of CX3CR1GFP/GFP mice with huGFAP-CCL2 hi tg + mice produced huGFAP-CCL2 hi tg + · CX3CR1GFP/GFP mice (huGFAP-CCL2 hi tg + /GFP + ) and huGFAP-CCL2 hi tg + /GFP + littermate controls, both of which contained in vivo GFP-labeled cells that expressed functional CX3CR1. Mating offspring of CX3CR1GFP/GFP and homozygous CCR2-deficient (CCR2 −) mice yielded CCR2 −/GFP − mice and littermate control CCR2 +/GFP + mice. All experiments were done by comparing tg + mice with their tg − littermate controls.

**Behavioral monitoring**

huGFAP-CCL2 hi tg + mice were monitored every other day for neurological impairment: loss of weight (a reduction of ≥8% was regarded as significant); hunched posture with reduced grooming; hypo- or hyper-reactive-to-tactile stimuli; abnormal gait with diminished righting reflex; or flaccid tail, limb weakness, single or bilateral hind-limb paresis. Mice that had at least loss of weight and hunched posture with reduced grooming or hypo- or hyper-reactive-to-tactile stimuli, abnormal gait with diminished righting reflex, or flaccid tail, limb weakness, single or bilateral hind-limb paresis were diagnosed with DESMO. All tg + mice were compared with their corresponding littermate control mice in the present studies. Animal experimental procedures were performed in accordance with National Institutes of Health (NIH) guidelines on animal care. Mice were maintained in micro-isolated, pathogen-free conditions in the animal facilities of The Cleveland Clinic Foundation (Cleveland, OH, USA).

**Quantitative immunohistochemical analyses of neural components**

Mice were deeply anesthetized and perfused through the heart with cold phosphate-buffered saline (PBS) followed by 4% paraformaldehyde in Sorenson’s buffer. The cervical spinal cords were removed and post-fixed overnight, and then free-floating cross-sections (30 μm) were cut. Axonal analyses
were performed as described previously (38). Briefly, spinal cord sections were immunostained with rabbit polyclonal antibodies against the 200-kDa neurofilament protein (1:10,000, Serotec, Raleigh, NC, USA). The bound primary antibodies were visualized with biotinylated secondary antibodies, avidin-peroxidase, and nickel-intensified diaminobenzidine. Images from defined areas were photographed using a Leica (Knollhill, UK) DMR microscope with an Optronics Magna Fire CCD camera and image acquisition system. Axonal counts were made in a blinded manner, using NIH Image computer software (Version 1.61). Numbers of axons in areas of the spinal cords from huGFAP-CCL2hi tg–/GFP– mice were compared with that in the corresponding areas in tg–/GFP– controls. At least six mice were included in each group. Enumeration of neurons in transverse sections from lumbar spinal cord was performed in thin slides of 1.0 µm. Mice were deeply anesthetized and perfused with cold PBS, followed by 2.5% glutaraldehyde and 4% paraformaldehyde in 0.08 M phosphate buffer. Spinal cords were post-fixed and embedded in Epon. Consecutive sections of 1.0 µm thickness were prepared, starting from levels of L1 of the spinal cords. The first of every 10 slices was collected. A total of eight slices from every mouse was stained with toluidine blue. Images from anterior and posterior horns of spinal cords were taken using a Leica microscope with an Optronics Magna Fire CCD camera and image acquisition system. Areas photographed were matched between huGFAP-CCL2hi tg–/GFP– and tg–/GFP– mice and examined for the number of neurons in a blind manner. Neuronal cell bodies without nuclei on the cross-sections were excluded. Results were expressed as the mean values of the eight slices per mouse. Three mice per group were analyzed. Synaptophysin, a glycoprotein component of synaptic vesicles, was detected using immunostaining in free-floating sections from cervical spinal cords. Briefly, free-floating sections from spinal cords were rinsed with PBS, treated with 10% Triton X-100, 3% H2O2, and washed in PBS. Primary rabbit antisynaptophysin antibodies (DAKO, Glostrup, Denmark) were applied and incubated overnight. Tissue sections were washed and incubated with biotinylated secondary anti-rabbit immunoglobulin G (IgG) antibody (BD PharMingen, San Diego, CA, USA), Neutraleit Avidin-Texas Red (Southern Biotechnology Association, Birmingham, AL, USA) was added after washing. Tissue sections were examined using confocal microscopy. Areas of anterior and posterior horns of the spinal cords from huGFAP-CCL2hi tg–/GFP– mice were photographed at 1.0 µm thickness and compared with corresponding areas from their littermate huGFAP-CCL2hi tg–/GFP– mice. Three or more mice per group were examined. Results were expressed as percentages of synaptophysin-positive areas vs. total areas examined. The myelin structure in huGFAP-CCL2hi tg–/GFP– was evaluated with immunohistochemistry using polyclonal anti-myelin basic protein (anti-MBP) antibodies (1:2000 dilution, DAKO). Spinal cord sections were incubated with anti-MBP antibodies at 4°C overnight. Biotinylated anti-goat IgG antibodies (Vector Laboratories, Burlingame, CA, USA) were applied after extensive washing. Sections were examined after incubation with avidin-peroxidase and 3,3′-diaminobenzidine (DAB; Sigma-Aldrich, St. Louis, MO, USA). At least six mice per group were included in the studies.

Immunohistochemical analyses of microglia and infiltrating cells

Free-floating, transverse sections of spinal cords (30 µm) were incubated with CD45, anti-IgM (Sigma-Aldrich), CD3, CD80, and CD86 antibodies (BD PharMingen) at 4°C overnight. Biotinylated secondary antibodies, avidin-peroxidase, and DAB (Sigma-Aldrich) were performed as described previously (38, 39). For Ig deposit in the CNS tissues, free-floating sections were incubated with biotinylated anti-mouse Ig (Vector Laboratories) at 4°C overnight, followed by avidin-peroxidase and DAB.

Fluorescent labeling of microglia was done using the B4 isoelectin (IB4) from Griffonia simplicifolia seeds (Molecular Probes, Eugene, OR, USA) as described previously (40). Colocalization of IB4 and GFP was visualized using confocal microscopy (Leica).

**Electric microscopic analysis**

Mice were anesthetized and perfused with cold PBS and followed by 2.5% glutaraldehyde and 4% paraformaldehyde in 0.08 M phosphate buffer. CNS tissues were dissected out, post-fixed, and embedded in Epon. Ultra-thin sections were prepared and mounted on Formvar-coated grids.

**Microglial activation in cortical slice preparations**

All experiments were done with tg+ mice or their littermate (tg–) controls: huGFAP-CCL2hi tg–/GFP–, tg–/GFP+, or CCR2+/GFP+ mice were anesthetized and perfused with cold PBS. CNS tissues were dissected out, and 300 µm brain slices were cut at 4°C in Hanks’ solution immediately using a vibratome (Leica). Brain slices were incubated in Dulbecco’s modified Eagle’s medium (DMEM) with 10% horse serum (Invitrogen, Carlsbad, CA, USA) in the presence or absence of an obligate monomeric MCP-1 mutant P8A (41) or a dominant-negative inhibitor of MCP-1 (27° (0.1, 1.0, 5.0, and 10.0 µg/mL for each; ref 42) for 1, 2, 3, 4, 5, 7, 9, 11, and 15 h and were fixed in 4% paraformaldehyde for 24 h. Tissue slices were then washed with PBS and mounted on slides using Vectashield (Vector Laboratories) and imaged using a confocal microscope (Leica). For three-dimensional reconstruction of an entire microglia (cell body+processes), z-stacks of 40 optical sections (1.0 µm/section) were collected. Forty cells per time point were analyzed using ImagePro.

**Real-time video confocal microscopic analyses**

huGFAP-CCL2hi tg–/GFP+ and huGFAP-CCL2hi tg–/GFP+ littermate control mice at 2 months were used in the current experiment. The maintenance of live cells in brain slices and acquisition of confocal microscopic images were performed as described previously (43–45) using a Leica TCS SP scanning laser confocal microscope. Dynamic processes of microglial activation were acquired by collecting stacks of confocal optical sections at 15-min intervals for 15 h. Each stack contained 30 sections spanning 60 µm in z dimension to provide adequate data for the reconstruction of the microglia.

**RNA preparation and mRNA quantification using reverse transcriptase (RT) -coupled polymerase chain reaction (PCR) analysis**

Mice at the age of 9 months were anesthetized with isoflurane and perfused through the left ventricle with ice-cold PBS. CNS tissues were harvested and immediately snap-frozen in liquid nitrogen. Samples were kept at −80°C until RNA extraction. Total RNA was extracted using TRIZOL™ reagent (Gibco-BRL, Life Technologies, Grand Island, NY, USA) according to the manufacturer’s instructions. Concentrations of RNA were determined by ultraviolet spectroscopy at 260 nm. Primers cd4f 5′GAG AGT CGG AGT TCT C3′ and cd4r
Detection of anti-MBP, myelin oligodendrocyte glycoprotein (MOG)35–55, proteolipid protein (PLP)139–151, and neurofilament antibodies was performed as described previously (10, 46) with modification. Briefly, MBP (0.1 mL/well, 5.0 µg/mL, Sigma-Aldrich), MOG35–55 peptide (MEV GWY RSP FSR VVH LYR NGK; 0.1 mL/well, 3.0 µg/mL, Bio Synthesis), PLP139–151 (HSL GKW LGH PDK F; 0.1 mL/well, 3.0 µg/mL, Bio Synthesis), and neurofilaments (0.1 mL/well, 5.0 µg/mL, Sigma-Aldrich) were added to corresponding 96-well microtiter plates (Nunc, Denmark) in coating buffer and were incubated at +4°C overnight. After being washed three times with PBS-Tween (0.1%), the plates were blocked with 5% fetal calf serum in PBS for 2 h at room temperature. Diluted sera (0.1 mL/well, 1:25, 1:50, 1:100, and 1:200) were added and incubated at room temperature for 2 h, followed by washing with PBS-Tween three times. Biotinylated anti-mouse IgG (1:3000, Sigma-Aldrich) were added and incubated at room temperature for 2 h. After incubation with ABC Vectastain (Vector Laboratories), the reaction was developed with 2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) peroxidase substrate (Vector Laboratories) and read at 405 nm in an enzyme-linked immunosorbent assay reader. In each assay for reactivity against MBP, MOG35–55, PLP139–151, and neurofilaments, all the samples were measured in duplicate. Data are expressed in optical density (OD) units at a specified dilution.

Isolation of microglia and flow cytometric analysis

Isolation of microglia and infiltrating leukocytes from mouse brains was performed as described previously (47). Brain tissues from four to five mice per group were pulled together to obtain sufficient microglia for one experiment. Cells isolated were washed with PBS and resuspended in fluorescein-activated cell sorter buffer. Anti-mouse CD4-phycoerythrin isothiocyanate (FITC), CD8-FITC, MHC-II-FITC (clone: 10-3.6), CD11b-fluorescein (PE), CD11c-PE, CD40-PE, and CD45-Cy-Chrome antibodies were purchased from BD PharMingen and titrated using mouse peripheral blood.

Statistical analyses

The Instat 2.02 software was used for the analyses of the difference between the huGFAP-CCL2hi tg+ and their littermate control tg− mice. The Mann-Whitney U-test was used for the comparisons of levels of mRNA, levels of serum antibodies against myelin components, and neurofilaments, T cell recall proliferations, and cumulative areas of microglia at different stages. A χ2 test was used for the comparisons of DESMO prevalence in groups of huGFAP-CCL2hi tg+ and their corresponding littermate control tg− mice. A P value <0.05 was considered significant.

RESULTS

Delayed neurological impairment in huGFAP-CCL2hi tg+ mice

Protein expression driven by the huGFAP promoter can be detected during the entire lifespan in mouse CNS tissue (48). Before 6 month of age, huGFAP-CCL2hi tg+ mice were free of neurological impairment (36). A neurological syndrome was observed in huGFAP-CCL2hi tg+ mice older than 7 months. Signs included loss of weight (a reduction of ≥8% was regarded as significant), hunched posture with reduced grooming, slow responses to tactile stimuli, flaccid tail, difficulty in righting reflex, limb weakness, and hind-limb paralysis. The onset of the syndrome was insidious and slowly progressive with no apparent remissions and relapses. This syndrome was designated as DESMO. The prevalence of DESMO was age-dependent (Fig. 1). Hind-limb paralysis did not occur in mice younger than 12 months and had 6.7% (3/45) prevalence in 15-month-old mice.

Morphological changes of microglia in CNS tissues from huGFAP-CCL2hi tg+ mice

Microglia, CNS resident phagocytes derived from bone marrow progenitors during development, function in part as CNS scavengers. The activation of microglia in vivo is under tight control, as overactivation of microglia is believed to be detrimental. The impact of CCL2 on microglial activation is unknown. Therefore, we examined the morphology of microglia using anti-CD45 antibody staining, which identifies infiltrating...
leukocytes and microglia (47). CNS tissues from huGFAP-CCL2hi tg+ mice contained increased CD45 immunoreactive microglia (Fig. 2). Compared with microglia in huGFAP-CCL2hi tg− mice, huGFAP-CCL2hi tg+ microglia displayed reduced numbers of processes that were short, thick, and with darkened CD45 staining. Thus, overexpression of CCL2 in the CNS is associated with morphological features of microglial activation. Such morphological changes of microglia were evident in mice 7 months old and progressed over the time-period examined (Fig. 2).

CCR2 is essential for DESMO

We obtained huGFAP-CCL2hi tg+ mice deficient for CCR2 (huGFAP-CCL2hi tg+·CCR2−) as described previously (36), huGFAP-CCL2hi tg+·CCR2− mice remained healthy, free from DESMO within the period observed (15 months). As expected, no perivascular lesions were observed in huGFAP-CCL2hi tg+·CCR2− mice, confirming the observation that CCR2 is the major CCL2-responsive leukocytic receptor (Fig. 2). It is surprising that CNS tissues from huGFAP-CCL2hi tg+·CCR2− mice contained minimally activated microglia with no significant difference compared with wild-type (huGFAP-CCL2hi tg−) mice (Fig. 2), strongly suggesting that CCR2 is the major receptor for CCL2 on microglia in huGFAP-CCL2hi tg+ mice, as morphologic alterations of microglia in CCR2+ animals occurred before detectable, hematogenous infiltrates.

Perivascular lesions in CNS tissues from huGFAP-CCL2hi tg+ mice

CNS tissue sections from huGFAP-CCL2hi tg+ mice showed moderate perivascular infiltration but rare parenchymal inflammatory cells. Consistent with the observation that monocytes are the major cell type responsive to CCL2, the perivascular lesions in huGFAP-CCL2hi tg+ mice contained numerous monocytes/macrophages (Fig. 3 and Supplemental Fig. 1) associated with the vessel wall or in perivascular lesions. Leukocytes tethering on the endothelium were not uncommon (Fig. 3). Macrophages with phagolysosomes of different stages were evident in the lesions. Some phagocytic vacuoles contained membranous structures (Fig. 3 and Supplemental Fig. 1). Costimulatory molecules such as CD80 and CD86 were expressed by infiltrating cells in the perivascular lesions (data not shown), consistent with results from flow cytometric analyses of cells isolated from brain tissues of huGFAP-CCL2hi tg− mice (see below). These lesions were present in huGFAP-CCL2hi tg+ mice older than 7 months with or without clinical DESMO.

Figure 2. CCR2 is essential for morphologic alteration of microglia in huGFAP-CCL2hi tg− mice. No significant morphologic features of microglial activation were observed in huGFAP-CCL2hi tg− control mice during the period examined (A, D, G; 3, 7, and 12 months, respectively). Spinal cord tissue sections from huGFAP-CCL2hi tg+ mice of a 3-month-old (B) showed a similar CD45 staining pattern compared with that in the controls (A). Sections from huGFAP-CCL2 hi tg+ mice at the age of 7 months (E) contained microglia with fewer, shortened, and thickened processes but larger cell bodies. These changes continued to be present in huGFAP-CCL2hi tg+ mice later in their life (H, 12 months). Spinal cord sections from huGFAP-CCL2hi tg+·CCR2− mice (C, F; 3, 7, and 12 months, respectively) showed no difference as compared with their wild-type controls (A, D, G). This graphic is representative of similar results from experiments with at least five mice in each group (original, ×100).

Figure 3. Perivascular infiltrates in the CNS of huGFAP-CCL2hi tg+ mice. Compared with sections from huGFAP-CCL2hi tg− controls (A), spinal cord sections from huGFAP-CCL2hi tg+ mice (B) showed thickened vessel wall and substantial leukocyte infiltrates in the perivascular area. In the vessel lumen (V), leukocytes were found to tether to the endothelia in perfused tissues (arrowheads). White matter remains largely normal in the spinal cords of huGFAP-CCL2hi tg− mice (B). Electric microscopic analyses showed perivascular area in huGFAP-CCL2hi tg− (C) and accumulation of mononuclear cells in the corresponding area in huGFAP-CCL2hi tg+ mice (D). These infiltrating monocytes displayed numerous processes with excessive interstitial fluid. An infiltrating macrophage (E) contained phagolysosomes of different stages. This set of graphic is representative of data with similar results from experiments containing four mice in each group.
Although no detectable CD3 or CD4 mRNA was found in CNS tissues from wild-type control, healthy mice by RT-PCR, spinal cord tissues from huGFAP-CCL2hi tg+ mice contained detectable mRNA specific for CD3 and CD4 (Supplemental Fig. 1). Mb1 mRNA was consistently detectable in the CNS tissues of huGFAP-CCL2hi tg+ mice (Supplemental Fig. 1). B cells expressing the membrane-bound form of IgM were found in CNS perivascular lesions of these mice (Supplemental Fig. 1). CNS perivascular lesions of huGFAP-CCL2hi tg+ mice contained plasma cells (Supplemental Fig. 1), which are terminally differentiated, nonrecirculating B cells, usually found in the secondary lymphoid organs. These results suggest that astrocytic overexpression of CCL2 leads to transendothelial migration of monocytes/macrophages, T cells, and B cells as well, thus providing the necessary, local environment for B cell proliferation and differentiation into plasma cells.

**Peripheral immune reactivity against myelin protein and neurofilaments in huGFAP-CCL2hi tg+ mice**

Microglia are CNS mononuclear phagocytes, and activated microglia can present myelin antigens to T cells (15, 19). Therefore, there existed the possibility that DESMO resulted from autoimmunity against CNS components. Sera from hu-GFAP-CCL2hi tg+ and their littermate control mice were measured for immune reactivity against myelin protein and neurofilaments. huGFAP-CCL2hi tg+ mice had higher levels of anti-PLP139–151 IgG in the circulation (0.508±0.332 OD units at serum dilution of 1:100, n=47) than did huGFAP-CCL2hi tg- mice (0.333±0.146, n=21, P=0.04). No circulating anti-PLP IgG was detected in huGFAP-CCL2hi tg+/-CCR2-/- mice (data not shown). There was no difference in the levels of anti-MOG and anti-MBP antibodies between huGFAP-CCL2hi tg+ and tg- mice, consistent with the MHC-restricted immune responses against dominant residues of PLP in SWXj mice (49). Neither huGFAP-CCL2hi tg+ nor tg- mice contained antineurofilament IgG in the circulation (Supplemental Table 1).

Upon stimulation with PLP139–151, MOG35–51, and MBP in vitro, mononuclear cells from peripheral blood, spleens, and deep cervical lymph nodes of huGFAP-CCL2hi tg+ and tg- mice failed to exhibit antigen-specific T cell proliferation (data not shown). T cells from huGFAP-CCL2hi tg+ mice did not transfer disease to naïve recipients, following stimulation by culturing with mixed or individual myelin and IL-12 (data not shown).

**Microglia in huGFAP-CCL2hi tg+ mice do not up-regulate MHC-II, CD11b, CD11c, CD40, and CD45**

Microglia activated in a proinflammatory environment can express features of professional APCs. huGFAP-CCL2hi tg+ mice, however, did not have a significantly elevated T cell response against myelin proteins, despite the highly activated microglia revealed by immunocytoLOGY (Fig. 2). To further characterize the activation status of microglia in huGFAP-CCL2hi tg+ mice, we examined the levels of MHC-II, CD11b, CD11c, CD40, CD80, CD86, and CD45 expressed by microglia and brain infiltrates in huGFAP-CCL2hi tg+ mice. Although brain leukocytes (CD45high) isolated from huGFAP-CCL2hi tg+ mice expressed higher levels of MHC-II, CD11b, CD11c, CD40 (Fig. 4D, upper panel), CD80, and CD86 (data not shown) as compared with leukocytes in the circulation, microglia (CD45low) of huGFAP-CCL2hi tg+ mice had identical levels of MHC-II, CD11b, CD11c, CD40, and CD45 as compared with those of littermate control huGFAP-CCL2hi tg- mice (compare Fig. 4C, upper and lower panels). These results suggest that chronically elevated levels of CCL2 do not promote microglial acquisition of APC characteristics, despite activated morphology of microglia (Fig. 2) in huGFAP-CCL2hi tg+ mice. Conversely, infiltrating CD45hi leukocytes that responded to CCL2 did up-regulate these molecules, (Fig. 4D, upper panel).

**Minimal alterations of axons, neuronal somata, and synapses in the CNS of huGFAP-CCL2hi tg+ mice with DESMO**

The presence of anti-PLP139–151 antibodies in the circulation of huGFAP-CCL2hi tg+ mice raised the possibility that the neurological dysfunction in huGFAP-CCL2hi tg+ mice might result from antibody-mediated myelin destruction. As a result of the large reserve of CNS function, a significant reduction of the number of neurons and/or demyelination is usually required for the expression of symptoms and signs of diseases such as HAD, AD, and MS. However, examination of the integrity of myelin structure in CNS tissues from huGFAP-CCL2hi tg+ mice revealed only a slight reduction of the intensity of MBP staining in a small proportion (1/6) of huGFAP-CCL2hi tg+ mice (Fig. 5A, D). The changes were, however, not typical of demyelination as observed in SWXj mice with PLP-induced EAE (Fig. 5C, F).

In huGFAP-CCL2hi tg+ mice, neurological impairments progress with no remission and relapse, suggesting the possibility of neuronal damage and loss. We evaluated the number of axons in axial sections of cervical spinal cords from huGFAP-CCL2hi tg+ mice with DESMO. Compared with littermate controls, CCL2hi tg+ mice with DESMO had equal numbers of axons in the cervical spinal cords (Supplemental Table 2). We quantitated neurons in axial sections of lumbar spinal cords. No significant difference was found between mice with DESMO and tg- control mice (Supplemental Table 2).

We considered whether activated microglia in the CNS of huGFAP-CCL2hi tg+ mice might mediate non-physiological synaptic stripping. Syntrophophins is a synaptic vesicle glycoprotein widely used as a marker for synapses. Sections of cervical spinal cords from huGFAP-CCL2hi tg+ mice contained numbers of syntrophophin-immunoreactive puncta comparable with
those in their tg^{−} control littermates (Supplemental Table 2).

Taken together, these results suggest that adaptive autoimmunity and neural tissue destruction do not account for the neurological impairment in mice with DESMO.

**Impaired microglial activation in cortical slices from huGFAP-CCL2_{hi} tg^{−} mice**

Given the lack of pathogenic autoimmunity and minimal disruption of CNS tissue elements in the CNS of mice with DESMO, it seemed plausible that microglial overactivation might underlie the observed phenotype. However, given that activation markers associated with APC function were not up-regulated on microglia of huGFAP-CCL2_{hi} tg^{−} mice, it was important to develop additional assays for microglial activation. Transformation of microglia in cortical slice preparations provides an incisive means to assess the response to tissue damage and culminates in rapid (\sim 12 h) acquisition of amoeboid appearance following a stereotyped sequence of well-characterized, morphological changes (40). Previous studies using this model assessed microglial morphology by immunohistochemistry using antibodies to surface or cytoplasmic markers. As microglia constitutively express high levels of CX3CR1 (thus, GFP in CX3CR1-deficient mice used in the current studies), CX3CR1 heterozygous (+/GFP) mice (37) can serve as a tool for the examination of microglial activation with high resolution, using cytoplasmic GFP as a reporter of microglial morphology. Using IB4 from G. simplicifolia, a classical marker for microglia, we first confirmed that all GFP^{+} cells in normal CNS tissue were positive for IB4 staining (Fig. 6A) and ionized calcium-binding adapter molecule 1 as well (our unpublished data).

Confocal microscopic analyses were used in the current experiment. Resting microglia (Fig. 6B and Sup-
Supplemental Table 3, 0 h) in huGFAP-CCL2hi tg+ and tg− mice showed an equal cumulative cellular area, a parameter that closely correlates with microglial activation. Time-lapse confocal microscopy culture revealed evident differences between microglia of huGFAP-CCL2hi tg+ and tg− mice during the period of observation in slice culture (Fig. 6B and Supplemental Table 3). Microglia from huGFAP-CCL2hi tg+ mice retracted their fine processes faster and had a smaller area than did microglia from huGFAP-CCL2hi tg− mice during the initial stage (de-ramification) of culture in 1 h. Among the time points examined, microglia in huGFAP-CCL2hi tg+ mice exhibited less fluctuation in mean cellular area, suggesting reduced capability of microglia in remodeling cell bodies. Although microglia in huGFAP-CCL2hi tg− mice generated numerous new processes after 11 h incubation (Fig. 6B), microglia from huGFAP-CCL2hi tg+ mice extended fewer processes, and surrounding neurophil contained abundant GFP+ debris (Fig. 6B).

Video confocal microscopy was used to monitor the dynamic process of microglial activation in huGFAP-CCL2hi tg+ mice and littermate controls. The entire process of microglial activation in huGFAP-CCL2hi tg+ and their littermate control mice can be viewed at www.fasebj.org. Representative microglia in slices from huGFAP-CCL2hi tg+ and tg− mice are shown in Supplemental Fig. 2. Initially, microglia in tg+ and tg− mice exhibited dense networks of fine, ramified cytoplasmic processes radiating from a relatively small cell body. During the subsequent 4 h, the distal processes retracted progressively toward the cell body. As processes shortened, proximal regions of processes became wider and appeared brighter. In the late stage of transformation, microglia in huGFAP-CCL2hi tg− mice alternatively extended and then retracted pseudopodia. By 11 h, cells with amoeboid morphology were observed. At the initial phase of process retraction, microglia in huGFAP-CCL2hi tg+ mice followed a similar pattern as that in tg− mice. However, process

Figure 5. Immunohistochemical analyses with anti-MBP antibodies in spinal cord tissues. Spinal cord sections of only one mouse from a group of six huGFAP-CCL2hi tg+ mice (A, D) showed areas of reduced intensity of immunostaining using anti-MBP antibodies (arrows) as compared with the tg− controls (B, E). C, F) Spinal cord sections from mice with experimental autoimmune encephalomyelitis (EAE) showed extensive demyelinating areas (arrowheads) with reduced intensity of anti-MBP staining (A–C ×25 original magnification; D–F ×200 original magnification).

Figure 6. Chronic elevation of CCL2 in the CNS desensitizes microglial CCR2. A) Microglia are the sole type of CNS resident cells expressing enhanced GFP in CX3CR1+/GFP mice, which were perfused with cold PBS, and brains were dissected out. Vibratome sections were stained with IB4, which identifies microglia (red) and endothelia (red, network-like structure of microcirculation). This is representative of four experiments with similar results. B) Microglia of huGFAP-CCL2hi tg+ mice lack morphologic transformation. Microglia in brain cortical tissue sections from huGFAP-CCL2hi tg− controls (tg−) and tg+ mice (tg+) displayed dense, fine processes with small cell bodies when initiating the tissue culture (0 h). Two hours later, processes were retracted halfway to their cell bodies and became shorter and wider (2 h). Although microglia in huGFAP-CCL2hi tg− mice were finishing the process retraction and had larger and brighter cell bodies at 7 h, microglia in huGFAP-CCL2hi tg+ mice failed to execute this activation program. Instead, their processes became fragmented and their cell bodies, smaller and spherical at 11 h of the incubation when microglia in huGFAP-CCL2hi tg− control mice formed numerous pseudopodia (original, ×400). This graphic is representative of three experiments with similar results.
retraction was rarely complete in huGFAP-CCL2hi tg+ mice. Instead, microspike-like protrusions were observed to form a feathery perimeter around the soma. Further, pseudopodia that extended from microglia were rarely observed in huGFAP-CCL2hi tg+ mice. Distal processes of microglia in huGFAP-CCL2hi tg+ mice often became fragmented, and particulate debris accumulated, as the cell bodies of huGFAP-CCL2hi tg+ microglia became smaller and spherical. Taken together, these results indicated that overexpression of CCL2 in the CNS altered the microglial activation process, resulting in significantly impaired, morphologic transformation of microglia in cortical slice preparations.

**Prolonged overexpression of CCL2 in the CNS leads to desensitization of CCR2 in microglia**

Murine microglia express CCR2 (50–52). Overexpression of CCL2 in the CNS might overactivate microglia via CCR2. Alternatively, desensitization of CCR2 as a result of chronic exposure to CCL2 might play a role in the failure of amoeboid transformation in cortical slice preparations of huGFAP-CCL2hi tg+ mice, as well as defective up-regulation of surface markers associated with APC function. We prepared cortical tissue sections from CCR2-deficient (CCR2−) GFP+ mice and CCR2 wild-type (CCR2+) GFP+ littermate controls. The microglial activation pattern in CCR2− CNS tissue slice culture (Supplemental Fig. 3) differed from that in CCR2+ controls and was similar to that observed in huGFAP-CCL2hi tg+ mice (Fig. 6B). These findings were further confirmed in CCR2+ CNS tissue slice culture experiments using the obligate monomer P8A and the receptor antagonist 7ND, which can antagonize signaling through CCR2 in vivo under defined circumstances (Supplemental Fig. 3). We concluded that the lack of microglial remodeling in huGFAP-CCL2hi tg+ mice was a result of receptor desensitization of CCR2 on microglia that were chronically exposed to high levels of CCL2.

**BBB is impaired in huGFAP-CCL2hi tg+ mice with advanced DESMO**

An intact BBB is critical to the normal function of CNS and is commonly disrupted during chronic inflammation. Immunohistochemical analyses (Fig. 7) using anti-mouse Ig revealed massive IgG deposition in the CNS of huGFAP-CCL2hi tg+ mice at the ages of 12 and 15 months. However, huGFAP-CCL2hi tg+ mice at 10 months did not have significantly increased Ig immune reactivity in the CNS as compared with that in their littermate tg− controls. These data indicate that the presence of CCL2-responsive hematogenous leukocytes and the dysfunction of microglia were associated with the degradation of BBB function in mice with DESMO.

**DISCUSSION**

Our results from the current studies demonstrated for the first time an impact for elevated CNS levels of CCL2 on local physiology, leading to progressive neurological impairment. Overexpression of the CCL2 gene in the CNS resulted in DESMO without additional stimulus. These results carry significant implications in understanding neurodegenerative, infectious, and inflammatory/autoimmune CNS diseases.

Young huGFAP-CCL2hi tg+ mice hosted in a pathogen-free environment are healthy and exhibit no overt CNS pathology. However, systemic injections of PTx led to massive infiltration of leukocytes accompanied by the transient encephalopathy, PREMO (36). Although a concentration gradient of CCL2 is essential for the migration of CCL2-responsive cells, an intact BBB reduces the likelihood of migration. The activity of the huGFAP promoter can be detected in early prenatal life and is sustained postnatally (48). In mice with DESMO, despite the protection by the BBB, leukocytes, including monocytes/macrophages and T cells, entered into the perivascular region. In view of the insidious onset and slowly progressive course of DESMO, the accumulation of leukocytes is likely to be a slow process.

CCL2 is active toward monocytes/macrophages, den-
dent arrest of rolling monocytes (60). Endothelial venules, where it triggers integrin-dependent migration in CNS tissues. Transcytosis and surface presentation by perivascular cells of another chemokine, IL-8, have been studied carefully (59). It is of interest to note that CCL2 derived from inflamed skin is transported to draining lymph nodes and the luminal surface of high endothelial venules, where it triggers integrin-dependent arrest of rolling monocytes (60).

It is remarkable that microglia in huGFAP-CCL2 hi tg+ mice, despite the appearance of activation and increased CD45 immunoreactivity (Fig. 2), were defective in their response to environmental stimuli. In view of the unchanged levels of CD45 revealed by flow cytometry in huGFAP-MCP-1 hi tg+ mice, the increased CD45 immunoreactivity is likely a result of the morphologic transformation of microglia. In contrast to monocytes in the perivascular lesions of mice with DESMO (Fig. 3 and Supplemental Fig. 1) and those in CNS tissue of mice with PREMO (36), microglia in huGFAP-CCL2 hi tg+ mice with DESMO have been exposed continuously to CCL2. Such chronic exposure impaired multiple microglial functions including expression of APC-associated surface markers.

CCL2 transgenic mice, which express CCL2 under control of the mouse mammary tumor virus long repeat, contain high levels (ng/mL) of CCL2 in the systemic circulation and are susceptible to intracellular pathogens including _Listeria monocytogenes_ (61). The mechanisms of this effect include monocyte CCR2 desensitization, as shown by elegant genetic studies (62). Using an in situ microglial activation system, we demonstrated that upon tissue injury, microglial activation in CNS tissues from huGFAP-CCL2 hi tg+ and CCR2− mice failed to culminate in amoeboid morphology. The similarity of microglial activation in cortical slice preparations from CCL2 tg+ and CCR2− mice suggests a failure of CCR2 signaling to microglia of huGFAP-CCL2 hi tg+ mice. Results of experiments using 7ND to block signaling to CCR2 on the microglia of wild-type mice argue that other factors that might influence microglial function during the development of huGFAP-CCL2 hi tg+ and/or CCR2− mice are unlikely to account for our results. Thus, it is proposed that chronic elevation of CCL2 in the CNS leads to desensitization of CCR2 and microglial dysfunction. It is interesting that double-transgenic huGFAP-CCL2 hi tg+ mice younger than 6 months, when altered morphological transformation of microglia (videos at www.fasebj.org), but not perivascular lesions, could be detected. Perivascular inflammation may generate inflammatory products and impair BBB function; the increased accumulation of serum components as a result of leaky BBB may further impair the microglial function and aggravate the process of DESMO.

In human, a genetic variation in the promoter region of the gene encoding CCL2 has been found to be associated with an increased risk of dementia and accelerated disease progression in patients with HIV infection (22). Increased monocyte/macrophage infiltration may play a role in the disease pathogenesis; the function of microglia in patients of CCL2 high-secretor phenotype deserves further attention. With the ample, consistent evidence of elevated levels of CCL2 in CNS tissues from chronic diseases such as AD, HAD, ALS, and MS, which have been reported independently by several different research groups, our current results provide new insights into the mechanisms of CCL2-associated dysfunction of the CNS and lay the groundwork for therapeutic strategies targeting the CCL2/CCR2 pathways.

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