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CXCL10 Triggers Early Microglial Activation in the Cuprizone Model

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A broad spectrum of diseases is characterized by myelin abnormalities and/or oligodendrocyte pathology. In most, if not all, of these diseases, early activation of microglia occurs. Our knowledge regarding the factors triggering early microglia activation is, however, incomplete. In this study, we used the cuprizone model to investigate the temporal and causal relationship of oligodendrocyte apoptosis and early microglia activation. Genome-wide gene expression studies revealed the induction of distinct chemokines, among them Cxcl10, Ccl2, and Ccl3 in cuprizone-mediated oligodendrocyte apoptosis. Early microglia activation was unchanged in CCL2- and CCL3-deficient knockouts, but was significantly reduced in CXCL10-deficient mice, resulting in an amelioration of cuprizone toxicity at later time points. Subsequent in vitro experiments revealed that recombinant CXCL10 induced migration and a proinflammatory phenotype in cultured microglia, without affecting their phagocytic activity or proliferation. In situ hybridization analyses suggest that Cxcl10 mRNA is mainly expressed by astrocytes, but also oligodendrocytes, in short-term cuprizone-exposed mice. Our results show that CXCL10 actively participates in the initiation of microglial activation. These findings have implications for the role of CXCL10 as an important mediator during the initiation of neuroinflammatory processes associated with oligodendrocyte pathology. The Journal of Immunology, 2015, 194: 3400–3413.

Myelin abnormalities and/or oligodendrocyte pathology are present in many CNS diseases, including multiple sclerosis (MS) (1), schizophrenia (2), Alzheimer’s disease (3), amyotrophic lateral sclerosis (4), or leukodystrophies (5, 6) such as Canavan’s disease (7). Affected brain regions in these disorders are characterized by the activation of microglia and/or astrocytes to a variable extent (8–12). Microglial cells are the brain-resident immune cells and represent ~10% of the total brain cell population.

Classically, microglia are classified as either resting or activated cells, depending on their morphology (13). Despite their role as sentinel cells of the brain, resting microglia are critically involved in the maintenance of brain homeostasis, neuroprotection, and are an important source of various growth factors such as brain-derived neurotrophic factor (BDNF) or TGF-β (14, 15).

Activation of microglia cells is mediated by receptors for various inflammatory mediators, such as pathogen-associated molecular pattern (e.g., bacterial wall LPSs, viral envelopes, and bacterial or viral DNAs or RNAs) or danger-associated molecular patterns such as neurotransmitters, peptides, cytokines, chemokines, complement externalized phosphatidylserine on apoptotic cells, or intracellular constituents released from necrotic cells. Microglial receptors therefore provide multiple converging signals that control the transition from surveillance to the different activated states (13).

Microglia cells can as well be activated indirectly by, for example, astrocytes. Astrocytes not only facilitate the activation of distant microglia, but can also inhibit microglial activities. Molecules contributing to this cell–cell communication include IL-1, ATP, or TGF-β (16). A better understanding of the cross-talk between astrocytes and microglia would be helpful to elucidate the role of glial cells in pathological conditions, which could accelerate the development of treatment for various diseases (17).

The chemokines CCL2, CCL3, and CXCL10 have been shown to induce chemotaxis in a variety of cell types, including microglia/monocytes (18–21). Chemokines also contribute to tissue homeostasis [reviewed in (22)] and can promote cell migration of nonimmune cells in the absence of inflammation (23). The expression and release of chemokines are induced in numerous de-
myelinating diseases and animal models, including experimental autoimmune encephalomyelitis (EAE), Thielé’s and hepatitis virus-induced demyelinating disease, experimental autoimmune neuritis, a murine model of globoid cell leukodystrophy called twitcher (24), and the toxic demyelinating cuprizone model (25, 26). Furthermore, the importance of chemokines has been demonstrated in other diseases that are characterized by myelin abnormalities and/or oligodendrocyte pathology, including amyotrophic lateral sclerosis (27), Alzheimer’s disease (28), leukodystrophies (29), or schizophrenia (30).

In the past years, we and others have extensively characterized the cuprizone model with respect to demyelination properties and the complex cell–cell interactions during these processes. Feeding of cuprizone induces demyelination of many CNS white and gray matter regions, most importantly of the corpus callosum (CC) (31). In this model, a primary oligodendrogliopathy results in significant oligodendrocyte apoptosis with concomitant microglial activation, astrogliosis, and demyelination (31), and it has been successfully used to study the role of microglia under well-defined pathological conditions (26). In the current study, we used this model to identify potential contributing factors for early microglia activation in the context of oligodendrocyte pathology. Our results demonstrate that oligodendrocyte apoptosis is paralleled by early microglial activation, and that this early activation is mediated in part by the chemokine CXCL10, released from astrocytes. This has implications for the role of CXCL10 as an important mediator during the initiation of neuroinflammatory processes associated with oligodendrocyte pathology.

Materials and Methods

Mice and demyelination
C57BL/6J mice were obtained from The Jackson Laboratory stock (000664) at Charles River (Sulzfeld, Germany) and served as controls for C2L2, C3L3, and CCL2/CCL3 knockout (CCL2+/−, CCL3+/−, CXCL10+/−) mice. CCL2+/− and CCL3+/− mice were obtained from The Jackson Laboratory (B6.129S4-Ccl2tm1Rol/J, B6.129P2-Ccl3tm1Unc/J). CXCL10−/− mice were provided as a gift of U. Christen (Pharmazentrum Frankfurt/ZAFES, Klinikum der Goethe Universität, Frankfurt/Main, Germany) (32). Male mice were housed in the animal house of the University of Frankfurt. Our laboratory conditions were governed by the Federation of European Laboratory Animal Science Association’s recommendations. The procedures were approved by the Review Board for the Care of Animal Subjects of the district government (Nordrhein-Westfalen, Germany) and performed according to international guidelines on the use of laboratory mice. Demyelination was induced by feeding 8-wk-old (19–21 g) mice a diet containing 0.2% cuprizone (bis-cyclohexanone oxadithydrazone; Sigma-Aldrich) in ground standard rodent chow for the indicated period (31, 33). Control mice were fed standard rodent chow.

Tissue preparation
Preparation of tissues was performed, as previously described (34–36). For histological and immunohistochemical (IHC) studies, mice were transcardially perfused with 2% paraformaldehyde. After overnight postfixation in the same fixative, brains were dissected, embedded in paraffin, and then coronally sectioned into 5-μm sections at the levels 215–275 according to the mouse brain atlas of Sidman et al. (http://www.hms.harvard.edu/research/brain/atlas.html). For gene expression analyses, mice were transcardially perfused with ice-cold PBS, brains quickly removed, and the entire CC dissected from the cortex, as described previously (37, 38). Tissues were immediately frozen in liquid nitrogen and kept at −80 °C until further use. For the preparation of myelin-entrenched brain debris, snap-frozen CC tissue was homogenized in ice-cold PBS, as published (11). After centrifugation at 1000 × g for 10 min at 4 °C, supernatant was collected and protein content was determined by bicinchoninic acid assay (Pierce Biotechnology, Germany).

IHC and evaluation
For IHC, sections were placed on silane-coated slides, deparaffinized, rehydrated, heat unmasked if necessary, blocked with PBS containing 1% horse or normal goat serum, and incubated overnight at 4 °C with the primary Abs diluted in blocking solution. Primary Abs and dilutions used in the study are given in Table I. After washing and blocking of endogenous peroxidase with 0.3% hydrogen peroxide (in PBS) for 30 min, sections were incubated with biotinylated secondary horse anti-mouse or goat anti-rabbit Abs (1:50; Vector Laboratories) for 1 h, followed by peroxidase-coupled avidin–biotin complex (ABC kit; Vector Laboratories). The diaminobenzidine reaction (DAKO Deutschland) was used to visualize immunoreact–avidin–biotin complex. Sections were counterstained with standard hematoxylin to visualize cell nuclei. Secondary Abs and dilutions used are given in Table II. H&E as well as luxol fast blue/periodic acid–Schiff (LFB/PAS) staining was performed on deparaffinized sections for evaluation of apoptosis (H&E) and myelin status (LFB/PAS), respectively.

Evaluation of apoptosis on H&E stains was performed using well-defined morphological criteria, such as condensed and fragmented nuclei (33, 39). Myelination of the CC was evaluated by two independent and blinded researchers in two different regions (area 225 and 265, according to the mouse brain atlas by Sidman et al.) on a scale from 0 (complete demyelination) to 10 (normal myelination). Results were averaged and expressed as percentage of control. IHC stains were examined with a Nikon ECLIPSE 80i microscope. For cell parameter quantifications, two consecutive sections per mouse were evaluated in the medial and lateral part of the CC, and values of both regions and sections were averaged. IBA1+ and glial fibrillary acidic protein (GFAP)+ cells with a clearly visible nucleus were counted. Cell numbers are given in cells per mm².

In situ hybridization of tissue sections
Commercial fluorescence in situ hybridization kits (QuantGene View RNA in situ hybridization (ISH) tissue assay; Affymetrix-Panomics) were used for single and double labeling of formalin-fixed, paraffin-embedded tissue, according to the manufacturer’s recommendations. Probe digestion/titration was adjusted to 20 min. Probes for Ccl2, Ccl3, Cxcl10, and Atp3 as well as the cellular markers Gfap and Plp1 were purchased from Affymetrix (Affymetrix-Panomics). Radiosotope in situ hybridization on brain sections was performed as described previously (40). Riboprobes were synthesized from linearized plasmids containing a fragment of mouse Ccl2, Ccl3, or Cxcl10 cDNA. Confocal and Z-stack confocal images were captured using the LSM710 laser-scanning microscope system (Carl Zeiss).

Cell culture and treatment
Primary rat oligodendrocytes were prepared from mixed glia cell cultures after 10–12 d, as described by Richter-Landsberg and Vollgraf (38, 41, 42) with minor modifications. Briefly, oligodendrocyte precursor cells (OPCs) were separated from the underlying astrocytic cell layer by vigorous shaking (for 16 h at 240 rpm) after microglia were detached by gentle shaking. Precursor cells were replated on poly-ornithine–coated culture dishes (105 cells/well) in a 6-well plate and kept for 2 h in DMEM supplemented with 10% FCS. Medium was then changed to serum-free DMEM containing 1× N2 supplement (Life Technologies), 10 nM bixin (Sigma-Aldrich), 0.1% BSA (Roth, Germany), 1% P/S, 1× N-acetyl-t-cysteine (1000× = 5 mg/ml; Sigma-Aldrich), and 10 ng/ml recombinant fibroblast growth factor 2 (FGF2) and platelet-derived growth factor α (both PeproTech). To obtain highly purified and proliferating OPC cultures, cells were cultured under these conditions for 3 d, and half of the medium was changed on the second day. On the third day, the medium was changed to differentiation medium to generate mature oligodendrocytes. Differentiation medium consisted of DMEM, 1× N2 supplement, 10 nM bixin, 15 nM 3,5’-triiodo-t-thyronine (T3; Sigma-Aldrich), 0.1% BSA, 1× P/S, 1× N-acetyl-t-cysteine, and 10 ng/ml rat recombinant cilium myelinotropic factor (Peptide Institute). Cells were allowed to differentiate until morphological changes (high number of cellular processes) were clearly visible (43). Purity of primary oligodendrocyte cultures was determined by immunocytochemistry (ICC). Cultures contained 0.367% ± 0.367% IBA1+ microglia and 8 ± 1.225% GFAP+ astrocytes (values are given as arithmetic mean of n = 3 experiments ± SEM). Mouse embryonic stem cell–derived microglial precursors (ESdM) were prepared as published previously (44). Cells were cultivated in serum-free DMEM/F12 medium (Life Technologies) containing 1× N2 supplement, 0.48 mM l-glutamine (Life Technologies), 5.3 μg/ml n-glucose (Roth, Germany), and 100 μg/ml P/S. The murine oligodendrogial cell line OliNeu (45) was cultured in SATO medium containing 2% FCS. For treatment, cells were seeded in 96-well plates (15,000 cells/well) or 6-well plates (400,000 cells/well), and the medium was replaced by SATO medium containing 0.5% FCS. For additional 24 h, cells were treated with sodium azide (40 μM) or CXCL10 (1, 10, or 100 ng/ml) for 24 h. Ninety-six–well plates were used for lactate dehydrogenase (LDH) release and cell titer blue assays. Supernatant was collected from 6-well plates for ELISA, and
remaining cells were washed with 1× Dulbecco’s PBS and lysed in PeqGold for subsequent quantitative RT-PCR (qRT-PCR) analysis.

Primary mouse astrocytes were prepared, as published previously (46). For treatment, astrocytes were seeded on 6-well plates at a density of 400,000 cells/well and cultured for 24 h in DMEM containing 10% FCS. After this, medium was changed to DMEM containing 1% FCS for additional 24 h. Cells were treated with 10 nM sodium azide (SA) or 100 U/ml IFN-γ for 24 h. Vehicle-treated cells served as control. Supernatant was collected for ELISA, and cells were washed in 1× Dulbecco’s PBS and lysed in PeqGold for qRT-PCR analysis.

Migration and phagocytosis assays

Migration and phagocytosis assays were performed according to the protocols of Beutner et al. (44). Briefly, for the migration assay, 1 × 10^6 ESDm cells were seeded into the upper chamber of a transwell system (8 μm pore size; Merck Millipore), and the lower chamber was filled with medium containing 50 ng/ml recombinant mouse CXCL10 (R&D Systems). Cells were incubated for 3 h, and migrated cells were counted. Results were normalized to control wells without CXCL10.

For phagocytosis assays, microglia were stimulated with either 500 ng/ml LPS, 10 ng/ml CXCL10, or LPS and CXCL10 in combination for 24 h and then incubated with fluoresbrite polychromatic red 1.0-LPS, 10 ng/ml CXCL10, or LPS and CXCL10 in combination for 24 h and normalized to control wells without CXCL10.

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Microarray analysis and real-time qRT-PCR

Regulation of gene expression in different treatment groups was analyzed using Affymetrix GeneChip microarrays, as published previously (36, 47, 48). For each treatment group, mRNA was isolated, and the quality was assessed using RNA NanoChips with the Agilent 2100 Bioanalyzer (Agilent). Probes for the GeneChip Mouse Gene 1.0 ST Arrays (Affymetrix) were prepared and hybridized to the arrays according to the Affymetrix GeneChip Whole Transcript Sense Target Labeling Assay Manual. Raw image data were analyzed with AGCC (Affymetrix), and gene expression intensities were normalized and summarized with RNA algorithm (49). Only genes expressed >3-fold compared with array internal negative controls were taken into consideration. Genes upregulated in the 2-d cuprizone-treated group versus the control group were identified according to the following criteria: expression of genes in cuprizone-treated sample is at least 2-fold higher compared with control samples, and the adjusted p value for changes in expression is <0.05. Gene expression levels were further determined using the qRT-PCR technique (Bio-Rad), SensiMix SYBR Green (Bioline), and a standardized protocol, as described previously (34, 48, 50). Primer sequences and respective annealing temperatures are given in Table III. The 18s, Gapdh, or Cycb were used as reference genes.

ELISA

CXCL10 rat and mouse ELISAs (PeproTech) were performed according to the manufacturer’s protocol. Color development of the ABTS substrate (Sigma-Aldrich) was monitored with a Tecan infinite 200 plate reader at 490 nm. In vivo samples

results were calculated using the following formula: Absorbance of treated sample / Absorbance of control sample × 100.

Results

Chemokines are dynamically regulated in cuprizone-treated mice

In previous studies, we and others were able to demonstrate that oligodendrocyte apoptosis with concomitant activation of microglia and astrocytes is manifest days after initiation of the cuprizone diet (25). In a first step, we aimed to identify the factors that are involved in early microglia activation in this model. To this end, total mRNA samples from the CC of control mice and mice-fed cuprizone for 2 d were analyzed using Affymetrix GeneChip arrays (n = 3 per experimental group, one single experiment). Our analysis revealed an increased transcription of numerous proinflammatory genes, including genes coding for chemokines (Fig. 1A). From the 21 CCL chemokines in the array, three were significantly increased, namely CCL2 (Mcp1), CCL3 (Mip1α), and CCL7 (Mcp3), and from the 13 CXCL chemokines analyzed, only Cxcl10 (Ip10) displayed an increased expression after 2 d of cuprizone treatment. The transcription of no other investigated chemokine transcript was increased at day 2. To confirm these results, qRT-PCR was performed with independent samples to quantify gene transcript levels of the three most upregulated chemokines, namely Ccl2, Ccl3, and Cxcl10 (Fig. 1B). In line with our microarray data, the mRNA levels of these three chemokines were found to be increased after cuprizone exposure compared with untreated controls, with CXCL10 revealing the most prominent fold change (~170-fold induction). Furthermore, significantly higher levels of CXCL10 protein were detected by ELISA in brain lysates from cuprizone-treated compared with control mice (Fig. 1C). To relate early chemokine expression to distinct brain regions, brain sections from cuprizone-treated mice were analyzed by in situ hybridization. As demonstrated in Fig. 1D, Ccl2 mRNA was evenly distributed in the CC, including the myelin- and oligodendrocyte-rich deep cortical layers (arrows in Fig. 1D). Within the hippocampal formation, the polymorph layer of the dentate gyrus displayed some positive signals (arrowheads in Fig. 1D). Ccl2 signal intensity was weak in superficial cortical layers. In comparison, the hybridization signal for Ccl3 was much weaker in general. We detected some spots in the CC and adjacent cortex region (Fig. 1E). In line with our data obtained by qRT-PCR, the strongest in situ hybridization signal was seen for Cxcl10. Highest signal intensities were found in the CC, deep cortical layers, and distinct hippocampal subregions, such as the polymorph layer of the dentate gyrus (arrowheads in Fig. 1F) or the stratum oriens and radiatum of the hippocampal cornu ammonis region (arrows in Fig. 1F). Remarkably, no Cxcl10 signal was observed in the neuronal granule cell layer of the dentate gyrus or the pyramidal layer of the cornu ammonis (asterisks in Fig. 1F), implicating that neurons are not the major source of CXCL10 in this animal model. In summary, we found a predominant induction of CXCL10, CCL2, and CCL3 chemo-
kines in response to cuprizone-induced oligodendrocyte stress, and we thus assumed that these chemokines orchestrate the observed concomitant microglia attraction/activation.

CXCL10- but not CCL2- or CCL3-deficient mice display less severe early microgliosis

To examine whether chemokines functionally contribute to early microgliosis activation in this model, CCL2−/−, CCL3−/−, and CXCL10−/− mice were fed cuprizone for 1 wk, and the extent of microgliosis was determined in IBA1-stained sections. Although numbers of IBA1+ microglia were comparable in CCL2−/−, CCL3−/−, and wild-type (WT) animals, a ∼40% reduction in the number of IBA1+ cells was found in CXCL10-deficient mice (Fig. 2). In detail, the number of microglia was reduced in the CC from 448.131 ± 615.53 cells in WT mice to 285.816 ± 18.33 cells in the CXCL10−/− mice (Fig. 2B). Thus, CXCL10, but not CCL2 or CCL3, regulates early microgliosis in this model.

Recently, it has been reported that cultured oligodendrocytes die when exposed to CXCL10 in a concentration-dependent manner (51). The lower extent of microglia activation in CXCL10−/− mice after cuprizone treatment therefore might simply be due to less severe oligodendrocyte loss in the absence of CXCL10. To investigate whether CXCL10 influences initial oligodendrocyte death during cuprizone treatment, the number of oligodendrocytes was quantified in the CC of WT and CXCL10−/− mice after 1 wk of cuprizone exposure. As shown in Fig. 3A, the cuprizone-induced loss of oligodendrocyte transcription factor 2–expressing oligodendrocytes was comparable between both genotypes. In line with these findings, the number of apoptotic cells (as determined in H&E-stained sections) was not significantly different in both strains (Fig. 3B). Furthermore, treatment of primary OPCs and mature oligodendrocyte cultures (Fig. 3C) and the murine oligodendroglial cell line OliNeu (Fig. 3D) with recombinant CXCL10 for 24 h did not induce significant cell death, as determined

FIGURE 1. Gene transcription and protein expression of chemokines in cuprizone-treated mice. (A) Evaluation of Affymetrix GeneChip arrays shows that Ccl2, Ccl3, Ccl7, and Cxcl10 transcripts are upregulated in the CC after 2-d (2d) cuprizone exposure. Hybridization signal and relative transcription levels for each mouse, as determined by Affymetrix GeneChip array, are presented in the table (n = 3). (B) mRNA expression levels of Ccl2, Ccl3, and Cxcl10 after 2-d (2d) cuprizone treatment (fold induction over control: Ccl2, 29.74 ± 2.98; Ccl3, 15.54 ± 1.57; Cxcl10, 180.7 ± 17.44, n = 4), determined by RT-qPCR. Note the strong upregulation of Cxcl10 expression (∼170-fold; unpaired t test with Welch’s correction). (C) CXCL10 protein levels in homogenized brain samples were determined by ELISA in control and 2-d cuprizone-treated mice (co, 0.189 ± 0.0177; 2 d, 0.28 ± 0.024; n = 3, unpaired t test). (D–F) Dark-field photographs of in situ hybridization-labeled brain sections (2d cuprizone) for the respective chemokines taken at the level of the dorsal hippocampus. Hybridization signals are shown as white grains. (D) Arrows mark the deep cortical layer 6, and arrowheads the hippocampal polymorph layer of the dentate gyrus. (F) Arrows indicate strati oriens and radiatum of the hippocampal cornu ammonis region, and arrowheads the polymorph layer of the dentate gyrus. Note that a signal is observed in neither the stratum granulare, nor in the neuronal granule cell layer of the dentate gyrus or the pyramidal layer of the cornu ammonis [asterisks in (F)]. Values are given as arithmetic mean ± SEM. Scale bar, 100 µm. *p < 0.05, **p < 0.01.
by LDH release. Thus, CXCL10 is not a master regulator of oligodendrocyte loss, at least in the applied model(s).

**Impaired early microglia activation results in myelin preservation and neuroprotection in the cuprizone model**

To investigate the functional relevance of impaired microglia activation in CXCL10−/− mice, another set of animals was treated with cuprizone for 3 wk and tissues were processed for histochemistry or IHC. LFB/PAS staining was performed to study the myelin status within the CC. As shown in Fig. 4A, demyelination was evident in the midline of the CC in WT, but not CXCL10−/− mice. As expected, preservation of myelin was paralleled by preservation of oligodendrocytes (Fig. 4B) and a lower extent of concomitant microglia activation (Fig. 4C). Extent of astrocyte activation was comparable in both genotypes (Supplemental Fig. 1).

We, furthermore, analyzed the extent of acute axonal damage by means of antiamyloid precursor protein IHC. As shown in Fig. 4D, widespread acute axonal damage was evident in the midline of the CC in WT animals exposed to cuprizone for 3 wk. In sharp contrast, axonal damage was almost absent in CXCL10−/− mice (primary and secondary Abs used are listed in Tables I and II).

In summary, these results clearly show that CXCL10 orchestrates early microglia activation in this model, and amelioration of early microgliosis results in neuroprotection.

**Astrocytes are a potential source of CXCL10**

The pivotal function of CXCL10-expressing astrocytes for remyelination processes in this model has recently been highlighted (52). To characterize the source of CXCL10 during initial microglia activation, we performed IHC double labeling of GFAP and CXCL10. Fig. 5A shows the lateral CC of an animal fed cuprizone for 1 wk. A clear colocalization of GFAP and CXCL10 suggests astrocytes to be the major source of CXCL10 in this model. To further confirm this finding, double ISH labeling for Gfap and Cxcl10 mRNA was performed on paraffin-embedded tissue of control and cuprizone-fed mice (Fig. 5B). As shown in Fig. 5B, numerous Cxcl10-expressing cells were visible in cuprizone-intoxicated animals (green signal). Approximately 60% of all Gfap-expressing cells were found to be positive for Cxcl10, confirming our IHC observation that astrocytes are a major contributor of Cxcl10 synthesis after short-term cuprizone exposure (arrows in B, yellow merge signal).

It was recently shown that stimulated oligodendrocytes can secrete a variety of chemokines, among them CXCL10 (53), and thus might be an additional source of chemokines. To investigate this aspect, double ISH labeling for Plp1 and Cxcl10 mRNA was performed. Because the signal intensity of Plp1 mRNA dramatically decreases as a response to cuprizone treatment, the detection of oligodendrocytes by Plp1 probes is somewhat difficult. However, some colocalization of Cxcl10 and Plp1 mRNA was evident in cuprizone-treated animals (Fig. 5B, lower left picture). To further strengthen our observation that, besides astrocytes, oligodendrocytes express Cxcl10, we performed double ISH labeling for Atf3 and Cxcl10 mRNA. The stress-related transcription factor activating transcription factor 3 was recently shown to be selectively expressed by oligodendrocytes in this model (54). As
highlighted in Fig. 5B, some stressed oligodendrocytes (i.e., Atf3+), expressing Cxcl10, underpinning the relevance of oligodendrocytes as an additional source of this chemokine under defined stress conditions. Cuprizone’s mode of action is the inhibition of the mitochondrial chain (respiratory chain complex-2 inhibition) with a subsequent cellular stress response (31, 54). To further address the potency of astrocytes and oligodendrocytes to respond on complex-2 inhibition of the respiratory chain by CXCL10 induction, primary astrocyte cultures and OliNeu cells were treated side by side for 24 h with sublethal doses of sodium azide (SA). SA inhibits the complex-2 of the mitochondrial respiratory chain and causes cell death in a dose-dependent manner (55). IFN-γ-treated cells served as a positive control for the inducibility of CXCL10 in the applied cell culture systems. As shown in Fig. 5C, and in line with our in vivo data, astrocytes were the more potent source of CXCL10 expression compared with oligodendrocytes.

**CXCL10 induces microgliosis, attraction and activation, but not phagocytosis**

To date, our results suggest that CXCL10 is critically involved in early microgliosis. Microgliosis comprises distinct cellular properties, among them 1) microglia attraction, 2) production of proinflammatory cytokines, 3) microglia-mediated phagocytosis, and 4) proliferation of microglia cells. In a next set of experiments, we addressed the question as to whether CXCL10 directly plays a role in microgliosis and which aspect of microgliosis is mediated by this chemokine. In this part of the study, we used a microglial cell line for all following experiments (44). First, we assessed promigratory effects of CXCL10. Using the Boyden chamber assay (44), recombinant CXCL10 was applied in the lower compartment, and number of migrated cells was analyzed after a 4-h migration period. As demonstrated in Fig. 6A, recombinant CXCL10 induced directed microglial cell migration. Furthermore, we were interested in whether recombinant CXCL10 induces a proinflammatory phenotype in microglia cells. To this end, microglia were stimulated and distinct gene expression levels were analyzed. Recombinant CXCL10 induced 18- and 7-fold increases in the expression of Inos and Tnfα mRNAs, respectively (Fig. 6B). These in vitro results implicate that CXCL10 induces a proinflammatory phenotype in microglia cells. To further address this aspect, we decided to analyze the expression of distinct pro- and anti-inflammatory cytokines in dissected CC samples from WT and CXCL10−/− animals after a 1-wk cuprizone exposure period, rather than relying on an artificial cell culture system. As shown in Fig. 6C, the expression of TNF-α was significantly induced in the CC of cuprizone-treated WT animals, and this induction was significantly less pronounced in CXCL10−/− mice. Other proinflammatory cytokines, such as IL-6 or CD11b, a component of the C3 complement receptor, were as well higher expressed in...
cuprizone-exposed animals. However, expression levels were similar in both genotypes (Supplemental Fig. 1B). Cuprizone-induced early microglia activation was as well paralleled by the induction of anti-inflammatory cytokines such as IL-10 or FGF2 (Supplemental Fig. 1B). Again, however, no difference was observed in WT versus CXCL10潮流/2 mice. Interestingly, BDNF expression was significantly lower in cuprizone-exposed WT animals compared with controls, and this reduction was ameliorated in CXCL10-deficient mice (Fig. 6C). These data indicate that CXCL10 fine-tunes the pro- and anti-inflammatory response during cuprizone-induced demyelination (primers used are shown in Table III).

Phagocytosis is another cardinal function of microglia, and this aspect of microgliosis might as well be regulated by CXCL10. Cuprizone-induced early microglia activation was as well paralleled by the induction of anti-inflammatory cytokines such as IL-10 or FGF2 (Supplemental Fig. 1B). Again, however, no difference was observed in WT versus CXCL10潮流/2 mice. Interestingly, BDNF expression was significantly lower in cuprizone-exposed WT animals compared with controls, and this reduction was ameliorated in CXCL10-deficient mice (Fig. 6C). These data indicate that CXCL10 fine-tunes the pro- and anti-inflammatory response during cuprizone-induced demyelination (primers used are shown in Table III).

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WT mice. Percentage of IBA1-expressing cells positive for the proliferation marker PCNA was, however, similar in both genotypes (Fig. 7B). Additional in vitro experiments showed that CXCL10 treatment does not result in increased metabolic activity in microglia cells, indicating that CXCL10 does not induce microglia proliferation in vitro (Fig. 7C). Taken together, our studies indicate that CXCL10 promotes microglia chemotaxis and the induction of a proinflammatory (M1) phenotype, but does not regulate microglial phagocytosis or proliferation.

Discussion
In the current study, we demonstrate the following: 1) that early oligodendrogliopathy in the cuprizone model is paralleled by the induction of various chemokines, predominantly CXCL10; 2) that early microglia activation is regulated by CXCL10; and 3) that demyelination and concomitant axonal damage are ameliorated in later lesion stages in CXCL10−/− mice. Furthermore, we identified astrocytes as the predominant source of CXCL10 in this model. These data strongly suggest that astrocyte-derived CXCL10 contributes to the development of brain-intrinsic inflammatory processes.

Significance of the study using the cuprizone model
The cuprizone model is a frequently used tool to study regenerative processes in the brain, that is, remyelination (26, 35, 52, 58, 59). These studies are many times reported in the context of the demyelinating disorder MS; however, principal mechanisms underlying reappearance of new myelinating oligodendrocytes are most likely relevant for a broad spectrum of diseases in which myelination is disturbed. Relatively few groups address early pathological events in the cuprizone model. As previously shown, feeding of cuprizone to young adult mice induces an early and selective apoptosis of oligodendrocytes (25) that is closely followed by microglia activation (60). The underlying molecular pathways of cuprizone-induced oligodendrocyte cell death are not well understood, but it is widely assumed that a cuprizone-induced copper deficit is detrimental to mitochondrial function in the brain, and that the subsequent disturbance of energy metabolism in oligodendroglia leads to apoptosis. There is not enough evidence to relate this model to a specific disease to date. As stated in the introduction of this manuscript, various brain disorders are characterized by myelin abnormalities and/or oligodendrogliopathy. Although some similarities exist between early MS lesions and cuprizone-induced oligodendrocyte death, such as absence of T cells (61) or oligodendrocyte apoptosis with concomitant activation of microglia (62), there is not enough evidence to date to state that a disturbance of oxidative phosphorylation, as operant in the cuprizone model, is causally linked to MS lesion development or progression. However, because oligodendrocyte apoptosis can be highly reproducible induced, this model is an ideal tool to study the relation of oligodendrocyte death and the activation of glia cells. By genome-wide array analyses, we could recently demonstrate that the expression of a broad spectrum of genes is significantly altered after short-term cuprizone exposure (63). In this study, we re-evaluated this gene expression study focusing on chemokine expression levels, and subsequently examined the role of CXCL10 in the context of early microgliosis.

Table I. Primary Abs used for IHC/ICC

<table>
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<tr>
<th>Ab</th>
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<th>Ag Retrieval</th>
<th>Supplier Catalogue No.</th>
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<td>Tris/EDTA</td>
<td>Millipore, MAB 348</td>
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<td>Tris/EDTA</td>
<td>Wako, 019-19741</td>
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<td>MAC3</td>
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<td>PCNA</td>
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Table II. Secondary Abs for IHC/ICC

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<th>Dilution</th>
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<td>Biotinylated anti-rabbit IgG</td>
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<td>Biotinylated anti-rat IgG</td>
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<tr>
<td>Biotinylated anti-goat IgG</td>
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<td>DAKO Deutschland E0466</td>
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<td>Anti-mouse Alexa Fluor 488</td>
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Histopathological effects of CXCL10 deficiency in the cuprizone model

Chemokines are significantly induced after short-term cuprizone intoxication. Although some of these chemokines appear to have redundant functions (such as CCL2 and CCL3), at least with respect to microglia activation, knockout experiments revealed that the chemokine CXCL10 is critically involved in early microglia responses. Whereas the number of apoptotic cells is unchanged in CXCL10−/− mice in early cuprizone lesions (i.e., 1-wk exposure), a reduced loss of oligodendrocytes was found in later lesion stages (i.e., 3-wk exposure). These results indicate that, despite direct toxic effects of cuprizone on oligodendrocytes, microglia actively contribute to lesion formation and progression in this model. Furthermore, the early impact of CXCL10 deficiency on microglia manifests in an amelioration of demyelination at later lesion stages, as indicated by a reduced loss of myelin. Reduced demyelination is accompanied by reduced axonal damage, as indicated by lower amount of anti-amyloid precursor protein+ bulbs within the CC of CXCL10−/− mice in comparison with WT animals. Whether or not reduced axonal degeneration is causally linked to the absence of CXCL10 is hard to address. Because activated microglia are well known to mediate axonal damage in MS (64, 65), we consider it more likely that the initial inhibition of microglia activation in CXCL10−/− animals finally results in less demyelination with less severe axonal damage.

Source of CXCL10 in the cuprizone model

Astrocytes are well described as potential source of diverse chemokines, among them CCL5/RANTES (66), CCL2/fractaline (67), CCL2/MCP1, CCL7/MCP-3 (68), CCL3/MIP-1α (69), CXCL1/CINC-1 (70, 71), or CXCL8/IL-8 (72). With respect to CXCL10, it has been demonstrated that astrocytes express and secrete this particular chemokine under various experimental conditions among virus-associated myelopathy (73), Lyme disease (74), Rasmussen encephalitis (75), EAE (76), coronavirus-induced encephalomyelitis (77), experimental African trypanosomiasis (78), or Aicardi-Goutières syndrome (79).

FIGURE 5. Astrocytes express CXCL10 in the early phase of cuprizone intoxication and in vitro. (A) Shows immunohistochemistry double labeling for CXCL10 (red) and the astrocyte marker protein GFAP (green) of mice treated with cuprizone for 1 wk. Cell highlighted by the inset is shown in a higher magnification. Note that the CXCL10 signal colocalizes with the astrocyte marker GFAP in the CC. Upper pictures in (B) show in situ hybridization using fluorophore-labeled probes against Cxcl10 mRNA (green) and Gfap mRNA (red), respectively, after 2-d cuprizone exposure. Slides have been analyzed by confocal laser-scanning microscopy. Arrows indicate double-positive cells. Lower left picture in (B) shows in situ hybridization against Cxcl10 mRNA (green) and oligodendrocyte marker protein Plp1 (red), respectively, after 2-d cuprizone exposure. Lower right picture in (B) shows double in situ hybridization against Cxcl10 mRNA (green) and mRNA marking stressed oligodendrocytes, i.e., Atf3 mRNA (red). Double-positive cells are marked with arrows. In (C), the quantification of mRNA (qRT-PCR) and protein expression (ELISA) of CXCL10 are shown in cultures of primary mouse astrocytes and the murine cell line OliNeu. Cells were treated with SA or IFN-γ as positive control. Values are given as arithmetic mean ± SEM. Scale bars, 50 μm in (A) and 10 μm in (B). *p < 0.05, **p < 0.01, ***p < 0.001.
We were recently able to demonstrate that CXCL10, derived from activated astrocytes, orchestrates myelin repair after cuprizone-induced demyelination (52). In this study, we further investigated the source of CXCL10 during initial oligodendrocyte apoptosis. In situ hybridization and IHC double-labeling experiments clearly showed extensive Cxcl10 mRNA and protein expression in astrocytes. Furthermore, we and others were recently able to show that astrocytes in vitro secrete CXCL10 under various proinflammatory conditions such as stimulation with LPS, IFN-\(\gamma\), or TNF-\(\alpha\) (52). Because cuprizone is believed to mediate its toxic effect by a disturbance of mitochondrial function in the brain, the mitochondrial toxin SA was used to mimic in vivo effects of cuprizone in cell culture. In line with our finding that astrocytes are the main source of CXCL10 after early cuprizone intoxication, no longer than 24 h of SA exposure was sufficient to induce Cxcl10 mRNA expression and protein release in astrocyte cultures. In contrast, cultured oligodendrocytes just moderately induced Cxcl10 mRNA expression, whereas CXCL10 protein release was not detected after 24 h of SA exposure. These results suggest that astrocytes are the first-line producers of CXCL10, whereas other cell types, such as microglia or oligodendrocytes, can synthesize Cxcl10 during later stages of lesion progression. Results of ISH double-labeling experiments indeed showed that some PLP\(^+\) oligodendrocytes express Cxcl10 after short-term cuprizone exposure (compare Fig. 5B). It remains, however, to be determined whether mature or premature oligodendrocytes are more potent in Cxcl10 production. In either case, our results indicate that cells from the oligodendrocyte lineage might actively modulate the inflammatory microenvironment and, thus, contribute to lesion formation and progression. Further studies have to show the potency of oligodendrocytes to function as an immunomodulatory cell type. Beyond, experiments using conditional knockout mice will have to show whether oligodendrocytic CXCL10 functionally contributes to lesion formation in the cuprizone model. Finally, other chemokines than CXCL10 might be expressed by stressed oligodendrocytes.

Role of CXCL10 in neuroinflammation

CXCL10 exerts pleiotropic effects on various immune and non-immune cells. For example, CXCL10 performs homing functions to attract CXCR3-positive cells, including macrophages, dendritic cells, NK cells, and activated T lymphocytes (CD4\(^+\) Th cells, CD8\(^+\) Tc cells) toward inflamed, infected, and/or neoplastic areas. In

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**FIGURE 6.** CXCL10 induces chemotaxis and a proinflammatory phenotype in microglial cells, but does not increase phagocytic activity. (A) In vitro migration two-chamber assay of cultured microglial cells (ESdM). Note that recombinant CXCL10 (50 ng/ml) induced directed migration of microglial cells. (B) qRT-PCR of cultured microglial cells stimulated with CXCL10 for 24 h. Note the induction of inos, Tnfa, and Cxcl10 transcripts in CXCL10-stimulated microglia. (C) In vivo qRT-PCR analysis of mRNAs for Tnfa and Bdnf of CC samples of control animals and animals treated with cuprizone for 1 wk. (D) Flow cytometry histograms show representative results obtained with the bead phagocytosis assay. Only cells that phagocytosed three or more beads were counted as phagocytically active microglial cells (ESdM). Numbers in the histograms indicate the percentage of phagocytically active microglial cells. Control: untreated microglia; LPS: microglia treated for 24 h with 500 ng/ml LPS; CXCL10: microglia treated for 24 h with 10 ng/ml CXCL10; LPS/CXCL10: microglia treated for 24 h with both LPS and CXCL10. (E) Quantification of phagocytic activity of microglial cells. Note that CXCL10 does not change the phagocytic activity of microglial cells, whereas LPS increased the uptake of beads. Results are presented as fold induction over controls. (F) Anti-MAC3-stained sections of untreated control, WT, and CXCL10\(^{-/-}\) mice treated for 1 wk with cuprizone. Note that MAC3 immunoreactivity is similar in WT and CXCL10\(^{-/-}\) animals. Scale bar, 20 \(\mu\)m. *p < 0.05, **p < 0.01, ***p < 0.001.
particular, it has been shown that CXCL10 acts as a chemoattractant for activated CD8+ T cells (83), plays a role in effector T cell generation and trafficking (84), generates a Th1-favored, proinflammatory response (85), appears to be involved in NK cell recruitment (86), drives plasma cell differentiation (87), and has been linked to the trafficking of immune cells into malignant disease sites (88). Furthermore, CXCL10 can inhibit vascular smooth muscle cell proliferation (89), or endothelial cell proliferation in vitro independently of CXCR3 (90).

Functional studies that aimed at defining the role of CXCL10 in EAE have produced conflicting results. Ab-mediated neutralization of CXCL10 protein has been shown to be protective in certain disease stages of murine hepatitis virus-infected mice (91) or during adoptive EAE (92). Wojcik et al. (93) showed that intrathecal infusion of antisense oligonucleotides against Cxcl10 reduced clinical signs in Lewis rats with myelin basic protein–induced EAE. In contrast, blocking of CXCL10 has been reported to exacerbate EAE (94). Moreover, CXCL10−/− mice display similar induction and severity of EAE as compared with WT controls (95). In line with these preclinical studies, various reports indicate that CXCL10 is involved in MS pathogenesis. For example, it has been shown that CXCL10 protein levels are increased in the cerebrospinal fluid (CSF) of MS patients during an acute relapse (96–98). Regression analysis indicated a significant linear relationship between CSF cell count and CXCL10 concentrations in the CSF (97). Comini-Frota et al. (99) reported that serum levels of CXCL10 are elevated in MS patients and correlated positively with relapses and IFN-β-1a/b-immunomodulatory therapy. Histopathological studies revealed that CXCL10 immunoreactivity is mainly associated with astrocytes around inflammatory lesions (97, 100–102), as described previously in EAE (103–105). CXCR3, the receptor of CXCL10, was found to be expressed in the CNS of MS patients. CXCR3+ cells were found to comprise up to 25% of perivascular mononuclear cells in MS lesions. Different from other chemokines, CXCL10 binds exclusively to its receptor

Table III. List of primers used for qRT-PCR

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<th>Name</th>
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<th>Reverse Sequence 5'-3'</th>
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<td>Cxcl10 mouse</td>
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<td>Cxcl10 rat</td>
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<td>Tspo mouse</td>
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<tr>
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<td>CAG AGG AGG CCT TGT TC</td>
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<tr>
<td>Irs mouse</td>
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<td>Gapdh mouse</td>
<td>ACA ACT TTA GCA TTA TGG AA</td>
<td>GAT GCA GGG GAT AGT TTT GC</td>
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</table>

FIGURE 7. CXCL10 does not regulate microglia proliferation. (A) Anti-IBA1 (red)– and anti-PCNA (green)–stained sections of WT and CXCL10−/− mice after 1-wk cuprizone treatment (arrowheads indicate double-positive cells; nuclei are marked in blue). (B) Relative quantification of anti-IBA1/PCNA double-positive cells in the CC of cuprizone-treated mice. (C) Treatment of cultured microglial cells (ESdM) with CXCL10 for 24 h. Note that CXCL10 did not change the metabolic activity of cultured microglial cells as determined by the cell titer blue assay. Scale bars, 50 μm.
CXCR3, shared by two other IFN-inducible proteins, that is, CXCL9 and CXCL11 (106-108).

Most studies link CXCL10 expression to the recruitment and distribution of T cells (84, 91, 92, 109). However, monocyte recruitment has been reported as well (110, 111). Evidence arises that microglia are critically involved in the initiation and progression of a variety of neurodegenerative diseases, among them MS and Alzheimer’s disease (112, 113). Although increased Cxcl10 expression levels in this model have already been reported by Biancotti et al. (81), not much is known about the impact of CXCL10 on microglia function in general and in the cuprizone model in particular. Despite the impact of CXCL10 deficiency on histopathological changes in cuprizone-fed mice, our in vivo results demonstrate effects of CXCL10 on microglia activation status. Stimulation of cultured microglia cells with recombinant CXCL10 induces TNF-α expression, whereas the cuprizone-induced rise in brain TNF-α levels is less intense in CXCL10-deficient mice. These results indicate that CXCL10 selectively regulates the expression of distinct proinflammatory cytokines in microglia. Furthermore, we were able to demonstrate that BDNF, a growth factor that is believed to limit brain damage and contribute to the repair process (114), is less intensively downregulated in CXCL10−/− mice. Other anti-inflammatory cytokines such as FGF2 or IL-10 are, in contrast, not under the control of CXCL10. Together with the notion of inflammatory cytokines such as TGF-β and IL-10 are, in contrast, neuroinflammation and tissue destruction. In this manuscript, the importance of astrocytes as ambiguous, as they have the capacity to both stimulate and restrain cellular events, such as further induction of astrocyte activation and/or sustenance of microgliosis.

Interestingly, peak Cxcl10 expression was observed at week 1 with a significant decline at week 2 after initiation of the cuprizone diet. In another study, we were able to show that, similar to Cxcl10, Ccl2 induction is a transient phenomenon in this model, whereas the mRNA levels for Ccl3 continuously increased (25). One might speculate that CCL2 and/or CXCL10 mediate early induction is a transient phenomenon in this model, whereas the mRNA levels for CCL3 continuously increased (25).

Conclusion
To our knowledge, this is the first report showing that CXCL10 is critical for microglia activation in a nonimmune driven demyelination model. Furthermore, the importance of astrocytes as modulators of neuroinflammatory processes is highlighted. The role of astrocytes in neuroinflammatory disorders is rather ambiguous, as they have the capacity to both stimulate and restrain neuroinflammation and tissue destruction. In this manuscript, we provide evidence strong that astrocytes participate in neuroinflammatory processes during early stages of CNS tissue damage, and their modulation might, thus, be an appropriate strategy to prevent neuroinflammation and CNS tissue destruction in the future.

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Disclosures
The authors have no financial conflicts of interest.

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