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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), Thieberger’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 de- and remyelination 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, astrocytosis, 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 No. 000664) at Charles River (Sulzfeld, Germany) and served as controls for CCL2, CCL3, and CXCL10 knockout (CCL2+/−, CCL3+/−, CXCL10+/−) mice. CCL2+/− and CCL3+/− mice were obtained from The Jackson Laboratory (B6.129S4-Ccl2−/−, B6.129P2-Cc3m1OdxJ), CXCL10+/− mice were provided as a gift of U. Christen (Pharmazentrum Frankfurt/ZAFES, Klinikum der Goethe Universität, Frankfurt/Main, Germany) (32). Male mice were used for all experiments. The mice were housed under standard laboratory conditions according to 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 (biss-cyclohexamone oxaddehydron; 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 cardially perfused with 2% paraformaldehyde. After overnight postfixation (1000 g for 10 min at 4˚C), supernatant was discarded. For acetylcholinesterase (AChE) or biocytin staining, brains were homogenized in ice-cold PBS, as published (37). Tissue preparation was performed, as previously described (34–36). For gene expression analyses, mice were assessed 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.

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 peroxidase–avidin–biotin complexes. 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 in H&E stained sections was performed using well-defined morphological criteria, such as condensation and fragmentation of 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 fluorescent in situ hybridization kits (Quantitative Gene 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 time was adjusted to 20 min. Probes for Ccl2, Ccl3, Cxcl10, and Afp as well as the cellular markers Gap and Ppil1 were purchased from Affymetrix (Affymetrix-Panomics). Radioisotopic 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 station (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 replenished 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 biotin (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 human 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 a differentiation medium to generate mature oligodendrocytes. Differentiation medium consisted of DMEM, 1× N2 supplement, 10 nM biotin, 15 nM 3,3′,5-triiodo-L-thyronine (T3; Sigma-Aldrich), 0.1% BSA, 1% P/S, 1× N-acetyl-t-cysteine, and 10 ng/ml rat recombinant ciliary neurotrophic factor (PeproTech). After 9 d, the cells were allowed to change their morphology (high number of cellular processes) were clearly visible (43). Purity of primary oligodendrocyte cultures was determined by immunocytochemistry (ICC). Cultures contained 0.3673 ± 0.3673% IBA1+ microglia and 8.1 ± 1.225% GFAP+ astrocytes (values are given as arithmetic mean of n = 3 experiments ± SEM). Mouse embryonic stem–derived microglial precursors (ESdM) were prepared as published previously (44). Cells were cultured in serum-free DMEM/F12 medium (Life Technologies) containing 1× N2 supplement, 0.48 mM t-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% FBS. For additional 24 h, cells were treated with sodium azide (SA; 10 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 titrer assays. Supernatant was collected from 6-well plates for ELISA, and
remaining cells were washed with 1× Dulbecco’s PBS and lysed in Peggold 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 mM 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 Peggold 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 × 105 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-latex beads (Polysciences) for 1.5 h. Flow cytometry was performed using the BD LSRFortessa cell analyzer (BD Biosciences), and results were analyzed using FlowJo 7.6.5 (Tree Star). To investigate, whether ESdM cells are comparable to microglia cells with regard to the phagocytosis of physiologically relevant cargo, ESdM cells were exposed to 15 μg protein/ml myelin-enriched mouse brain debris (see above). After 24 h, cells were fixed in 10% formalin for 10 min, stained for proteolipid protein (PLP) using a fluorescence-labeled secondary Ab (AlexaFluor 488; Invitrogen), and internalized PLP visualized using the confocal LSM710 laser-scanning microscope (Carl Zeiss AG).

CellTiter Blue Cell viability and CytoTox nonradioactive cytotoxicity assay

Cell viability and cytotoxicity assays were performed according to the manufacturer’s protocol (Promega). Briefly, cells were treated, as indicated, and supernatants were used for LDH-based cytotoxicity assay. After 30-min incubation at room temperature, LDH activity was monitored with a Tecan infinite 200 plate reader at 490 nm. Results were normalized to lysis control and subsequently expressed as percentage of control. The remaining cells were incubated with CellTiter Blue solution for ∼4 h. Fluorescence intensity of resorufin in supernatants was then measured at 560/590 nm, mean value of lysis control was subtracted from all samples as background, and treated samples were normalized to control samples.

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). Total RNA (n = 3 per treatment group) 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 RMA (Robust Multi-array Average) algorithms. Gene expression was quantified by the expression intensity, which was calculated as background, and treated samples were normalized to control samples as background. Gene expression 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 technology (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 Cyc3 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 405 nm with a wavelength correction at 650 nm. For in vivo samples, CXCL10 release was normalized to total protein content of cell lysate, determined with Pierce bicinchoninic acid protein assay (Thermo Fisher Scientific). For measurement of CXCL10 protein in protein lysates of mouse brain tissue, lysates were prepared from cortex and CC samples of control mice or mice treated for 2 d with cuprizone. Briefly, brains were homogenized in ice-cold PBS containing protease-inhibitor mixture (Roche) using precellly tissue homogenizer (PegLab). Brain lysates were subsequently centrifuged twice for 20 min at 12,000 × g at 4°C, and the supernatant was stored at −80°C until used.

Statistical analysis

If not stated otherwise, at least two independent experiments were performed with a group size of ≥5 animals per experimental group. Statistics were performed using absolute data. Intergroup differences were tested by ANOVA, followed by Tukey’s or Bonferroni post hoc tests using GraphPad Prism 5 (GraphPad Software), if not stated otherwise. All data are given as arithmetic means ± SEM. The p values are indicated as *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001. For further information, see figure legends.

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 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 ± 15.53 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
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).

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 *Cxl10* 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 *Aif3* and *Cxl10* 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$^+$) express 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 culture s 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 microglia 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 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 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.

**FIGURE 3.** CXCL10 does not affect oligodendrocyte apoptosis. (A) Anti-oligodendrocyte transcription factor 2 stained sections of the midline of CC in control (co), WT, and CXCL10$^−/−$ mice after 1 wk of cuprizone exposure. Note that WT (740.4 ± 94.75, n = 17) and CXCL10$^−/−$ (513.3 ± 44.35, n = 17) mice showed a reduction in the number of cells compared to control mice (1331 ± 91.49, n = 14). (B) Evaluation of apoptotic cells in H&E-stained sections within the CC after 1-wk cuprizone treatment. Note that no significant difference is evident between WT (69.34 ± 14.27, n = 11) and CXCL10$^−/−$ (102.1 ± 9.89, n = 12) mice. Black arrows indicate apoptotic cells. (C) Primary cultures of OPCs and mOL were treated with recombinant CXCL10 for 24 h. Treatment of primary oligodendrocyte cultures with CXCL10 (1, 10, or 100 ng/ml) did not induce cell death as determined by LDH release. Furthermore, the murine oligodendrocyte cell line OliNeu was treated with 1, 10, or 100 ng/ml CXCL10 for 24 h, and cell death was determined by LDH release. Results are shown in (D). Values are given as arithmetic mean ± SEM. Scale bar, 100 μm in (A) and 10 μm in (B).
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 CXCL10^2/2^ 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. It has been demonstrated that the phagocytic activity of the applied ESdM cell line can readily be measured by quantifying the uptake of labeled microsphere beads by flow cytometry (44). However, in a first step, we deemed it mandatory to investigate whether this particular microglia cell line can phagocytose physiological relevant cargos, such as myelin debris. Therefore, cells were treated with myelin debris for 24 h, and the uptake was visualized by means of anti-PLP ICC. As demonstrated in Supplemental Fig. 1C, PLP-positive myelin debris accumulates within the intravesicular compartment of cultured ESdM cells. In a next step, we quantified the effect of recombinant CXCL10 on the ability of microglia phagocytosis rate by means of polystyrene latex beads ingestion (44). As a positive control, we used LPS, which induced a 2-fold increase in phagocytosis activity. We found that CXCL10 did not affect phagocytosis of latex beads, neither under basal conditions nor after LPS stimulation (Fig. 6D, 6E). These in vitro results indicate that CXCL10 does not modulate microglia phagocytosis. To verify these findings in vivo, the CC of wild-type and CXCL10^2/2^ mice were stained for the MAC-3/lysosomal-associated membrane protein 2, which is a reliable marker for late-stage lysosomes and phagocytosis (56, 57). In line with our in vitro results, the amount of MAC-3–positive cells was similar in both strains after 1 wk of cuprizone exposure (Fig. 6F).

Finally, we addressed whether CXCL10 affects the proliferation of microglia cells. To this end, brain sections from WT and CXCL10^2/2^ mice were double stained for the proliferation marker proliferating cell nuclear Ag (PCNA) and the microglia marker IBA1. Numerous PCNA-expressing cells were found in the CC of cuprizone-exposed mice (Fig. 7A). As expected, numbers of IBA1^+ cells were lower in CXCL10^2/2^ compared with CXCL10-deficient mice were exposed to 0.2% (w/w) cuprizone for 3 wk. In (A), the myelin status of the CC as determined by LFB/PAS staining is shown. Results are given as percentage of myelination. Note reduced loss of myelin-staining intensity in CXCL10^2/2^ mice when compared with WT. Reduced loss of oligodendrocytes (B) as well as lower numbers of microglia (C) were found in cuprizone-fed CXCL10^2/2^ mice when compared with WT. (D) The number of APP^+ bulbs (arrows) indicating axonal damage was induced in cuprizone-fed mice when compared with controls. Axonal damage was ameliorated in cuprizone-fed CXCL10^2/2^ mice. In (E), representative pictures of the evaluated histological stains are shown for control as well as 3-wk cuprizone-fed animals. White arrows indicate axonal APP^+ bulbs. Values are given as arithmetic mean ± SEM. Scale bar, 100 μm. *p < 0.05, **p < 0.01, ***p < 0.001.
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. 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.

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 oligodendrogliocyte pathology. 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 oligodendrogliocyte 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>
<th>Host</th>
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<th>Dilution</th>
<th>Ag Retrieval</th>
<th>Supplier Catalogue No.</th>
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<td>IBA1</td>
<td>Rabbit</td>
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<td>Wako, 019-19741</td>
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<td>MAC3</td>
<td>Rat</td>
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<td>OLIG2</td>
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<td>PCNA</td>
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<td>Biotinylated anti-mouse IgG</td>
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<td>Biotinylated anti-rabbit IgG</td>
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<td>Goat</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/fractalkine (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).
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-γ, or TNF-α (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.

With respect to microglia, we observed that the stimulation with recombinant CXCL10 modestly induces the expression of Cxcl10 mRNA in ESDM cells, an observation that is in line with other reports (80). However, we did not identify microglia as a source of CXCL10 in early cuprizone-induced lesions. This discrepancy might be due to astrocytic modulation of microglia function in vivo, for example, by the production of ILs such as IL-4 or IL-10 (80, 81). Both ILs have been shown to be induced in cuprizone-fed animals (81, 82).

**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
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 proven 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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<td>GCT GGA ATC TCC CGG CTG TAG</td>
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<tr>
<td>Cxcl10 mouse</td>
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<td>Tifa mouse</td>
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<td>GCT AGC AGG TGC GCT ACA G</td>
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<td>Bdnf mouse</td>
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<td>ATC CAC CTT GCC GAC TAC AG.</td>
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<tr>
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<td>CAG AGG GAT CTT GTC CT.</td>
</tr>
<tr>
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<td>Cal11 mouse</td>
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<td>Cc5 mouse</td>
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<td>Cc5 mouse</td>
<td>ACA ACT TTG GCA TTG TGG AA</td>
<td>GAT GCA GGG ATG ATG TGG TTG</td>
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</tbody>
</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 FGF2 or IL-10 are, in contrast, prevent neuroinflammation and CNS tissue destruction in the biguous, as they have the capacity to both stimulate and restrain roles of astrocytes in neuroinflammatory disorders is rather am-

modulators of neuroinflammatory processes is highlighted. The cellular events, such as further induction of astrocyte activation and/or sustenance of microgliosis. One might speculate that CCL2 and/or CXCL10 mediate early results display highly specific effects of CXCL10 on microglia in the cuprizone model in particular. Despite the impact of 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 reduced axonal damage in CXCL10−/− mice, we provide substantial evidence for the assumption that CXCL10 particularly induces a detrimental phenotype in microglia cells. Beyond, our results display highly specific effects of CXCL10 on microglia in the context of early oligodendrogliopathy and highlight the complex intercellular communication of astrocytes, microglia, and—potentially—oligodendroglyocytes.

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 expressions in an experimental demyelination animal model and multiple scle-

References


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Conclusions

To our knowledge, this is the first report showing that CXCL10 is critical for microglial activation in a nonimmune driven demy-

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94. Narumi, S., T. Kaburaki, H. Yoneyama, H. Iwamura, Y. Kobayashi, and 
97. Sørensen, T. L., M. Tani, J. Jensen, V. Pierce, C. Luccinetti, V. A. Folick, 
Supplementary figure 1

In (A), representative pictures of stains against the astrocyte marker protein GFAP are shown for control animals and animals fed cuprizone for 3 weeks. Induction of GFAP+ cell numbers was comparable in cuprizone fed WT and CXCL10−/− animals when compared to controls. In (B), expression levels determined by RT-qPCR of Il10, Il6 and Fgf2 mRNA are shown in the CC of animals treated with cuprizone for 3 weeks. Cuprizone leads to a comparable induction of all genes in both WT and CXCL10−/− mice. In (C) a confocal picture of an ESDM cell treated with myelin enriched brain debris for 24 hours is shown. Internalized PLP is shown in green. *** = p < 0.001