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The role of monocytes in remyelination and repair processes in the CNS

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To my family

Contents

Chapter I: General introduction

1.1 Multiple sclerosis

Multiple sclerosis (MS) is an inflammatory demyelinating and neurodegenerative disease of the central nervous system (CNS). With around 2.5 million people worldwide being affected and a lifetime risk of one in 800, MS represents one of the primary causes of neurologic disability in young adults (Dutta and Trapp, 2007). As a distinct disease it was coined in 1868 when Jean-Martin Charcot reported characteristic scars in the brain, describing them as “la sclérose en plaques” (Talley, 2005). Some of the MS clinical features are cognitive impairment, vision loss, tremor, poor balance and coordination, weakness, etc (Compston and Coles, 2002). The majority of MS patients (~85%) initially develop a relapsing-remitting course of the disease (RRMS) characterized by clearly defined alternating episodes of neurologic disability (relapse) and recovery (remission) and many of them within a period of about 25 years exhibit a secondary progressive disease course (SPMS) characterized by steadily increasing permanent neurologic disability. Approximately 10% of patients suffer from gradual decline in neurologic function from disease onset (primary-progressive MS; PPMS) while the rest (~5%) experience progressive-relapsing MS (PRMS), having progressive neurologic decline accentuated with acute attacks with or without recovery (Dutta and Trapp, 2007; Bradl and Lassmann, 2009). Even if the precise etiology of MS is not yet known, this immunologically mediated disease seems to develop in genetically susceptible populations as a result of environmental exposures (Ramagopalan et al., 2010).

The histopathologic hallmarks of MS include breakdown of the blood-brain barrier (BBB), multifocal inflammation, demyelination, oligodendrocyte loss, reactive gliosis, and axonal degeneration which is the major cause of permanent neurologic disability (Brück and Stadelmann, 2003; Bjartmar et al., 2003). Recent studies show that mechanisms of tissue injury in MS are complex and involve numerous components of the immune system (Lassmann, 2008b). Showing significant variations between individual MS patients, active lesions largely consist of reactive astrocytes, phagocytic macrophages, T-cells (both CD4+ and CD8+), B-cells, and plasma cell infiltrates (Hu and Lucchinetti, 2009; Lucchinetti et al., 2000). The relative contribution of the different molecular players could account for lesion heterogeneity (Lassmann, 2008a). MS lesions are typically disseminated throughout the CNS, commonly affecting optic nerves, brainstem, spinal cord, cerebellum, and periventricular white matter (Compston and Coles, 2002). Although MS has traditionally been considered as a disease of the white matter, in recent years an extensive cortical and deep gray matter demyelination has been recognized and highlighted (Lassmann and Lucchinetti, 2008). Cortical lesions are especially frequent in chronic MS as opposed to patients with the relapsing course of the disease (Albert et al., 2007). In comparison to the white matter, demyelinating lesions of the gray matter are characterized by less extensive inflammation, less gliosis, and more efficient myelin repair (Bo et al., 2003a; Albert et al., 2007). This, together with the observation that the demyelinating process often stops at the cortex/white matter border (Bo et al., 2003b), raises the possibility that the pathogenesis of lesion development may differ in white and gray matter.

Demyelination together with consequent axonal and neuronal loss forms the major part of MS pathology. From animal models it is know that in a permissive environment spontaneous

remyelination efficiently occurs after demyelination (Stangel, 2008). Even if remyelination results in a thinner and shorter myelin sheath compared to developmental myelination for a given diameter of axon, it restores saltatory conduction and prevents secondary axonal damage, thus providing neuroprotection (Franklin and Kotter, 2008). A considerable proportion of MS patients in different stages of the disease acquire extensive remyelination (Patrikios et al., 2006). However, in some of the patients remyelination fails, leaving the axons vulnerable to degeneration (Franklin and Ffrench-Constant, 2008).

1.2 Animal models

Currently there is a collection of different animal models available to study specific aspects of MS pathogenesis. The first described and established as well as the most frequently used model of CNS autoimmune inflammation is experimental autoimmune encephalomyelitis (EAE). EAE can be induced in different animal species by immunization with spinal cord homogenate, myelin basic protein (MBP), proteolipid protein (PLP) or myelin oligodendrocyte glycoprotein (MOG). These proteins induce an autoimmune response which leads to the myelin loss (Gold et al., 2006). Classical models of EAE are mainly driven by major histocompatibility complex (MHC) class II-restricted CD4+ T-lymphocytes that initiate a cascade of events resulting in perpetuation of inflammation and CNS tissue damage (Schreiner et al., 2009). However, assessment of remyelination is especially complicated in EAE because de- and remyelination occur synchronously (Franklin and Ffrench-Constant, 2008).

For studying the potential viral component of MS etiology, comonly used models are Theiler's murine encephalomyelitis virus (TMVE), murine hepatitis virus (MHV) and Semliki

Forest virus (SFV), each having a distinct immunopathological mechanism. Intracerebral infection of susceptible mouse strains with TMEV causes an initial acute grey matter disease followed by a chronic progressive immune-mediated demyelination in the white matter of the spinal cord and persistent CNS infection (Ulrich et al., 2008; Ercolini and Miller, 2009).

While not attempting to absolutely mimic the pathogenesis of autoimmune CNS inflammation, several toxin-based models including the cuprizone, lysolecithin and ethidium bromide model, have been proven as useful for studying de- and remyelination (Schreiner et al., 2009). Feeding of young adult mice for several weeks with cuprizone (bis-cyclohexanone oxaldydrazone) leads to synchronous and anatomically reproducible demyelination, while its removal from the diet results in remyelination (Lindner et al., 2008). Susceptibility to cuprizone-induced demyelination is influenced by age, genetic background, gender, and toxin dosage (Taylor et al., 2009; Skripuletz et al., 2008). The underlying mechanism that leads to myelin loss in this model is not fully understood. Since cuprizone induces alterations in mitochondrial morphology and respiratory chain pathways, it has been assumed that this compound disturbes energy metabolism, making oligodendrocytes unable to mantain energy demanding myelin (Wakabayashi et al., 1977; Pasquini et al., 2007). However, it is still not clear whether copper deficiency is the reason for oligodendrocyte susceptibility since the administration of copper to cuprizone-fed animals failed to completely diminish pathological effects (Carlton, 1967). The regional cuprizone-induced demyelination pattern includes white matter tracts, mainly corpus callosum and cerebellar peduncles, and more recently it has been detected in hippocampus as well as in cerebral and cerebellar cortex (Lindner et al., 2008; Skripuletz et al., 2008; Skripuletz et al., 2010; Koutsoudaki et al., 2009). This animal model is characterized by an intact BBB

(Bakker and Ludwin, 1987), providing the advantage to selectively study factors and molecules that could affect the extent of de- and remyelination in the white and gray matter, without the influence of the peripheral immune system component.

1.3 The role of microglia

Considered as "long-lived population of tissue macrophages", microglia constitute 10-20% of the total glia and represent resident immune effector cells of the CNS. They were first described in 1919 by del Rio-Hortega and distinguished from astrocytes and oligodendrocytes (Rezaie and Male, 2002). The origin of microglial cells has been for a long time a matter of debate. Nowadays it is considered that microglial progenitors are of mesenchymal origin, having monocytic properties and deriving from primitive myeloid progenitors (Ginhoux et al., 2010). During embryonic and early postnatal development these progenitor cells (ameboid microglia) invade the CNS where they differentiate into the adult (ramified) microglia with small soma and highly branched processes (Kaur et al., 2007). In the healthy adult CNS ramified microglia sparsely express molecules associated with macrophage function and therefore have been characterized as "resting". However, this state of cells is far away from being inactive. As shown with *in vivo* two-photon imaging, these "vigilant CNS housekeepers" constantly survey their microenvironment, standing ready to support endangered neurons or tissue integrity (Nimmerjahn et al., 2005). Although long time considered as inevitably toxic, microglial activation is today regarded as a variable and adaptive process. Depending on activating conditions and stimulus nature and intensity, "alerted" microglia can be beneficial or destructive.

The ultimate result is determined by CNS tissue tolerance for those microglial reactive phenotypes (Schwartz et al., 2006; Biber et al., 2007).

Despite considerable heterogeneity in the pathogenesis of MS lesions for which four distinct patterns have been proposed, the accumulation of activated microglia and macrophages is common (Lucchinetti et al., 2000). Microglial activation and dramatic accumulation within demyelinated areas are also present in both the EAE and the cuprizone model (Gold et al., 2006; Gudi et al., 2009). Regarding different disease stages, in contrast to late-active MS lesions characterized by a homogenic population of phagocytic macrophages, early-active plaques contain a mixture of infiltrating hematogenous monocytes and microglia differentiated by morphology, localization, and surface markers (Brück et al., 1995). Microglia/macrophages are also present in inactive, demyelinated or remyelinated lesions, but to a lower extent (Brück et al., 1995). However, it is still not clear if these cells have an essential role in demyelination and axonal degeneration or whether they act as scavengers and restorers of damaged tissue (Fig. 1). It is generally accepted that neural antigen-reactive CD4+ T helper (Th) type 1 and Th17 subsets of T-lymphocytes mediate MS lesions initiation (McFarland and Martin, 2007). In these inflammatory conditions adult human microglia become competent antigen-presenting cells (APC). They upregulate the expression of MHC II and costimulatory molecules like CD40, CD80, and CD86 which in turn bind ligands expressed on T-cells, a reaction that is essential for optimal APC function (De et al., 1995; Aloisi, 2001). Microglia present endogenous myelin antigen to T-lymphocytes, resulting in activation of both cell types. In response to this interaction T-cells secrete pro-inflammatory cytokines like interferon-gamma (IFN-γ), interleukin (IL)-2, and tumor necrosis factor-alpha (TNF-α) that promote microglial phagocytosis and activate them

to further overexpress antigen-presenting and co-stimulatory molecules as well as lipolytic and proteolytic enzymes (e.g. matrix metalloproteinases; MMPs), reactive oxygen and nitrogen intermediates, excitotoxins, chemokines, pro-inflammatory/cytotoxic cytokines, all having potential to induce tissue injury and attract other immune cells to the inflammation site (Merson et al., 2010). Several studies demonstrated the importance of Toll-like receptors (TLRs) in MS pathology and their expression on microglia in active lesions (Aravalli et al., 2007; Bsibsi et al., 2002). TLRs are a family of pattern-recognition receptors which bind highly conserved structural motifs essential for the survival of pathogens, such as lipopolysaccharide (LPS) (Akira et al., 2006). They play a major role in infectious but also in non-infectious CNS diseases and their activation leads to oligodendrocyte and neuronal injury (Lehnardt et al., 2002; Lehnardt et al., 2003). There are a variety of endogenous ligands for TLRs which may be present within MS lesions, e.g. fibrin and oxidised lipids (Lassmann, 2008a). Stimulation of microglia with TLR agonists leads to increased secretion of many soluble immune mediators, including TNF-α, interleukin (IL)-1β, -10, -12, -6, nitric oxide (NO) as well as chemokines such as CCL2, CCL3 and CCL5 (Lehnardt, 2010). Engagement of TLRs on APC induces upregulation of costimulatory molecules representing the link between innate and adaptive immune responses (Zhang and Ghosh, 2001). This can reactivate infiltrating antigen-specific T-cells and, together with the recruitment of monocytes from peripheral blood that accumulate in the lesions and differentiate into microglia, may set off a domino effect of inflammation propagation (Jack et al., 2005; Mildner et al., 2007). However, MS lesions are self-limited and it is likely that local regulatory mechanisms contribute to the resolution of inflammation and promotion of tissue repair. Autoreactive T-cells ultimately undergo programmed cell death, being subjected to phagocytosis

by microglia (Pender, 1999). Fas (CD95) and Fas ligand (CD95L) are involved in this mechanism as well as in elimination of excessive microglia/monocytes (Kohji and Matsumoto, 2000). Microglial phagocytosis of apoptotic T-lymphocytes and myelin debris has shown to be neuroprotective, inducing downregulation of pro-inflammatory cytokines (TNF-α, IL-1β, IL-12, CCL5, MMP-9) and upregulation of anti-inflammatory factors (IL-10, transforming growth factor beta; TGF-β) (Chan et al., 2006; Boven et al., 2006). Associated with spontaneous remission in MS and recovery from EAE, Th2 cell derived cytokines e.g. IL-4, IL-10, IL-13, and TGF-β can directly inhibit pathogenic Th1 and Th17 cells and suppress pro-inflammatory and cytotoxic functions of microglia/macrophages (Nakayama and Yamashita, 2008; Merson et al., 2010). Moreover, efficient clearance of myelin debris generated during demyelination is critical for differentiation of oligodendrocyte precursor cells (OPC) and subsequent remyelination (Kotter et al., 2006). Two of the recently described microglial receptors whose activation antagonize pro-inflammatory activity resulting in tissue repair are CD200R and TREM-2 (triggering receptor expressed on myeloid cells-2), being proposed as attractive therapeutic targets (Koning et al., 2007; Takahashi et al., 2007). It is likely that many of the molecules produced by activated microglia referred as cytotoxic have dual and context-dependent effects. For instance, in the cuprizone-mediated demyelination model TNF-α has been shown to promote remyelination (Arnett et al., 2001), while mice carrying a deletion for MHC II or inducible nitric oxide synthase (iNOS) showed impaired remyelination and more severe demyelination, respectively (Arnett et al., 2003; Arnett et al., 2002). Similarly, matrix metalloproteinases have been shown to mediate pathogenesis of demyelinating diseases, although their role in neurogenesis and myelin formation has been demonstrated (Yong, 2005).

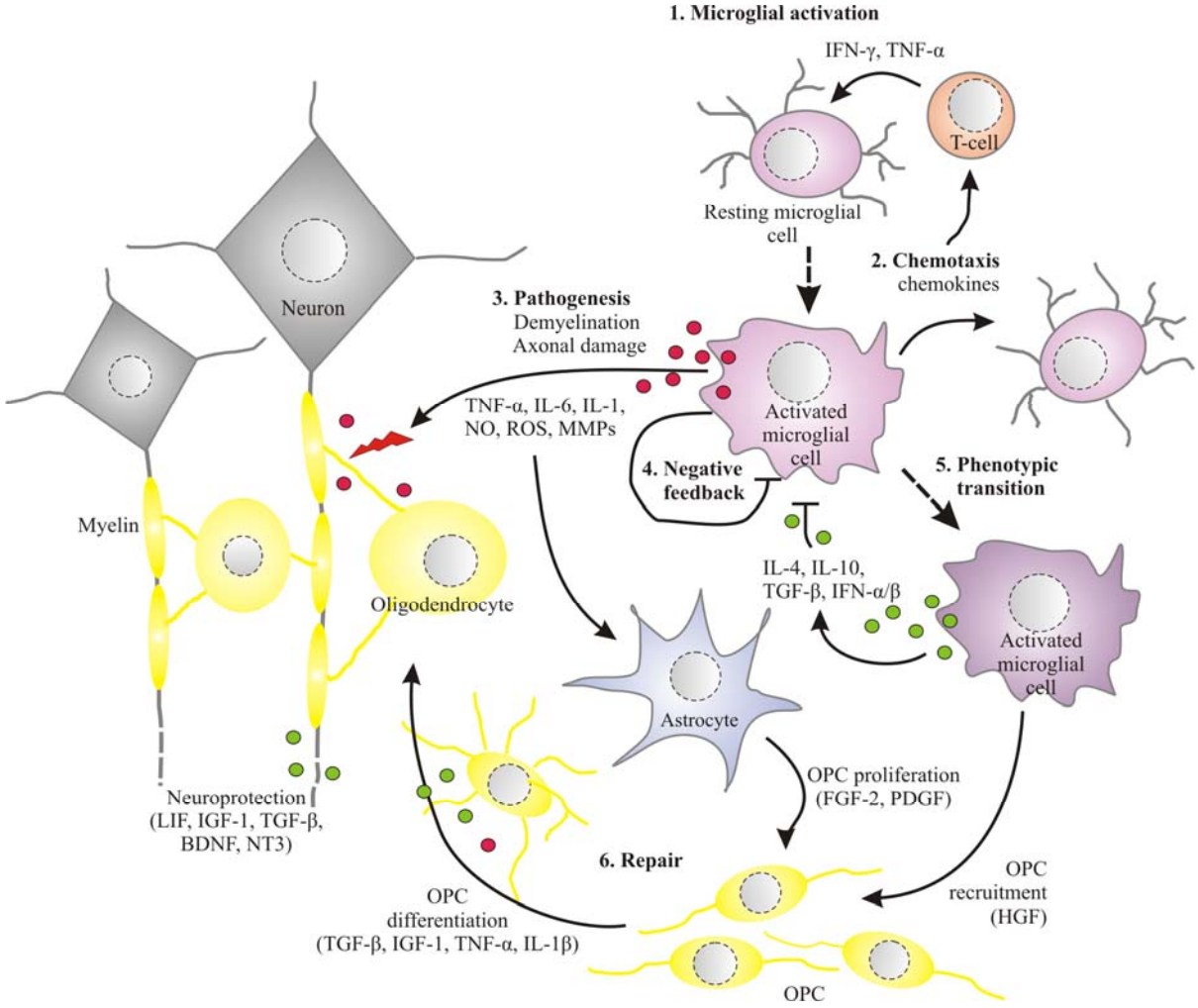

Figure 1. Microglial soluble mediators that contribute to neurodegeneration and repair during CNS inflammatory demyelination (modified from Merson et al., 2010). Abbreviations that are not in the text: FGF, fibroblast growth factor; PDGF, platelet-derived growth factor; HGF, hepatocyte growth factor; IGF, insulin-like growth factor; LIF, leukemia inhibitory factor; NT, neurotrophin.

Microglia seem not to be necessarily primary mediators of myelin destruction. Contrary to the long-standing view, a study on MS lesions formation introduced a different hypothesis (Barnett and Prineas, 2004). Accordingly, extensive oligodendrocyte programmed death precedes

microglial myelin phagocytosis, while T-cell infiltration and inflammation exacerbation take place as a result of postapoptotic necrosis, caused by exceeded clearance mechanisms. While most of the information referring to the pathogenesis promoting role of microglia/monocytes is gained from EAE, this pattern of MS plaque development is more comparable with the cuprizone toxin-induced model where microglial response most probably occurs as a secondary response to oligodendrocyte injury (Gudi et al., 2009).

There is a growing body of evidence about the importance of microglia in promoting remyelination and CNS tissue regeneration. The studies from both humans and experimental models showed that the inflammatory response and microglial activity in particular are a prerequisite for efficient oligodendrocyte maturation and remyelination (Foote and Blakemore, 2005; Merson et al., 2010). Nevertheless, the exact mechanisms are still not clear. Deciphering the role of microglia is an important challenge and a prerequisite for developing specific therapeutics that will prevent neurodegeneration and/or facilitate neural repair.

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Chapter II:

CCL5 induces a pro-inflammatory profile in microglia *in vitro*

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2.1 Abstract

Chemokines are a family of small secreted proteins that recruit immune cells to areas of inflammation and that are implicated in diverse events like development and physiology of the central nervous system. The chemokine receptors CCR1, CCR2, CCR3, CCR5, and CXCR2 have been found to be expressed on microglia in many neurodegenerative diseases, such as multiple sclerosis and Alzheimer's disease. There is emerging evidence that chemokines, besides chemoattraction, might directly modulate reactive profiles of microglia. To address this hypothesis we have investigated the effects of CCL2, CCL3, CCL5, and CXCL1 on cytokine and growth factor production, NO synthesis, and phagocytosis in non-stimulated and lipopolysaccharide-stimulated primary rat microglia. The respective receptors CCR1, CCR5, and CXCR2 were shown to be functionally expressed on microglia. All tested chemokines stimulated chemotaxis whereas only CCL5 increased NO secretion and attenuated IL-10 as well as IGF-1 production in activated microglia. Based on these findings we propose that besides its chemoattractant function, CCL5 has a modulatory effect on activated microglia.

2.2 Introduction

Microglia are resident immunocompetent cells of the central nervous system (CNS). In their activated state they release a wide range of soluble mediators like nitric oxide (NO), tumor necrosis factor-alpha (TNF-α), interleukin-10 (IL-10), and insulin-like growth factor-1 (IGF-1). In different diseases these mediators contribute to either tissue damage or neuroprotection and repair processes within the surrounding brain tissue. Another hallmark of microglia is phagocytosis. Efficient clearance of apoptotic cells, myelin debris, or amyloid deposits may contribute to resolution of inflammation, neuroprotection, and remyelination (Boven et al., 2006). One of the earliest microglial responses to an activating stimulus is the recruitment to the site of neuronal injury or inflammation (Garden and Moller, 2006).

Chemokines are a group of small secreted proteins (8-14 kDa) which interact with highly conserved seven-transmembrane domain G-protein-coupled receptors that are grouped according to the position of cystein residues (XCR, CCR, CXCR and CX_3CR). Initially identified as regulators of leukocyte migration, chemokines show a wide spectrum of actions in the CNS (Ambrosini and Aloisi, 2004). Microglia, astrocytes, neurons, and endothelial cells have been characterized as endogenous sources of chemokines and as chemokine receptor expressing cells (Cartier et al., 2005). CCR1, CCR2, CCR3, CCR5, and CXCR2 are constitutively expressed at low levels on microglia (Bajetto et al., 2002). Besides their involvement in brain development (Rezaie et al., 2002; Luan et al., 2001) and physiological functions like the fine-tuning of the neuronal-microglial communication, neuromodulation and neuro-endocrine regulation (Asensio and Campbell, 1999; Adler and Rogers, 2005; Callewaere et al., 2007), these receptors and their ligands are upregulated in numerous CNS pathologies. In Alzheimer‘s disease (AD) many of the

CCR3, CCR5, and CXCR2 positive reactive microglia are associated with amyloid deposits (Xia et al., 1998; Xia et al., 1997). Within multiple sclerosis (MS) lesions activated microglia/macrophages express CCR1, CCR2, CCR3, CCR5, and CXCR2 (Szczucinski and Losy, 2007; Filipovic et al., 2003) and the temporal CCR5 expression correlates with early remyelination (Trebst et al., 2008). At the same time, increased presence of their ligands has been detected in patient's cerebrospinal fluid and lesion sites (Filipovic et al., 2003; Sorensen et al., 1999). They also seem to play an important role in other neurodegenerative diseases, namely HIV-1-associated dementia, HIV-encephalitis, meningitis, traumatic brain injury, Behcet's disease, myelopathy, spinal cord contusion injury, and macular degeneration, as well as in stress and neuropathic pain (Biber et al., 2002; Callewaere et al., 2007). Information about these chemokine receptors and their ligands obtained from experimental animal models are controversial suggesting both neuroprotective (El et al., 2007; Gamo et al., 2008; Chiu et al., 2010) and neurodegenerative functions (Rottman et al., 2000; McMahon et al., 2001; Izikson et al., 2000; Carlson et al., 2008). Furthermore, several lines of evidence suggest that chemokines directly modify pro- and anti-inflammatory responses of microglia (Rankine et al., 2006; Hughes et al., 2002; Perrin et al., 2005; Semple et al., 2009) which has already been shown for macrophages and monocytes (Villalta et al., 1998; Jiang et al., 1992; Aliberti et al., 1999).

Although being implicated in regulation of various microglial actions, the exact role of CCR1, CCR2, CCR3, CCR5, CXCR2, and their ligands remains to be elucidated. The aim of this study was to investigate whether CCL2 (ligand for CCR2), CCL3 (ligand for CCR1 and CCR5), CCL5 (ligand for CCR1, CCR3, and CCR5) and CXCL1 (ligand for CXCR2) can modulate microglia functions *in vitro*.

2.3 Materials and Methods

2.3.1 Cell culture

Microglia were isolated from neonatal Sprague-Dawley rats cerebra as described previously (Giulian and Baker, 1986; Stangel et al., 2000). Following mechanical and enzymatic dissociation cells from 1-2 brains were plated per poly-L-lysine (Sigma-Aldrich, St. Louis, USA) coated culture flask (75 cm^2; Sarstedt, Numbrecht, Germany) and cultured in Dulbecco's Modified Eagle Medium (DMEM; Invitrogen, Karlsruhe, Germany) supplemented with 10% fetal bovine serum (FBS; Biochrom AG, Berlin, Germany) and 1% penicillin/streptomycin (Sigma-Aldrich, Steinheim, Germany). After 7 days in primary mixed culture, microglia were harvested by shaking on an orbital shaker-incubator (Edmund Bühler, Hechingen, Germany) and plated on culture dishes (Nunc, Roskilde, Denmark). After an attachment period, cells were washed with culture medium and kept in culture for 24 h before experimentation. Microglial purity was more than 95% as determined by OX42 (AbD Serotec, Kidlington, UK) immunoreactivity (data not shown).

2.3.2 Cell viability assay

The metabolic activity of cells, as measured by alamarBlue® assay, serves as an indicator for cell viability (Nociari et al., 1998). After 24 h incubation of microglia (4×10^4 cells/well in 96-well plates) in medium containing CCL2, CCL3, CCL5, or CXCL1 (PeproTech, Hamburg, Germany) at concentrations of 1, 10, and 100 ng/ml, in the presence or absence of 10 ng/ml lipopolysaccharide (LPS; L 2654, Sigma-Aldrich, Steinheim, Germany), the supernatants were replaced with medium containing 10% alamarBlue® (TRECK Diagnostic Systems, Cleveland,

USA) and incubated for 4, 6, and 24 h. In three independent experiments optical densities (OD) from duplicates were measured at 620 nm emission wavelength on a plate reader (Spectra, SLT Labinstruments, Crailsheim, Germany).

2.3.3 Polymerase chain reaction

After 24 h of incubation in the medium and medium containing 10 ng/ml LPS, microglia were collected and resuspended in lysis buffer. Total RNA was extracted using RNeasy Mini Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. The samples were treated with RNase-free DNase (Qiagen, Hilden, Germany). 1 µg of each RNA sample was reverse-transcribed using M-MuLV reverse transcriptase and random hexanucleotides (Applied Biosystems, Darmstadt, Germany). 2 µl of this reaction were used in a PCR with 1 unit Taq polymerase (Promega, Mannheim, Germany). For the detection of CCR1, CCR2, CCR3, and CCR5 35 cycles and for CXCR2 40 cycles were performed. Primer pairs used were based on the published rat chemokine receptor sequences (Table 1) and their selectivity was verified using a BLAST database search. After electrophoresis in an agarose gel containing ethidium-bromid, PCR products were visualized under UV-light. The experiment was repeated two times with similar results.

2.3.4 Calcium imaging recordings

For determination of intracellular calcium concentration ($[Ca^{2+}]_i$), microglia were seeded (3×10^4 cells per 12 mm glass coverslip in 24-well plates) and cultured for 48 h in culture medium. Coverslips were incubated for 20 min with 4 µM of the membrane-permeable high-affinity ratio-metric calcium dye Fura-2-acetoxymethyl (Sigma-Aldrich, Steinheim, Germany) at

37˚C in the dark and allowed to de-esterify for 15–30 min in standard extracellular solution at room temperature. Microglia were placed in a recording chamber (3 ml), which was continuously background superfused (10 ml/min). Standard extracellular solution contained HEPES 20 mM, Na^+ 140.8 mM, Cl^- 146 mM, K^+ 5.8 mM, Mg^{2+} 0.9 mM, Ca^{2+} 1.3 mM, and glucose 5.6 mM at pH 7.3 (NaOH). Fluorescent images were obtained at high spatial resolution (0.09 Am2 pixel size, Till Vision Imaging System; TillPhotonics, Graefelfing, Germany) at recording rates of 1 Hz for CCL5 and 4 Hz for all other chemokines. For the analysis of Ca^{2+} transients, background subtraction was used and subcellular regions of interest were defined over the cytosol. Chemokines were applied at a concentration of 100 ng/ml for 120 s using a custom-made solution applicator attached to the objective (Achroplan 0.75 W; Zeiss, Jena, Germany) of the upright microscope (Axioskop 2 FS Plus; Zeiss, Jena, Germany), as described (Grosskreutz et al., 2007). The perfusion rate was adjusted using a custom water-column based air pressure system fitted on 125 ml reservoir syringes. For each chemokine, Ca^{2+} transients were measured in five cells per field in at least three independent experiments.

2.3.5 Chemotaxis assay

Cell migration in response to chemokines was assessed using a 48-well microchemotaxis chamber (Neuro Probe, Gaithersburg, USA) as previously described (Chen, 2005; Maysami et al., 2006). Lower wells were loaded with control medium or medium containing chemokines (100 ng/ml), lower and upper wells were separated by a polyvinylpyrrolidone-free polycarbonate membrane with 8 μm pore size (Neuro Probe, Gaithersburg, USA) and 5 x 10^4 microglial cells resuspended in medium were added into each compartment of the upper chamber. After 5 h of

incubation at 37 °C and 5% CO_2 the non-migrated cells were wiped off from the upper side of the membrane and migrated cells were fixed and stained with the Diff-Quik Set (Dade Behring, Deerfield, USA). Cells were counted in a blinded manner under a light microscope (Olympus BX61; Olympus, Tokyo, Japan) in ten random high-power fields (400x magnification) in each of the three wells per condition. Data from four independent experiments are represented as ratios with the respective unstimulated control.

2.3.6 Nitric oxide assay

In the cell supernatants obtained from control and stimulated microglia (4 x 10^4 cells/well in 96-well plates) nitrite levels were assessed using the Griess reaction (Stangel and Compston, 2001). 100 µl of culture medium was mixed in a 96-well plate with equal volume of 0.2% N-(1-Naphthyl)ethylenediamine dihydrochloride in H_2O and 1% sufanilamide (Merck, Darmstadt, Germany) in 5% H_3PO_4. After colour development (10 min at room temperature in the dark), OD was measured at 540 nm on a plate reader. As an indicator of NO release, the amount of its stable breakdown product nitrite was determined by linear regression from a standard curve using known concentrations of sodium nitrite (Merck, Darmstadt, Germany). The measurement of duplicates was repeated in five independent experiments.

2.3.7 Enzyme-linked immunosorbent assay

For this assay microglia were cultured in 96-well plates (4 x 10^4 cells/well). After 24 h treatment with chemokines, the amount of TNF-α, or IL-10 in the supernatants of non-stimulated and LPS-challenged cells were determined using the rat BD OptEIA™ ELISA sets (BD Biosciences, Heidelberg, Germany) and IGF-1 using a mouse/rat IGF-I ELISA kit (Diagnostic

Systems Laboratories, Webster, USA). All sandwich type immuno-assays were performed according to the manufacturer's protocol. Recombinant rat TNF-α, IL-10, and IGF-I were used as standards and absorbances were read at 450 nm. Data were obtained from duplicates repeated in five (TNF-α and IL-10) and three (IGF-I) independent experiments.

2.3.8 Phagocytosis assay

Twenty-four hours after seeding microglia in 24-well plates (1 x 10^5 cells/well), cells were stimulated with chemokines for the next 24 h. Phagocytic activity was determined using flow cytometry as previously described (Stangel et al., 2000). Fluorescein isothiocyanate (FITC)-labelled latex beads (1 μm in diameter, Fluoresbrite™ YG carboxylate microspheres; Polysciences, Warrington, USA) were added to the supernatants in a final dilution of 1:200. After 30 min of incubation at 37 °C cells were washed four times to terminate microglial phagocytosis and remove cell surface-bound beads. Adherent cells were collected, washed, resuspended in FACS-flow (BD Biosciences, Franklin Lakes, USA) and measured on a Becton-Dickinson FACSCalibur flow cytometer. Microglia were gated using forward/side scatter characteristics and data were analysed using CellQuest™ software. Mean fluorescence intensities (MFI) and percentages of positive cells were determined in a total of 10000 events. MFI is considered to be an equivalent of the total number of latex beads phagocytosed by a given number of cells. Results from four independent experiments are shown as relative phagocytosis as compared to control conditions.

2.3.9 Statistical analysis

Statistical analysis was performed using SPSS 17.0 (SPSS, Chicago, USA). All analysed data were normalized to respective controls (medium or LPS) and represented as arithmetic mean ± standard error of the mean (SEM). For all experiments one sample *t*-test was performed and values of $p < 0.05$ (*), $p < 0.01$ (**) and $p < 0.001$ (***) were considered as statistically significant.

2.4 Results

2.4.1 CCR1, CCR5 and CXCR2 are functionally expressed on microglia

The presence of CCR1, CCR5, and CXCR2 expression on microglia was determined by RT-PCR (Fig. 1A). Transcripts of rat CCR2 and CCR3 were completely absent or under the detection limit in both untreated and LPS-treated microglia (data not shown).

After application of 100 ng/ml chemokine for 120 s, Ca^{2+} transients were observed in 40%, 41%, 40%, and 47% of microglia for CCL2, CCL3, CCL5 and CXCL1, respectively (Fig. 1B), demonstrating functional expression of chemokine receptors.

2.4.2 CCL2, CCL3, CCL5 and CXCL1 attract microglia and do not influence cell viability

Alamarblue® assay was performed to assess if chemokines and/or LPS applied on primary rat microglia influence cell viability. The results showed that none of the chemokines (at concentrations of 1, 10, and 100 ng/ml) or LPS (10 ng/ml) were cytotoxic (data not shown). Since Alamarblue® also represents an indirect cell proliferation indicator, the data imply that chemokines did not influence microglia proliferation. To test responsiveness of microglia to

chemokines, chemoattractive properties of CCL2, CCL3, CCL5, and CXCL1 were investigated using a microchemotaxis chamber. Under control conditions microglia showed considerable random migration. As compared to unstimulated control cells, all tested chemokines at a concentration of 100 ng/ml induced a significant increase in chemotaxis (Fig. 1C).

2.4.3 Nitric oxide production after stimulation with CCL2, CCL3, CCL5, and CXCL1

To test whether CCL2, CCL3, CCL5, and CXCL1 influence NO formation in non-stimulated and LPS-stimulated (10 ng/ml for 24 h) microglia, the Griess assay was performed. In comparison to the medium control, LPS strongly induced NO synthesis, as expected (Stangel and Compston, 2001). Baseline NO release was not affected by any of the chemokines (not shown). However, LPS-induced NO release was significantly increased by co-stimulation with 1 and 10 ng/ml CCL5 ($p < 0.05$ and $p < 0.001$, respectively), while co-stimulation with 100 ng/ml of CCL5 showed only a tendency towards the same effect (Fig. 2A). Concerning other chemokines, CCL2 at a concentration of 10 ng/ml slightly but significantly increased LPS-induced NO production ($p = 0.048$). Taking into consideration relative arithmetic mean values of NO production after co-stimulation with CCL2 and CCL5, their effect appears as dose-dependent (Fig. 2A). Nevertheless, high standard deviations at 100 ng/ml might be the reason for the absence of a statistically significant result.

2.4.4 CCL2, CCL3, CCL5 and CXCL1 show no influence on microglia-derived TNF-α secretion

After 24 h stimulation of primary rat microglia with chemokines in presence and absence of 10 ng/ml LPS, TNF-α release was measured by ELISA. LPS elevated TNF-α production as formerly shown (Stangel and Compston, 2001), while the baseline soluble TNF-α amount was

below the detection limit. However, as compared with medium control, chemokine co-stimulation did not significantly change TNF-α levels in the supernatants (Fig. 2B).

2.4.5 CCL5 attenuates microglial IL-10 and IGF-1 production

IL-10 and IGF-1 protein production was investigated by ELISA after 24 h treatment with chemokines with and without LPS co-stimulation (10 ng/ml for 24 h). LPS stimulated IL-10 and suppressed IGF-1 secretion in microglia as previously shown (Mizuno et al., 1994; Pang et al., 2010). While baseline IL-10 levels were below the assay sensitivity threshold, LPS-induced IL-10 secretion was significantly attenuated by 100 ng/ml of CCL5 ($p < 0.05$) (Fig. 2C). A similar effect of CCL5 (100 ng/ml) in combination with LPS was seen on IGF-1 release ($p < 0.01$; Fig. 2D). However, CCL5 alone did not alter baseline production of microglia-derived IGF-1 (data not shown).

2.4.6 CCL2, CCL3, CCL5 and CXCL1 have no effect on microglial phagocytotic activity

We further investigated whether CCL2, CCL3, CCL5, or CXCL1 influence microglial phagocytic activity. Cells were treated with chemokines for 24 h. LPS co-stimulation with chemokines was excluded because pilot experiments showed that 24 h incubation of microglia with 10 ng/ml LPS had no influence on phagocytosis. Results from four independent experiments revealed that none of the chemokines changed either the uptake rate (Fig. 2E) or the percentage of cells having phagocytosed fluorescent latex beads (data not shown).

2.5 Discussion

In the present study we explored the reactive profiles of microglia in response to the chemokines CCL2, CCL3, CCL5, and CXCL1. Using primary rat cultures we found transcriptional and functional expression of the chemokine receptors CCR1, CCR5, and CXCR2 whose activation induced Ca^{2+} transients and chemotaxis (Fig. 1). Under our culture conditions, no expression of CCR2 and CCR3 could be found. Although moderate in amplitude, both CCL5 and CCL2 stimulated NO production in LPS-activated microglia, whereas only CCL5 suppressed IL-10 and IGF-1 secretion. None of the chemokines influenced TNF-α production or phagocytic activity (Fig. 2). While the basal levels of IGF-1 and NO production in the supernatants were characteristic for microglia grown *in vitro*, we did not observe any direct effect of chemokines in non-LPS-activated microglia probably because of the necessity for a certain stimulus that primes microglia.

In our experimental setting microglia expressed CCR1 and CCR5, suggesting that one of these receptors might be responsible for changing the secretion levels of IL-10, IGF-1, and NO when engaged by CCL5. However, even though CCL3 has been shown to interact with the same receptors (Blanpain et al., 2003), we did not observe similar effects. It should be noted that despite the promiscuity of chemokine receptors and structural similarities between CC chemokines binding to the same receptors, different domains of ligands interact with specific receptor sites (Blanpain et al., 2003). For instance, in CCR1 expressing transfected COS-7 cells, both CCL3 and CCL5 inhibited cyclic adenosine monophosphate (cAMP) accumulation but only CCL3 promoted inositol phosphate (IP) formation (Tian et al., 2004). Accordingly, mutation

studies revealed the second extracellular loop of CCR5 as the main structural determinant involved in differential binding of CCL3 and CCL5 (Blanpain et al., 2003).

Initially considered as a T cell-specific protein, CCL5 has been found to be produced by many cell types including monocytes/macrophages, microglia, astrocytes, and neurons (Appay and Rowland-Jones, 2001; Kremlev and Palmer, 2005; Kim et al., 2004; Sanchez et al., 2009). In addition to being essential in leukocyte recruitment, CCL5 seems to affect several other immunological phenomena, i.e. T cell proliferation (Taub et al., 1996b), secretion of IL-2 and the expression of IL-2 receptors (Taub et al., 1996b), production of TNFα (Qiu et al., 2009), regulation of cytotoxic T lymphocytes (Taub et al., 1996a), modulation of the cytolytic activity of natural killer cells (Taub et al., 1996a), production of NO (Villalta et al., 1998), increasing in intracellular reactive oxygen species (Qiu et al., 2009), expression of SOD1 (Qiu et al., 2009), and phagocytosis of Trypanosoma cruzi by human macrophages (Villalta et al., 1998). However, although peripheral macrophages are highly effective in inducing pro-inflammatory responses, microglia do not always react in the same way (Carson et al., 2007). Here we show for the first time that CCL5 decreases the secretion of the LPS-induced anti-inflammatory IL-10 suggesting that this chemokine acts pro-inflammatory. LPS has been shown to stimulate microglial glycogen synthase kinase-3β (GSK-3β) which induces IL-10 secretion. Treatment with GSK-3β inhibitor blocked LPS-elicited NO and CCL5 but increased IL-10 production. GSK-3β overexpression in turn upregulated NO and CCL5 but downregulated IL-10 production in LPS-stimulated cells (Huang et al., 2009). Taking into consideration that our data also offer such a divergent pattern of IL-10 and NO (Fig. 2A, C), it may be possible that CCL5 exerts its effects via GSK-3β.

Another novel result that we observed was the decrease of IGF-1 production in LPS-activated microglia after treatment with higher concentration of CCL5 (Fig. 2D). In the CNS, IGF-1 is generally known for regulating cell proliferation, differentiation, and survival during development, myelination, and phagocytosis (reviewed in Aberg, 2010). This growth factor acts via the high-affinity IGF-1 receptor linked to tyrosine kinase activity and various intracellular messenger cascades such as phosphatidylinositol-3 (PI3) kinase and MAP kinases (Aberg, 2010). In cardiomyocytes GSK-3β has been shown to be a negative regulator of IGF-1 (Seimi et al., 2004). It remains to be elucidated whether this kinase could also mediate CCL5 induced IGF-1 downregulation in microglia.

Interestingly, CCL2 selectively elevated NO without affecting other soluble immunomodulatory parameters suggesting that CCL2 might not influence the microglial immune response as extensively as CCL5 does. However, this result appear to be controversial as CCR2 expression on our cells was not detectable. Although CCL2 is described as a specific ligand of CCR2, it is known that this chemokine also binds to CCR1 with lower affinity (Neote et al., 1993; Sozzani et al., 1993). Thus, we presume that the increase of NO was mediated by binding to CCR1.

In many autoimmune diseases CCL5 and CCR5 gene polymorphisms are associated with susceptibility or clinical course severity. For instance, MS patients with high-producer alleles for CCR5 and CCL5 have more extensive perivascular leukocyte infiltration, an increased T2 lesion volume, a higher black hole ratio on MRI, a lower percentage of lesions with signs of remyelination, and increased risk of severe axonal loss (van Veen et al., 2007). With respect to

our data, it is conceivable that in these patients higher amounts of CCL5 may contribute to a pro-inflammatory profile of microglia, thereby worsening the pathological signs.

Animal models and brain sections of MS affected tissue demonstrated an extensive upregulation of CCR1 and CCR5 by macrophages/microglia during episodes of active inflammation and demyelination (Trebst et al., 2003; Eltayeb et al., 2007). Here we show that CCL5, being a ligand for both of these receptors, renders microglia pro-inflammatory. However, these changes were modest and should be considered as a fine-tuning of microglia.

Arising conclusion from these data is that, besides chemotaxis, isolated microglia have clearly distinctive replies to different chemokines and their concentrations. Since chemokines are able to directly modulate microglial immune responses and consequently influence different neuropathological conditions, it is of great interest to elucidate their ultimate roles and underlying pathways.

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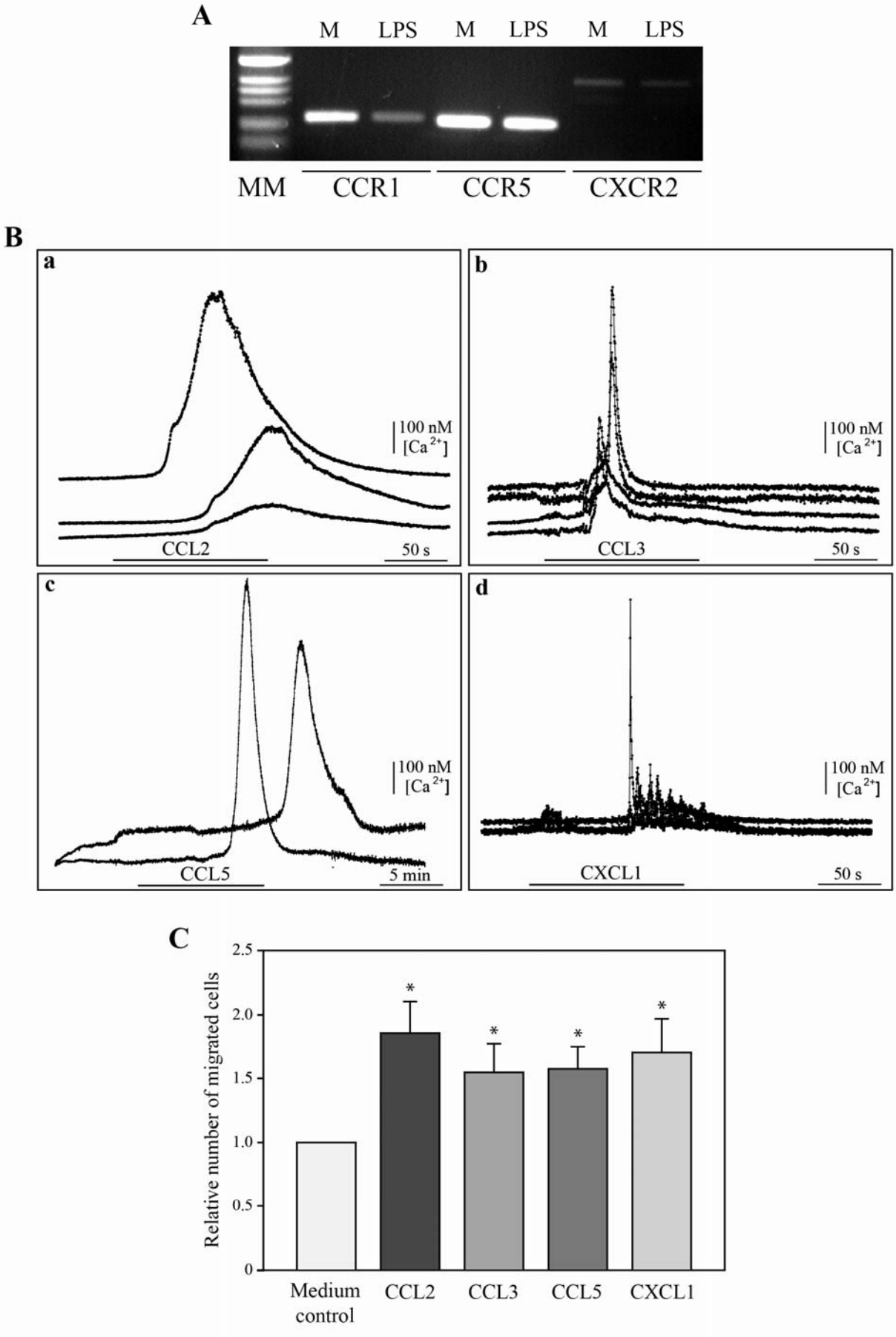
A
M
LPS
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CCR1
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CXCR2
B
a
100 nM
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50 s
b
100 nM
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CCL3
50 s
c
100 nM
[Ca 2+]
CCL5
5 min
d
100 nM
[Ca 2+]
CXCL1
50 s
C
Relative number of migrated cells
2.5
2.0
1.5
1.0
0.5
0
Medium control
CCL2
CCL3
CCL5
CXCL1

Figure 1: Cultured rat microglia express functional chemokine receptors. (A) RT-PCR products separated on agarose gel. cDNA obtained from untreated and LPS-stimulated microglia is marked "M" and "LPS", respectively. Results shown are representative of two independent experiments with similar results. MM - molecular weight marker. (B) Chemokines elicit calcium transients in microglia. Representative traces are induced by 100 ng/ml of CCL2 (a), CCL3 (b), CCL5 (c) and CXCL1 (d), applied at the times indicated by the horizontal bars. (C) CCL2, CCL3, CCL5 and CXCL1 at concentration of 100 ng/ml induced migration of microglia. Relative numbers of migrated cells are represented as mean ± SEM. The asterisks show significant difference from control ($p < 0.05$).

Table 1. Primer sequences and PCR conditions.

Gene	Primer sequences (5'→3')	Product size (bp)	Annealing temperature (°C)	Number of PCR cycles
Rat CCR1	GGAGTTCACTCACCATACCTGTAG GGTCCAGAGGAGGAAGAATAGAAG	232	62	35
Rat CCR2	GTAACTGTGTGGTTGACATGC CACTCGGTCTGCTGTCTCCCTA	206	57	35
Rat CCR3	ACTCTGCTTAGATGCCCCAAT CCTCTCACCAACAAAGGCATA	249	57	35
Rat CCR5	GCAAGTCAATCCTGATCGTGT TCAGCTTTCAAAGACCCAATC	197	55	35
Rat CXCR2	GCAAACCCTTCTACCGTAG AGAAGTCCATGGCGAAATT	414	51	40

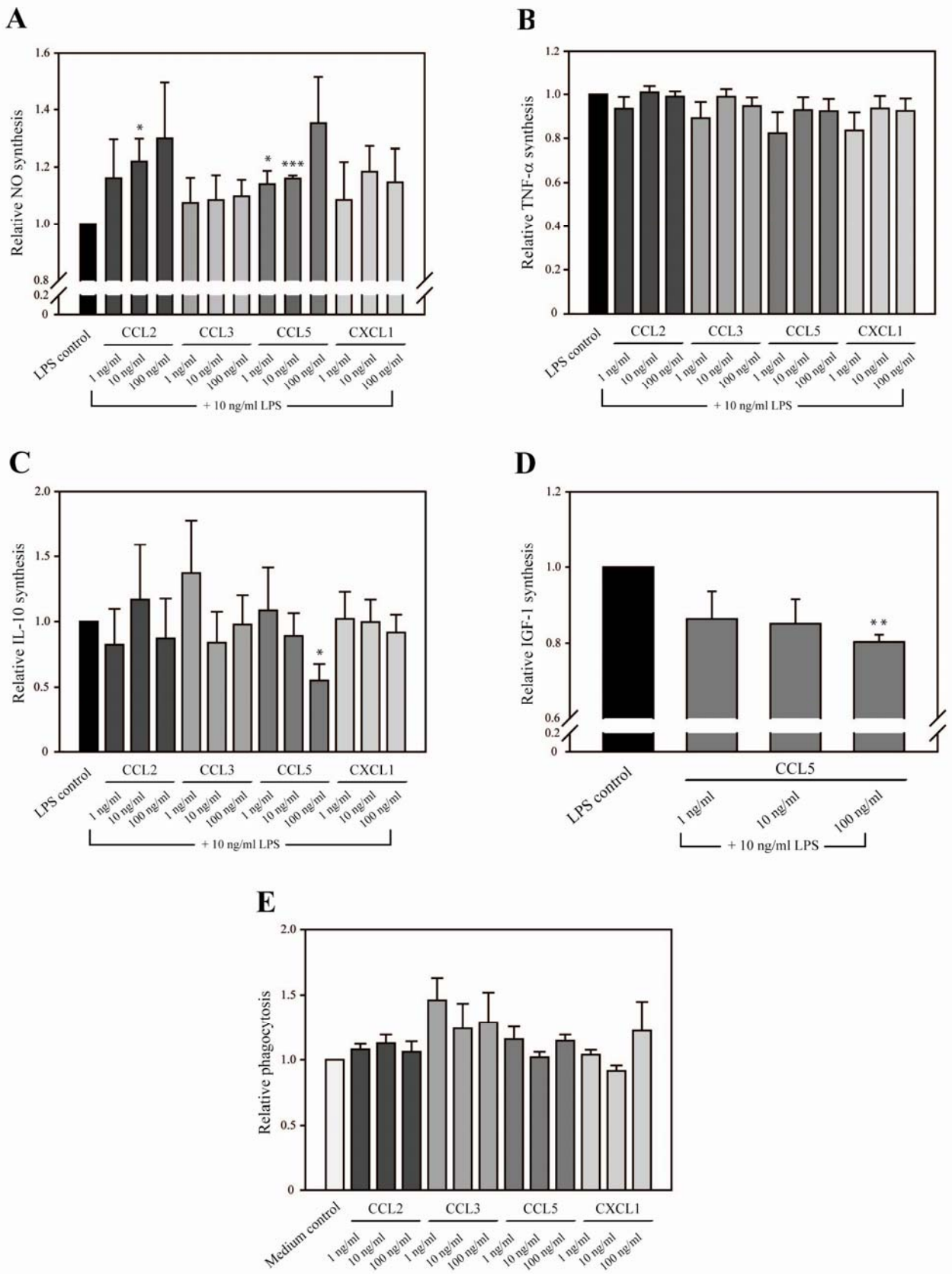
A
Relative NO synthesis
1.6
1.4
1.2
1.0
0.8
0.2
0
*
*
***
LPS control
CCL2
CCL3
CCL5
CXCL1
1 ng/ml
10 ng/ml
100 ng/ml
+ 10 ng/ml LPS
B
Relative TNF-α synthesis
1.2
1.0
0.8
0.6
0.4
0.2
0
C
Relative IL-10 synthesis
2.0
1.5
1.0
0.5
0
*
D
Relative IGF-1 synthesis
1.2
1.0
0.8
0.6
0.2
0
**
E
Relative phagocytosis
2.0
1.5
1.0
0.5
0
Medium control

Figure 2: Relative NO (A), TNF-α (B), IL-10 (C), and IGF-1 (D) production and phagocytosis (E) in primary rat microglia after treatment with chemokines. Data are represented as mean ± SEM and normalized with respective controls (LPS control - 10 ng/ml for 24 h; medium control). Significant differences are indicated with asterisks: (*) $p < 0.05$, (**) $p < 0.01$, (***) $p < 0.001$.

Chapter III:

Differential gene expression of matrix metalloproteinases and their tissue inhibitors during de- and remyelination of the brain white and gray matter

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In preparation

3.1 Abstract

Matrix metalloproteinases (MMPs) are a family of endopeptidases which together with tissue inhibitors of metalloproteinases (TIMPs) play an essential role in tissue remodeling. Apart from being involved in the pathogenesis of demyelinating diseases like multiple sclerosis (MS), there is emerging evidence that MMPs also promote remyelination and repair. Since differences between white and gray matter lesions have recently come into the focus of MS research, we investigated region specific expression patterns of eleven MMPs and four TIMPs in the cuprizone murine model in order to identify factors that may regulate regeneration in the central nervous system (CNS). Demyelination was induced in young adult C57BL/6 mice by feeding with 0.2% cuprizone following normal chow to allow remyelination. At nine different time points mRNA was extracted from microdissected cortex and corpus callosum and analyzed using quantitative PCR. In comparison with age-matched controls, MMP-12, TIMP-1, and MMP-14 were significantly upregulated in both areas during de- and remyelination. Interestingly, MMP-3, MMP-11, and MMP-14 were upregulated only in the white matter during remyelination, while MMP-24 was significantly downregulated during demyelination. Moreover, different expression patterns of TIMP-3 and TIMP-4 were observed in cortex and corpus callosum. These findings suggest that MMPs may play a role in the regulation of both de- and remyelination. Differences in MMP and TIMP expression levels in the white and gray matter reveal different molecular mechanisms between these areas.

3.2 Introduction

Matrix metalloproteinases (MMPs) are a family of zinc-dependent endopeptidases that cleave components of the extracellular matrix (ECM). Together with endogenous tissue inhibitors of metalloproteinases (TIMPs) that regulate their activity, MMPs play an essential role in tissue remodelling. In the central nervous system (CNS), subsets of MMPs and TIMPs are expressed by all cell types (Milward et al., 2007). Besides structural rearrangement of the ECM, these proteins regulate numerous physiological processes such as development, plasticity, cellular growth, survival and migration, programmed cell death, angiogenesis and signalling (Milward et al., 2007; McCawley and Matrisian, 2001). Uncontrolled and abundant expression of MMPs is considered to mediate harmful tissue destruction and, within the CNS, pathogenesis of inflammatory demyelinating diseases like multiple sclerosis (MS) (Kieseier et al., 1999). In addition to the role in opening the blood-brain barrier (BBB), perpetuation of inflammation and neurotoxicity, MMPs are able to degrade myelin proteins directly (Agrawal et al., 2006; English et al., 2000; Vos et al., 2000; Chandler et al., 1995). Recently MMPs and TIMPs have also been implicated in regenerative processes like axonal growth, oligodendrocyte maturation, remyelination and mantaining of healthy myelin (Larsen and Yong, 2004; Lehmann et al., 2009; Yong, 2005). An alteration in MMP/TIMP equilibrium has been found in MS (Lindberg et al., 2001; Yong et al., 2007) and in related animal models, e. g. experimental autoimmune encephalomyelitis (EAE) (Toft-Hansen et al., 2004; Nygardas and Hinkkanen, 2002), Theiler murine encephalomyelitis (TME) (Ulrich et al., 2006), and canine distemper virus (CDV) encephalomyelitis (Gröters et al., 2005).

In recent years, extensive cortical demyelination in MS patients has been recognized and highlighted (Albert et al., 2007; Bo et al., 2003). Demyelination and axonal injury in the cortex could account for clinically significant cognitive, motor and sensory impairment in MS (Bo et al., 2003). Since the pathology and remyelination capacity in the cortex differ from that in the white matter, different mechanisms of tissue injury in white and gray matter have been proposed (Lassmann and Lucchinetti, 2008).

The cuprizone model is a commonly used toxic experimental model of MS (Kipp et al., 2009). Feeding mice with cuprizone (bis-cyclohexanone oxalhydrazone) leads to oligodendrocyte depletion and reversible demyelination in the corpus callosum and cortex (Gudi et al., 2009; Skripuletz et al., 2008). Characterized by an intact BBB, this model is advantageous for studying remyelination of the CNS, without the influence of the peripheral immune system component (Bakker and Ludwin, 1987).

Since in the light of CNS demyelinating diseases both beneficial and detrimental functions for MMPs have been proposed, better understanding of their roles remains a challenge. The aim of the present study was to reveal time-dependent molecular and cellular expression patterns of MMPs and TIMPs in the brain white and gray matter during de- and remyelination.

3.3 Materials and Methods

3.3.1 Animals and induction of demyelination

C57BL/6 mice were purchased from Charles River (Sulzfeld, Germany). Animals underwent weekly routine cage maintenance and were microbiologically monitored according to the recommendations of the Federation of European Laboratory Animal Science Associations

(Rehbinder et al., 1996). Food and water were available *ad libitum*. All research and animal care procedures were approved by the Review Board for the Care of Animal Subjects of the district government (Lower Saxony, Germany) and performed according to international guidelines on the use of laboratory animals.

Experimental demyelination was induced in 8 week old male mice by feeding with 0.2% cuprizone (Sigma-Aldrich, Munich, Germany) mixed into a ground standard rodent chow for 4.5 weeks. To allow remyelination, animals were subjected to a normal diet for an additional 1.5 week.

3.3.2 Tissue preparation

At different time points (week 1, 2, 3, 3.5, 4, and 4.5 for demyelination and week 5, 5.5, and 6 for remyelination) mice were sacrificed and perfused via left cardiac ventricle with ribonuclease (RNase) free phosphate buffered saline (PBS) for gene expression analysis or with 4% paraformaldehyde (PFA) in PBS for immunohistochemistry. A group size of five (for gene expression analysis) and four (for immunohistochemistry) animals was investigated at each time point, parallel with the age-matched untreated controls. For gene expression analysis brains were removed, embedded in Tissue Tek® Compound (Sakura, USA), snap frozen in liquid nitrogen and stored at -80˚C until the following step. 30 µm thick serial coronal sections (bregma 0.98 mm to -2.46 mm, according to the mouse atlas by Paxinos and Franklin, 2001) were cut on polyethylene-naphtalate (PEN) membrane slides (P.A.L.M. Microlaser Technologies, Bernried, Germany) at -20˚C under RNase free conditions. Sections were shortly fixed in 70% ice-cold ethanol, rinsed with RNase free H_2O, stained in 1% cresyl violet acetate solution (Sigma-

Aldrich) in 50% ethanol for 30 s, following dehydration in 70% and 100% ethanol dilutions and air drying for 1-2 min. All solutions were prepared with 0.1% diethylpyrocarbonate (DEPC)-treated water (Carl Roth, Karlsruhe, Germany). For immunohistochemistry, brains were postfixed in 4% PFA in PBS at 4˚C overnight and paraffin embedded or cryoprotected in 30% sucrose in PBS for 24 h, embedded in tissue-freezing medium and snap frozen. 7 µm (paraffined) and 10 µm (frozen) thick serial coronal slices were cut and dried overnight. Regions between bregma -0.70 mm and -1.46 mm (Paxinos and Franklin, 2001) were analysed.

3.3.3 Immunohistochemistry

Paraffin embedded sections were dewaxed, rehydrated through graded alcohol concentrations and microwave treated for 5 min in 10 mM citrate buffer (pH 6.0). Endogenous peroxidase activity was inhibited with 3% H_2O_2 and unspecific binding was blocked for 1 h with PBS containing 3% normal goat serum and 0.1% Triton-X-100. H_2O_2 treatment was omitted for fluorescent visualization, and cooking for staining of frozen slices (Nogo-A). Tissue was incubated overnight at 4 ˚C with the following primary antibodies: myelin basic protein (MBP; 1:500, Sternberger Monoclonals, Lutherville, USA) as a myelin marker, fluorescein coupled *Ricinus communis* agglutinin I (RCA I; 1:1000, Jackson ImmunoResearch Laboratories, West Grove, USA) for activated microglia, glial fibrillary acidic protein (GFAP; 1:200, Chemicon/Millipore, Billerica, USA) for astrocytes, and Nogo-A (1:750, Chemicon/Millipore) for mature oligodendrocytes. Subsequently, slides were washed in PBS, incubated with biotinylated secondary antibodies (1:500; Vector Laboratories, Burlingame, USA) for 1 h, following peroxidase-coupled avidin-biotin complex (ABC Kit, Vector Laboratories) treatment

for an additional hour. Immunoreaction was visualized with 3,3'-diaminobenzidine (DAB Peroxidase Substrate Kit, Vector Laboratories) or Alexa Fluor® 555 dye (1:500, Invitrogen, Carlsbad, USA) for GFAP. Immunofluorescent sections were counterstained with 4',6-diamidino-2-phenylindole (DAPI) nuclear dye (Invitrogen).

3.3.4 Evaluation of de- and remyelination

For the determination of the myelin content in the corpus callosum and the cortex throughout and following cuprizone treatment, MBP immunostained sections from four animals per time point were evaluated by light microscopy (Leica Microsystems, Wetzlar, Germany). De- and remyelination were assessed by three blinded observers using a scale from 0 to 3 for corpus callosum, and from 0 to 4 for cortex, as previously published (Gudi et al., 2009; Skripuletz et al., 2008). Score 0 represents complete absence of MBP signal while 3 and 4 represent fully expressed MBP in the corpus callosum and cortex, respectively.

3.3.5 Laser microdissection

The PALM MicroBeam System (P.A.L.M. Microlaser Technologies) was used to precisely excise the medial part of the corpus callosum and the dorso-lateral parts of the left and right cerebral cortex from frozen coronal brain sections of cuprizone-treated and age-matched control mice (Fig. 1). Microdissected areas from different brain regions were separately collected with a sterile 21 gauge needle and stored at -80˚C until RNA extraction.

3.3.6 RNA isolation and quantitative RT-PCR

RNA was isolated with the RNeasy Micro Kit (corpus callosum) and the Mini Kit (cortex) according to the manufacturer's recommendations (Qiagen, Hilden, Germany). Concentrations were measured using a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific, Wilmington, USA). Final RNA dilutions were adjusted with RNase-free water (4 ng/µl for corpus callosum, 12 ng/µl for cortex, and 20 ng/µl for positive controls) and reversely transcribed using the High Capacity cDNA Reverse Transcription Kit with RNase Inhibitor (Applied Biosystems, Foster City, USA) in a MyCycler™ PCR Thermal cycler (Bio-Rad Laboratories, Hercules, USA). The primer pair sequences used for the detection of MMP (MMP-2, -3, -7, -9, -10, -11, -12, -13, -14, -15, -24), TIMP (TIMP-1, -2, -3, -4) and housekeeping gene (β-actin and glyceraldehyde-3-phosphate dehydrogenase; GAPDH) mRNA are described elsewhere (Ulrich et al., 2005). Quantitative PCR was performed with 1 µl cDNA per 25 µl reaction mix using the Brilliant SYBR Green qPCR Core Reagent Kit (Agilent Technologies, Santa Clara, USA) in a Mx3005P™ Real-Time PCR System (Agilent Technologies) with absolute external standards containing 10^2-10^8 copies/µl, as described (Ulrich et al., 2005). Correct product sizes were confirmed by gel electrophoresis (data not shown). The obtained measurements were analyzed using MxPro QPCR software version 4.10 (Agilent Technologies). Data were normalized with a normalization factor calculated from geometric averaging of housekeeping genes using geNorm software version 3.5 (Vandesompele et al., 2002).

3.3.7 Statistical Analysis

Statistical analysis was performed using SPSS Statistics Version 17.0 (SPSS Inc., Chicago, USA). Measured MMP and TIMP copy numbers per 1 ng RNA were normalized to housekeeping genes while unspecific samples according to the melting curve were excluded. Normal distribution was tested with the Kolmogorov-Smirnov test. Statistical differences between cuprizone treated and control animals were calculated independently for each time point using paired Student *t*-test and values of $p < 0.05$ (*), $p < 0.01$ (**) and $p < 0.001$ (***) were considered as statistically significant. For myelin protein expression significant effects are tested with ANOVA and indicated by asterisks (compared to controls) or rhombs (compared to the preceding time point): (*#) $p < 0.05$, (**##) $p < 0.01$, (***###) $p < 0.001$.

3.4 Results

3.4.1 De- and remyelination

The extent of demyelination and subsequent remyelination in the white and gray matter was judged by MBP immunostaining. In comparison with control mice, a severe loss of MBP was observed in both corpus callosum and cortex ($p < 0.001$) after 4.5 weeks of 0.2% cuprizone feeding (Fig. 2K, L). After toxin removal (weeks 5-6) MBP reexpressed until almost complete restoration of myelin. The peak of demyelination in the cortex was slightly delayed and less prominent as compared to the corpus callosum, as described (Gudi et al., 2009). This feeding regime was used because a detailed analysis of demyelination in the corpus callosum has demonstrated peak demyelination after 4.5 weeks of cuprizone feeding (Gudi et al., 2009).

3.4.2 Glial response

Microglial activation was visualized by fluorescent RCA I immunostaining. In the control animals, RCA I signal was not observed in either corpus callosum or cortex (Fig. 3C, G). The presence of activated microglia in the corpus callosum was noticed as early as 2 weeks of cuprizone treatment (data not shown), reaching its peak at week 4.5 (Fig. 3D). After cuprizone cessation, the microglial response gradually declined until week 6, when there were only some sparsely distributed activated microglia (Fig. 3E, F). In the cortex, microglia demonstrated similar dynamics as in the corpus callosum, but lower in magnitude (Fig. 3G-J). Unlike activated microglia, some of the GFAP positive astrocytes could be seen in the brains of untreated animals (Fig. 3K, O). During the cuprizone diet astrogliosis in the corpus callosum was becoming more prominent, reaching its maximum at week 4.5 and steadily declining during normal diet (Fig. 3L-N). Although reduced, a remarkable number of activated astrocytes was still present at week 6 (Fig. 3N). Similar to the white matter, astrogliosis was also prominent in the cortex during and after cuprizone treatment (Fig. 3P-R), considerably exceeding the amount of cortical microglia. To detect mature oligodendrocytes we used Nogo-A immunostaining. In control mice, a large amount of Nogo-A positive cells was present in the white matter (Fig. 3S, W). After 4.5 weeks of cuprizone diet, an obvious lack of Nogo-A positive cells was noticed (Fig. 3T, X). They reappeared after toxin removal in both analyzed areas, but constantly lower in number and slightly delayed in the cortex as compared with the white matter (Fig. 3U-V, Y-Z). Extensive microgliosis, astrogliosis and lack of oligodendrocytes corresponded to the peak of myelin loss as demonstrated by MBP staining (Fig. 2).

3.4.3 MMP and TIMP mRNA expression

The relative expression of MMP and TIMP mRNA is summarized in Table 1. For both regions (corpus callosum and cortex) at all time points (week 1, 2, 3, 3.5, 4, 4.5 for demyelination and week 5, 5.5, 6 for remyelination) values (normalized copy numbers/1 ng RNA) for each gene are represented as ratios of arithmetic means of treated versus control mice (n = 5 per group). The changes in MMPs and TIMPs expression were different depending on the phase and location (Fig. 4-6). During early phases of demyelination the most prominent upregulation was seen for MMP-3 in the corpus callosum, TIMP-4 in the cortex and MMP-12 and TIMP-3 in both areas. During peak demyelination elevated transcript numbers were seen for MMP-14 and TIMP-2 in the white matter and MMP-12 and TIMP-1 in both regions. In this phase MMP-15 and -24 were significantly downregulated. During remyelination, MMP-3, -11, -14, TIMP-3 and -4 were upregulated in the corpus callosum. The expression of MMP-12 was prominent in both areas with an increasing tendency in white matter and decreasing in the gray matter. Enzymes that were not or only minimally regulated were MMP-2, -7, -9, -10, and 13.

3.5 Discussion

In the present study we explored the expression profiles of eleven MMPs and four TIMPs within narrow time intervals during cuprizone induced demyelination and subsequent remyelination. Depending on the phase of the disease and the cerebral region, genes with altered transcript numbers could be categorized into groups. During early demyelination MMP-3 expression was elevated in the corpus callosum, TIMP-4 in the cortex and MMP-12 and TIMP-3 in both areas. During severe demyelination we detected MMP-24 downregulation and TIMP-2

upregulation in the corpus callosum as well as MMP-12, -14 and TIMP-1 upregulation and MMP-15 dowregulation in both areas. During remyelination we detected MMP-3, -11, -14, TIMP-3 and -4 upregulation and MMP-24 downregulation in the corpus callosum, while MMP-12 transcript numbers were still high in both areas. Throughout the cuprizone treatment MMP-2, -7, -9, -10 and -13 were minimally affected or not detected in both white and grey matter.

MMP-12 is found to be highly expressed in active demyelinating MS lesions and, to a lower extent, within chronic active and inactive lesions (Vos et al., 2003). This enzyme was also found to be upregulated during disease progression in EAE and chronic demyelination in TME (Ulrich et al., 2006; Dasilva and Yong, 2008) and to be capable of cleaving MBP (Shiryaev et al., 2009). These and our data strongly support the role of MMP-12 in myelin degradation. MMP-12 upregulation can be driven by pro-inflammatory stimuli like tumor necrosis factor-alpha (TNF-α) and interleukin (IL)-1β (Dasilva and Yong, 2008). Since these cytokines are also being expressed during demyelination in the cuprizone model (Arnett et al., 2001; Mason et al., 2001), they might contribute to the MMP-12 upregulation observed in our study. Macrophages isolated from MMP-12 knock-out mice had no migratory capacity in contrast to those from wild-type mice (Shipley et al., 1996). Since the highest number of RCA I and GFAP positive cells in both corpus callosum and cortex is present after 4.5 weeks of cuprizone feeding (Fig. 3D, H), strong MMP-12 upregulation could account for microglial and astrocytic migration towards the demyelinating site. Interestingly, during remyelination the increasing tendency of MMP-12 mRNA expression continued in the corpus callosum, while it declined in the cortex (Fig. 4). Long time considered as monocytic lineage restricted, MMP-12 secretion has recently been associated with neurons and oligodendrocytes/OPCs, inducing their maturation and processes extension *in vitro* (Larsen and

Yong, 2004; Wasserman and Schlichter, 2007). Thus, numerous Nogo-A positive cells seen in the corpus callosum during remyelination (Fig. 3U-V) could be the source of MMP-12 mRNA. Larsen et al. showed *in vivo* that MMP-12 drives myelogenesis via releasing insulin-like growth factor-1 (IGF-1) (Larsen et al., 2006). Since IGF-1 has been upregulated in the corpus callosum during cuprizone treatment (Taylor et al., 2010), this link could represent the mechanism for promoting remyelination.

MMP-3 was upregulated in the corpus callosum during early demyelination and, after returning to the basal level, its expression was again highly elevated during remyelination (Fig. 5). The initial MMP-3 upregulation prior to demyelination is consistent with findings in TME and spontaneously demyelinating transgenic mouse model (Ulrich et al., 2006; D'Souza et al., 2002). This protease is also implied in proteolytic breakdown of myelin from MS patients and found to be expressed in early reactive plaques (D'Souza and Moscarello, 2006; Lindberg et al., 2001). Nevertheless, the high upregulation in the corpus callosum that we detected during remyelination suggests involvement of MMP-3 in CNS tissue repair. Its proteolytic activity may be required for releasing soluble growth factors, thereby supporting remyelination and neural outgrowth (Fowlkes et al., 2004; Dubois-Dalcq and Murray, 2000; McCawley and Matrisian, 2001). Moreover, there is a possibility that MMPs which are shown to cleave myelin proteins (including MMP-3 and MMP-12) could facilitate the removal of myelin debris and thus promote remyelination (Kotter et al., 2006).

Interestingly, in the white matter we detected upregulation of MMP-14 and downregulation of MMP-15 (also in cortex) and -24 during demyelination. A similar trend for MMP-14 and -24 was also present during remyelination. Comparable expression patterns of these

membrane type (MT)-MMPs were observed in EAE and showed no association with the expression of the pro-inflammatory cytokines (Toft-Hansen et al., 2007). Besides cleaving a range of ECM molecules, MT-MMPs also function as cell surface sheddases, processing proMMPs and other bioactive molecules (Barbolina and Stack, 2008; Fillmore et al., 2001). Functional consequences of MMP-15 and -24 downregulation are still not clear, while the MMP-14 might facilitate cell motility. Their ability to modify pericellular microenvironment could influence degenerative and repair processes in the CNS on many different ways.

As our data also confirmed, in the adult brain TIMP 2-4 are constitutively expressed at high levels while the basal TIMP-1 expression is low (Young et al., 2002). In the present study the expression of all four TIMPs were altered as compared to the controls. However, along the course of de- and remyelination they displayed somewhat different patterns (Fig. 6). TIMP-1 (in both corpus callosum and cortex) and TIMP-2 (in corpus callosum only) significantly upregulated during the peak of demyelination and gradually declined during remyelination. The cellular source of TIMP-1 in the brain are mainly astrocytes (Gardner and Ghorpade, 2003), which explains its temporal correlation with abundant astrogliosis (Fig. 2). Myelin protective properties of TIMP-1 were suggested in EAE where TIMP-1 knock-out mice and TIMP-1 over-expressing transgenic mice showed severer and milder forms of the disease, respectively (Crocker et al., 2006; Althoff et al., 2010). In the serum and cerebrospinal fluid of MS patients decreased TIMP-1 and TIMP-2 levels as well as elevated MMP-9 and MMP-2 levels have been found, suggesting that high MMP-9/TIMP-1 and MMP2/TIMP2 ratios play an important role in the pathogenesis and progression of MS (Fainardi et al., 2006; Fainardi et al., 2009). Suprisingly, we did not detect a high upregulation of either MMP-9 or MMP-2 mRNA in the cuprizone

treated animals which could be explained by the lack of leukocyte infiltration. Nevertheless, the MMP-9 mRNA upregulation in the cortex was small but significant (Table 1). Since MMP-9 is able to degrade MBP (Shiryaev et al., 2009), in our experimental model this gelatinase could directly contribute to the mechanism of demyelination, or alternatively, even facilitate remyelination by processing proteoglycan that inhibits OPC maturation (Larsen et al., 2003).

Regarding other TIMPs, there was an elevation of TIMP-3 and TIMP-4 mRNA levels in the cortex early after toxin application which decreased after 3 weeks to the basic level. In the corpus callosum, there was a TIMP-3 mRNA peak during the first week of cuprizone feeding with a gradual downregulation. TIMP-4 was moderately but significantly upregulated throughout remyelination. Unlike TIMP-1, -2, and -4 which are secreted, TIMP-3 is bound to the cell surface and it inhibits several membrane-bound molecules, such as MMP-14, MMP-3, and TACE (Rosenberg, 2009). Thus, at the peak of demyelination in the corpus callosum, lack of TIMP-3 may interfere with MMP-14 upregulation (Table 1). In spite of being highly expressed in the brain, the function of TIMP-4 is largely unknown. Its upregulation in the white matter that we detected during remyelination could signal a role in this process. Moreover, numerous physiological functions of TIMPs, independent of their MMP inhibition, have recently come into the focus (Perez-Martinez and Jaworski, 2005; Seo et al., 2003; Chirco et al., 2006).

MMP-11 has been described to be constitutively expressed in astrocytes and present in chronic CDV-associated brain lesions (Kumnok et al., 2008; Miao et al., 2003). However, present literature about its role and target substrates in the CNS is poor. Concerning MMP-11 mRNA upregulation in the corpus callosum at week 5.5 and 6, we propose its possible involvement in promoting remyelination.

In the light of differences in pathology between the white and gray matter in the cuprizone model, MMP-3 and TIMP-4 deserve attention. Region-specific expression of MMPs and TIMPs in the brain has also been shown in CDV infected mice and associated with differential impairment of CNS integrity (Khuth et al., 2001). The reasons underlying molecular differences between cortical and white matter lesions of MS patients are not clear (Lassmann and Lucchinetti, 2008). Possible explanation might be the myelin amount, specific cellular interactions or even different effector mechanisms, e. g. alterations of glutamate reuptake regulation (Vercellino et al., 2009). Differential regulation of MMPs and TIMPs in the white and grey matter during de- and remyelination strongly implies these proteins as potential players in region-specific pathogenesis.

In summary, initiation, progression, and resolution of demyelination in the cuprizone mouse model were related with differential and region specific MMP and TIMP expression patterns. MMP-3 and MMP-12 were highly expressed during myelin recovery. This study highlights that MMPs expressed in a spatially and temporally restricted manner may have important roles in promoting CNS tissue repair.

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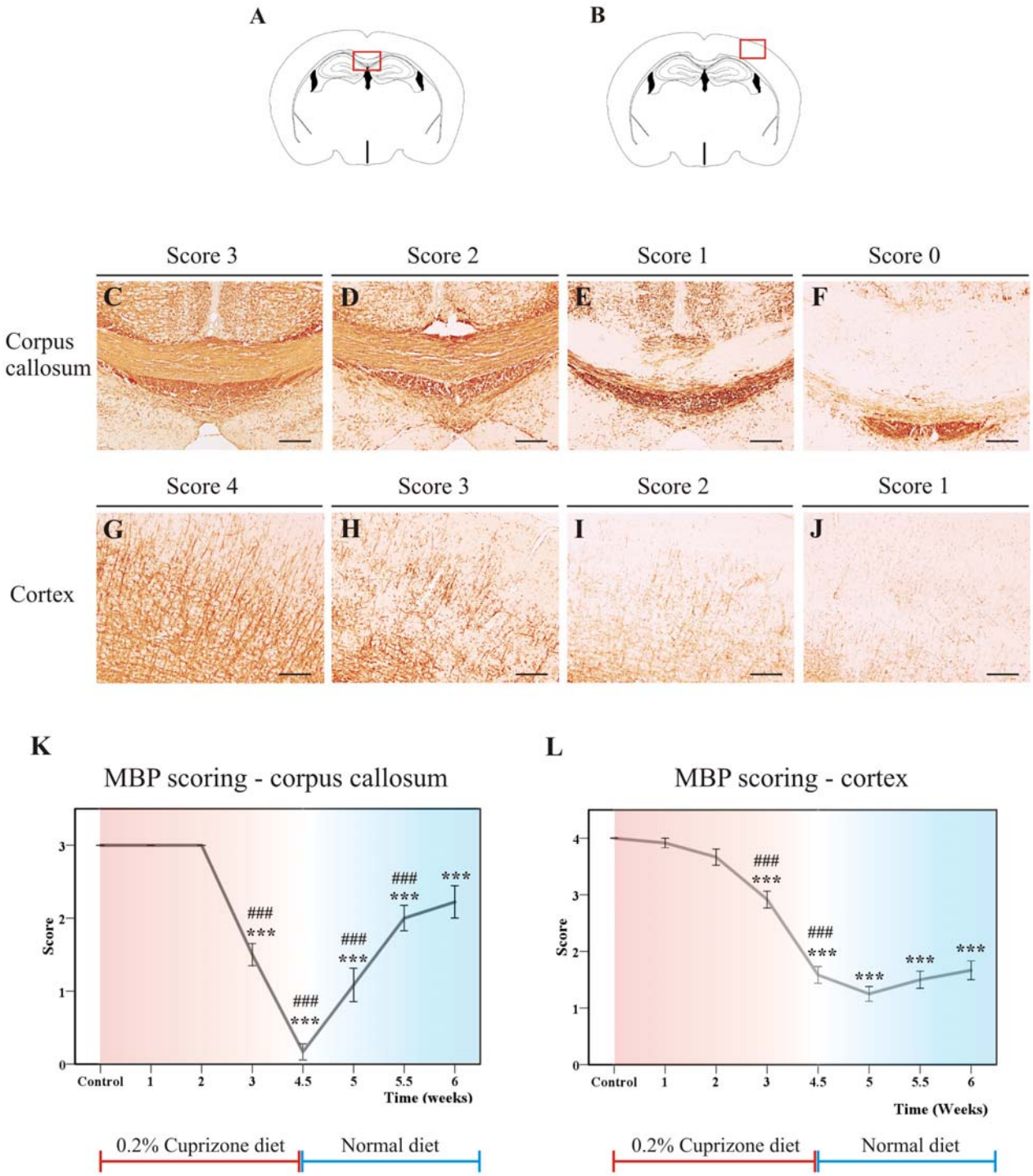
Figure 1: Laser microdissected areas. Medial corpus callosum (A) and dorsolateral cortices (B) excised from the cresyl violet stained coronal brain sections (C).


A
B
Score 3
Score 2
Score 1
Score 0
C
D
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Corpus callosum
Score 4
Score 3
Score 2
Score 1
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H
I
J
Cortex
K
MBP scoring - corpus callosum
Score
Time (weeks)
Control
0.2% Cuprizone diet
Normal diet
L
MBP scoring - cortex
Score
Time (Weeks)
Control
0.2% Cuprizone diet
Normal diet

Figure 2: Myelin protein expression during de- and remyelination. Red rectangles on the schematic drawings of the coronal mouse brain sections show examined parts of the corpus callosum (A) and cortex (B). Myelin amounts have been analysed by scoring of the MBP immunostained brain sections at different time points where score 0 stands for complete absence of MBP signal while score 3 and 4 represent fully expressed MBP in corpus callosum (C-F) and cortex (G-J), respectively. Demyelination was induced by 0.2% cuprizone diet for 4.5 weeks and subsequent remyelination took place after toxin withdrawal (week 5-6). At week 4.5 a severe loss of MPB can be seen in both corpus callosum (K) and cortex (L), demonstrating almost complete demyelination. Significant effects tested with ANOVA are indicated by asterisks (compared to controls) or rhombs (compared to the preceding time point): (*#) $p < 0.05$, (**##) $p < 0.01$, (***###) $p < 0.001$. Scale bar: 200 μm.

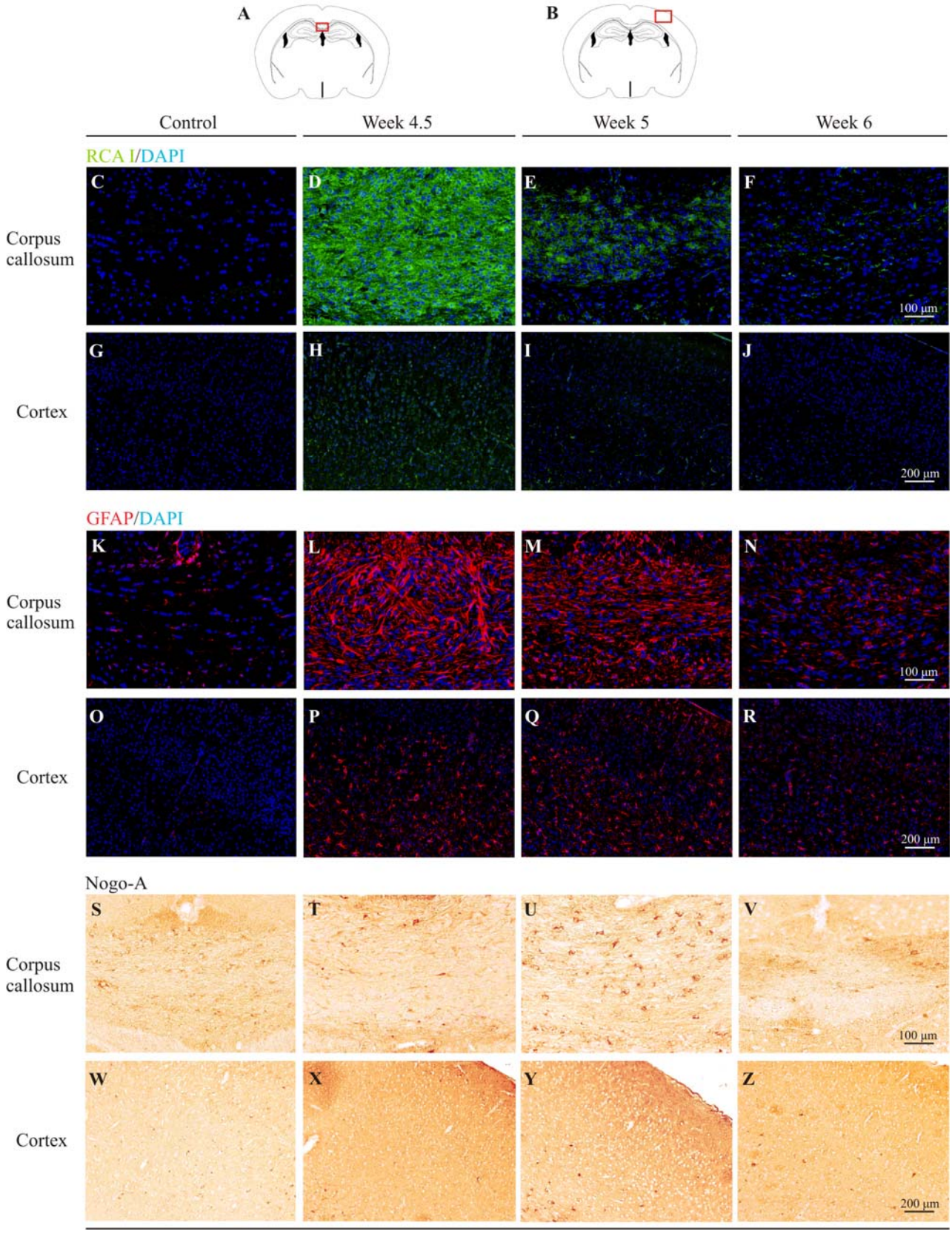
A
B
Control
Week 4.5
Week 5
Week 6
RCA I/DAPI
Corpus callosum
Cortex
C
D
E
F
G
H
I
J
100 µm
200 µm
GFAP/DAPI
K
L
M
N
O
P
Q
R
Nogo-A
S
T
U
V
W
X
Y
Z

Figure 3: Glial response during cuprizone-induced demyelination and subsequent remyelination. Red rectangles indicate analysed parts of the corpus callosum (A) and cortex (B). RCA-I (green), GFAP (red) and Nogo-A (visualized with HRP/DAB) immunostaining show extensive microglial (C-D, G-H) and astrocytic (K-L, O-P) response as well as loss of mature oligodendrocytes (S-T, W-X) in corpus callosum and cortex after 4.5 weeks of 0.2% cuprizone diet, as compared with controls. Following toxin withdrawal (week 5-6), the resolution of microgliosis (E-F, I-J), astrogliosis (M-N, Q-R) and oligodendrocyte reappearance (U-V, Y-Z) took place in white and gray matter, respectively. Nuclei in the immunofluorescent stainings (C-R) are counterstained with DAPI. Scale bars: 100 μm (corpus callosum); 200 μm (cortex).

Table 1. Relative MMP and TIMP expression during cuprizone induced de- and remyelination

		Time (weeks)								
Gene	**Region**	**1**	**2**	**3**	**3.5**	**4**	**4.5**	**5**	**5.5**	**6**
MMP-2	CC	0.9	2.6	0.6	0.7	1.6	1.3	1.4	1.3	1.6
	CTX	1.7	2.6	1.6	1.9	1.8	0.9	0.5	1.0	2.8
MMP-3	CC	2.6	4.2**	2.0	0.6	1.7	1.6	2.4	11.7**	8.6*
	CTX	2.8	1.8	1.8	1.1	1.3	1.8	1.4	0.5	1.1
MMP-7	CC	1.0	1.3	2.0	0.4	0.2	0.5	1.0	0.4	0.3
	CTX	1.0	1.5	1.0	0.7	0.9	1.0	1.0	0.7	1.0
MMP-9	CC	0.2	0.3	0.6	0.1	0.3	0.2	0.4	0.4	0.2
	CTX	1.4*	1.6*	1.5	1.8*	1.2	1.7*	1.4	1.0	1.4
MMP-10	CC	ND	ND	ND	ND	ND	ND	ND	ND	ND
	CTX	ND	ND	ND	ND	ND	ND	ND	ND	ND
MMP-11	CC	0.5	1.4	1.4	1.0	0.6*	0.4	1.1	2.6**	2.5*
	CTX	1.5	1.3	1.0	0.9	1.2	1.9	1.2	1.1	1.1
MMP-12	CC	2.3	24.3***	20.6**	10.9*	73.5**	19.6*	18.9**	29.8*	29.3**
	CTX	3.3*	24.5*	124.1**	92.5*	81.6*	62.3**	55.9**	8.9*	17.1*
MMP-13	CC	0.5	0.5	1.9	1.2	1.6	1.3	1.3	1.0	1.0
	CTX	2.0	1.2	0.7	2.2	1.6	2.9	1.5	2.6	1.1
MMP-14	CC	1.4	1.2	2.0*	2.1*	1.8	1.6	1.4	2.8*	2.4*
	CTX	1.6	1.1	1.0	0.9	1.0	1.7*	1.5	1.1	0.8
MMP-15	CC	0.7	0.4	1.1	0.4*	0.7	0.7	0.8	1.3	1.0
	CTX	0.7	0.8	0.7*	0.8	0.7*	0.8	1.2	0.9	0.8
MMP-24	CC	0.9	0.7	0.5	0.2**	0.2**	0.2**	0.3**	0.4**	0.4***
	CTX	1.1	1.2	1.0	0.9	1.0	1.0	1.1	0.9	1.0
TIMP-1	CC	1.0	2.3	1.0	5.2	6.8*	5.4*	5.8*	4.8	2.1
	CTX	2.1	2.4	2.8	2.9*	4.0*	5.9*	3.4	2.9**	1.4
TIMP-2	CC	1.1	0.8	1.6*	1.4	1.9*	2.6***	2.2**	1.9*	1.4
	CTX	0.8	1.0	1.2*	1.0	1.1	1.1	1.4	1.1	1.2
TIMP-3	CC	2.6*	1.2	1.0	0.8	0.4	0.3*	1.1	1.2	1.5**
	CTX	1.4*	1.5*	1.5*	1.1	1.0	0.9	1.0	0.9	1.1
TIMP-4	CC	1.0	0.8	0.7	0.7**	0.8	0.9	1.7	1.2	2.2**
	CTX	2.8*	1.8**	1.4	1.0	0.9	1.3	1.0	0.9	1.0

For each gene arithmetic means of normalized copy numbers / 1 ng RNA from cuprizone treated mice were compared with age-matched controls (n = 5 per group) and analysed with paired Student *t*-test. Significant differences are designated with asterisks as follows: (*) $p < 0.05$, (**) $p < 0.01$, (***) $p < 0.001$. Values in the table represent ratios of arithmetic means of treated versus control mice. Dashed line indicates the removal of cuprizone from diet and beginning of remyelination. CC = coprus callosum; CTX = cortex; ND = not detected.

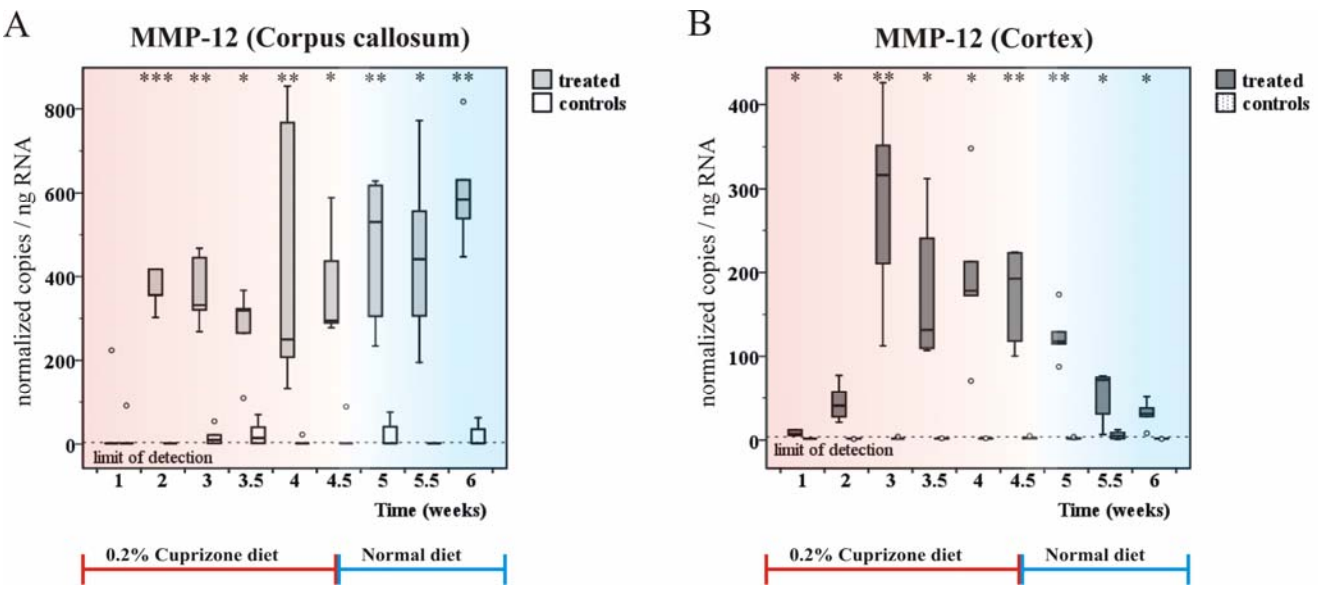

Figure 4. Matrix metalloproteinase (MMP)-12 expression measured by quantitative PCR. Box and whiskerplots show the median and quartiles of normalized copies/1 ng RNA. Extreme values are shown as circles. During demyelination high upregulation of MMP-12 is present in both corpus callosum (A) and cortex (B). During remyelination this trend continues in the corpus callosum, but not in the cortex. Significant differences between treated versus age-matched control animals were analysed with paired Student *t*-test and depicted as follows: (*) $p < 0.05$, (**) $p < 0.01$, (***), $p < 0.001$.

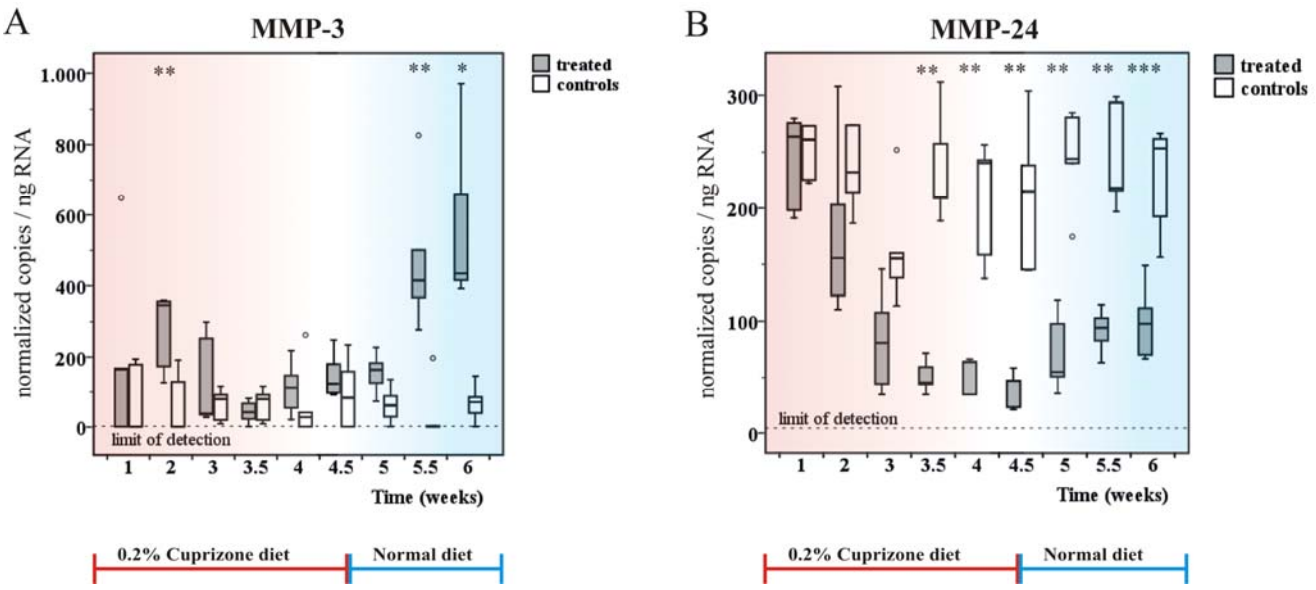

Figure 5. Matrix metalloproteinase (MMP)-3 (A) and -24 (B) expression in the corpus callosum measured by quantitative PCR. Box and whiskerplots show the median and quartiles of normalized copies/1 ng RNA. Extreme values are shown as circles. At week 2 MMP-3 demonstrates moderate upregulation which becomes particularly high during remyelination, at week 5.5 and 6 (A). Significant downregulation of MMP-24 takes place during demyelination, a trend which continues during remyelination (B). Significant differences between treated versus age-matched control animals were analysed with paired Student *t*-test and depicted as follows: (*) $p < 0.05$, (**) $p < 0.01$, (***) $p < 0.001$.

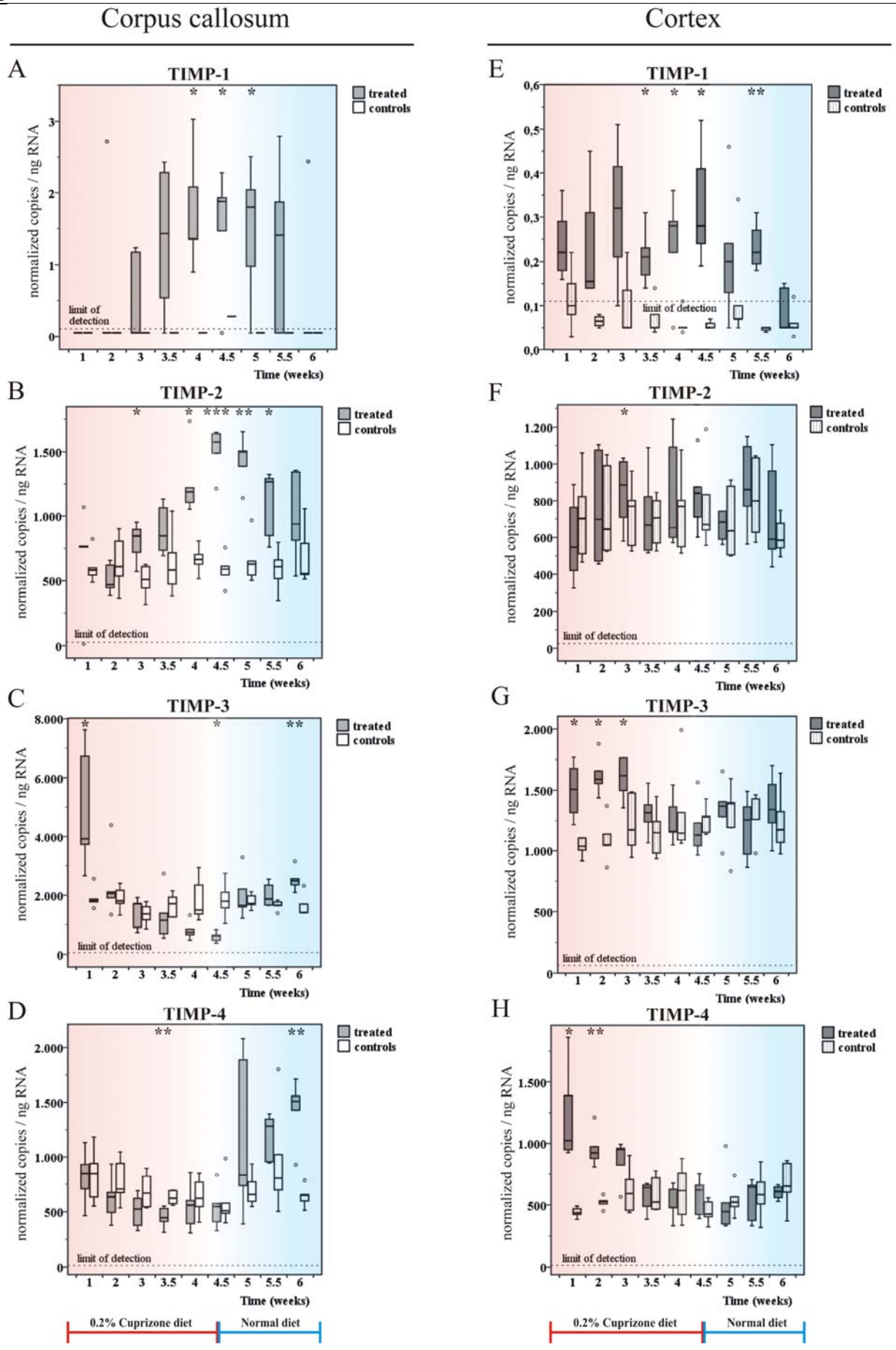
Corpus callosum
Cortex
A
TIMP-1
B
TIMP-2
C
TIMP-3
D
TIMP-4
E
TIMP-1
F
TIMP-2
G
TIMP-3
H
TIMP-4
normalized copies / ng RNA
limit of detection
Time (weeks)
treated
controls
control
0.2% Cuprizone diet
Normal diet

Figure 6. Tissue inhibitor of metalloproteinase (TIMP)-1, -2, -3 and -4 expression in the corpus callosum (A-D) and cortex (E-H) measured by quantitative PCR. Box and whiskerplots show the median and quartiles of normalized copies/1 ng RNA. Extreme values are shown as circles. During early demyelination there was high upregulation of TIMP-3 in the corpus callosum and TIMP-3 and -4 in the cortex. During peak demyelination the upregulation of TIMP-1 (corpus callosum and cortex) and TIMP-2 (corpus callosum) was prominent. Within the remyelination period in the white matter, only TIMP-4 was significantly expressed. Significant differences between treated versus age-matched control animals were analysed with paired Student *t*-test and depicted as follows: (*) $p < 0.05$, (**) $p < 0.01$, (***) $p < 0.001$.

Chapter IV:

Characterisation of microglia during de- and remyelination: evidence for creating a repair promoting environment

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In preparation

4.1 Abstract

Microglia play a key role in the initiation and perpetuation of de- and remyelination because of their ability to present antigens and to clear cell debris. Different factors expressed or secreted by microglia seem to play an important role in regenerative processes. However, it remains unclear which factors lead to a protective microglial phenotype. Moreover, recent data indicate region-specific differences within the central nervous system (CNS) for both de-/remyelination and microglial response. Here, we examined changes in microglial phenotypes in the cuprizone model in order to identify factors that promote neuroprotection. We undertook an extensive and detailed analysis of the expression of surface markers, cytokines and growth factors, and phagocytic activity of microglia. During demyelination we found an increase of microglial phagocytic activity, associated with an upregulation of phagocytic receptors, particularly TREM-2b. The expression of MHC II was only increased at the peak of demyelination but costimulatory molecules showed no significant changes. Interestingly, the proinflammatory cytokine TNF-α was upregulated while the anti-inflammatory cytokines IL-10 and TGF-ß remained unchanged. The expression of growth factors IFG-1 and FGF-2, both suggested to promote remyelination, was increased during demyelination. Our findings characterise changes of microglial markers during de- and remyelination suggesting that debris clearance mediated via TREM-2b may play an important role in the regulation of these processes. Microglial phagocytosis as well as production of TNF-α, IGF-1, and FGF-2 seem to be involved in promoting regeneration.

4.2 Introduction

Microglia represent resident cells of the central nervous system (CNS) that participate in the regulation of immune responses in the CNS due to their phagocytic function, ability to present antigens and secret immunoregulatory proteins such as cytokines, growth factors, and chemokines. Peripheral macrophages can be characterised into two subsets, the M1 and M2 phenotype (Gordon, 2003; Martinez et al., 2008; Mosser, 2003). While M1 macrophages are involved in T helper cell type 1 (Th1) responses and the elimination of microorganisms, the M2 macrophages promote the expression of anti-inflammatory molecules, the phagocytosis of debris, and tissue repair. It has been controversially debated whether microglia play a harmful or beneficial role in CNS diseases (Block et al., 2007; Hanisch and Kettenmann, 2007). Recent data suggest that microglia could fulfill both roles, and different phenotypes with distinct effector functions are involved in the regulation of de- and regenerative processes. Regional differences in microglial phenotypes may also play a role in the pathophysiology of CNS diseases (de Haas et al., 2008). In acute inflammatory lesions from patients with Multiple Sclerosis (MS), demyelination associated with infiltrates containing mainly T cell and activated macrophages (Adams et al., 1989; Hickey, 1999) led to the long prevailing hypothesis that a T cell dependent, macrophage-mediated autoimmune attack drives the disease pathogenesis (Prineas and Graham, 1981; Sriram and Rodriguez, 1997). However, not only in active MS lesions, but also in newly forming lesions, oligodendrocyte apoptosis in the absence of T cells and peripheral macrophages could be observed resulting in an infiltration and activation of local microglia (Barnett and Prineas, 2004; Gay, 2006; Henderson et al., 2009). Moreover, not only demyelination, but also

remyelination shows different patterns with regard to the extent and completeness (Goldschmidt et al., 2009; Patani et al., 2007; Patrikios et al., 2006). However, it still remains unclear why remyelination often fails in MS and consequently leads to the long term disability in MS patients (Franklin, 2002). Recent data suggest that the phagocytosis of myelin debris by microglia and macrophages plays an important role in the initiation of remyelination (Neumann et al., 2009). Kotter et al. (Kotter et al., 2001) showed that in the lysolecithin mouse model the depletion of macrophages reduces remyelination. Moreover, differentiation of oligodendrocyte precursor cells can be inhibited by the excessive myelin (Kotter et al., 2006). Under certain conditions the phagocytosis of apoptotic cells induces an anti-inflammatory cytokine profile (Liu et al., 2006; Magnus et al., 2001). Interestingly, pro-inflammatory cytokines tumour necrosis factor alpha (TNF-α) and interleukin-1ß (IL-1ß) have also been demonstrated to enhance remyelination in the cuprizone model (Arnett et al., 2001; Mason et al., 2001).

The better understanding of these multifaceted roles of microglia during de- and regenerative CNS processes can contribute to establishing new neuroprotective therapeutic strategies. Therefore, using the cuprizone induced de- and remyelination mouse model, we aimed to identify possible neuroprotective region-specific functions of microglia.

4.3 Materials and Methods

4.3.1 Animals and cuprizone treatment

C57BL/6 male mice were obtained from Charles River. All research and animal care procedures were approved by the Review Board for the Care of Animal Subjects of the district government (Lower Saxony, Germany) and performed according to international guidelines on

the use of laboratory animals. Experimental demyelination was induced by feeding 8-week-old mice with 0.2% cuprizone (bis-cyclohexanone oxaldihydrozone; Sigma-Aldrich, St. Louis, USA) mixed into a ground standard rodent chow. The cuprizone diet was maintained for 5 weeks. To induce spontaneous remyelination animals were put on normal chow for another week.

4.3.2 Immunohistochemistry

To investigate the amount of cuprizone induced de- and remyelination and accumulation of activated microglia, after 3, 4, 4.5, 5, 5.5, and 6 weeks we performed immunohistochemical stainings for the myelin protein proteolipid protein (PLP), surface protein expressed on all activated mouse mononuclear phagocytes Ricinus communis agglutinin 1 (RCA-1), and cell proliferation marker Ki-67. Mice were perfused with 4% paraformaldehyde (PFA) in phosphate buffer via left cardiac ventricle. The brains were removed, postfixed in 4% PFA and paraffin embedded. For light microscopy, 7 µm serial paraffin sections were cut and dried at 37°C overnight. Coronal sections between bregma -0.82 mm and bregma -1.82 mm (according to mouse atlas by Paxinos and Franklin, 2001) were analysed. A group size of six animals was investigated at each time point. For the immunofluorescent staining, paraffin embedded sections were de-waxed, rehydrated, and microwaved for 5 min in 10 mM citrate buffer (pH 6.0). Slides were blocked by incubation in PBS containing 10% normal goat serum and 0.1% Triton X-100 for 1 h. Sections were then incubated overnight with primary antibody. For myelin staining PLP antibody (1:500, Serotec, Kidlington, UK) was used. After washing, sections were further incubated with secondary antibody goat anti-mouse IgG (H+L) Alexa-555 conjugated (1:500, Invitrogen, Carlsbad, USA) for 1 h. For immunofluorescent double staining of microglia

fluorescein labelled RCA-1 antibody (1:1000, Vector Laboratories, Burlingame, USA) and anti-mouse Ki-67 antibody (1:50, Becton Dickinson, Franklin Lakes, USA) were used. Sections were then incubated for 1 h with the secondary antibody goat anti-mouse IgG (H+L) Alexa-555 conjugated (1:500, Invitrogen). Slides were mounted with Mowiol (Merck KGaA, Darmstadt, Germany) containing 4'6-diamidino-2-phenylindole (DAPI; 1:1000; Invitrogen).

In order to determine the extent of demyelination in the corpus callosum and cerebral cortex, brain sections were analysed by light microscopy (Leica Microsystems, Wetzlar, Germany). Brain sections from cortex were scored by three blinded observers using a scale from 0 (complete demyelination) to 4 (normal myelin) as previously described (Skripuletz et al., 2008). Corpus callosum was scored under the same conditions and graded on a scale from 0 (complete demyelination) to 3 (normal myelin) as previously described (Lindner et al., 2008).

Quantification of activated (RCA-1^+) and proliferating (Ki-67^+) microglia was performed in the central part of the corpus callosum within an area of at least 0.1875 mm^2 using a magnification of x 400 as previously described (Gudi et al., 2009). In the cerebral cortex counting was performed for the cell layers 1-6 in both left and right hemispheres within an area of at least 1.125 mm^2. Counted cells were expressed as number of cells per mm^2.

4.3.3 Isolation of microglia

Microglia were isolated from control and cuprizone treated mice after 3.5, 4.5, 5, and 6 weeks, according to the published protocol (de Haas et al., 2007). Mice were anaesthetised with ketamin/rompun s.c. and transcardially perfused with PBS to clear the intravascular compartment from blood cells. Brains were stored in ice-cold Hanks' balanced salt solution (HBSS, PAA,

Pasching, Austria) containing 15 mM HEPES buffer and 5% glucose. Corpus callosum and cortex were dissected under a light microscope from whole brains using ultra fine needle blade (Fine Science Tools, Foster City, USA). For one experiment examined areas from 8 to 10 mice were pooled. Tissue was mechanically dissociated in a tissue homogenizer (Wheaton, Millville, USA) and triturated using pasteur pipettes of decreasing diameter. Cell suspension was filtered through a 60µm cell strainer (Steriflip, Millipore, Billerica, USA), pelleted and resuspended in ice-cold 75% percoll (GE healthcare, Uppsala, Sweden). Units of 3.3 ml 75% percoll were overlayered with 5 ml 25% ice-cold percoll and 3 ml ice-cold PBS. Density gradient centrifugation was performed at 800 g without brake for 25 min at 4°C. After centrifugation the myelin containing 0/25 interface was discarded and the microglia containing 25/75 interface was collected and washed with ice-cold PBS. Viable cells were counted by trypan blue exclusion and cells from corpus callosum and cortex were further used for flow cytometry.

4.3.4 Flow cytometry

Flow cytometry was performed using a FACScalibur (Becton Dickinson). Microglia from the cortex and corpus callosum were identified according to their typical expression profile of $CD11b^{+}$ and $CD45^{low}$ and gated using forward and side scatter characteristics and (purity: 97.6 ± 0.6% for the cortex and 95.7 ± 1.5% for the corpus callosum in untreated control mice). For the each group $5x10^{4}$ cells were counted within the gate and mean fluorescence intensities (MFI) as well as percentages of positive cells were analysed with CellQuest software. Staining was performed with a combination of the three antibodies labelled with the fluorochromes phycoerythrin (PE), fluorescein isothiocyanate (FITC) and allophycocyanin (APC). For

intracellular cytokine and chemokine staining microglia were fixed with 4% paraformaldehyde and permeabilised with permeabilisation buffer (eBioscience, San Diego, USA) before fluorochrome-labelled antibodies were added.

Microglia were characterised by staining with the following anti-mouse antibodies: rat CD11b (PE, clone M1/70), hamster CD178 (FAS-L; PE, clone MFL3), hamster CD195 (CCR5; PE, clone 7A4), rat interleukin-12 (IL-12; PE, clone C17.8), hamster CD80 (PE, clone 16-10A1), rat CD45 (FITC, clone 30-F11), rat CD16/32 (Fcγ Receptor II+III; FITC, clone 93), rat CD86 (FITC, clone GL1), rat major histocompatibility complex class II (MHC class II; FITC, clone M5/114.15.2), rat IL-10 (FITC, clone JES5-16E3), hamster monocyte chemoattractant protein-1 (MCP-1/CCL2, FITC, clone 2H5), rat CD197 (CCR7; APC, clone 4B12), rat CD40 (APC, clone 1C10), rat F4/80 (APC, clone BM8), rat TNF-α (APC, clone MP6-XT22), rat interferon-gamma (IFN-γ; APC, clone XMG1.2) (all from eBioscience); hamster CD36 (APC, cloneHM36, Biozol, Eching, Germany); goat CD200 receptor 1 (CD200R; PE, clone not supplied, R&D Systems, Minneapolis, USA), rat macrophage inflammatory protein 1 alpha (CCL3; PE, clone 39624, R&D Systems), rat CD172a (signal-regulatory protein alpha (SIRP-α); FITC, clone P84, Becton Dickinson), rat triggering receptor expressed on myeloid cells 2b (TREM-2b; APC, clone 237920, R&D Systems), rat T-cell immunoglobulin domain and mucin domain 3 (Tim-3; APC, clone 215008, R&D Systems), Toll-like receptor 4 (TLR-4; FITC, clone 76B357.1, Novus Biologicals, Littleton, USA); corresponding isotype controls: (rat IgG_{2a} and IgG_{2b} PE, FITC and APC-labelled, rat IgG_1 APC-labelled, hamster IgG PE- and APC-labelled, all from eBioscience, and goat IgG PE-labelled, R&D Systems).

To study the phagocytic activity of microglia, fluorescent latex beads (Fluoresbrite YG carboxylate microspheres; 1 μm diameter; Polysciences, Eppelheim, Germany) were added to microglia in a final dilution of 1:100. After incubation for 1 h at room temperature, the cells were washed three times with PBS and resuspended in FACS-flow (BD Biosciences). MFI was measured in the FL1 channel to identify the amount of beads per phagocytosing cell and the percentage of microglia that phagocytosed latex particles.

4.3.5 RNA isolation and quantitative RT-PCR

The expression of growth factors was measured by quantitative PCR (qPCR). According to the manufacturer's recommendations total RNA was extracted from isolated microglia from the cortex and corpus callosum using the RNeasy®Mini Kit (Qiagen, Venlo, Netherlands). The RNA concentration was measured with BioPhotometer plus (Eppendorf, Hamburg, Germany). cDNA was synthesised using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, USA). RNA samples from the cuprizone treated and control mice were parallel processed under the same conditions. qPCR analysis was performed using the StepOne™ Real-Time PCR System and appropriate TaqMan assays (Applied Biosystems). All primers were intron-spanning (Table 1). A negative control containing PCR amplification mix without reverse transcribed cDNA template was included for each PCR plate. Gene expressions of brain-derived neurotrophic factor (BDNF), ciliary neurotrophic factor (CNTF), insulin-like growth factor 1 (IGF-1), fibroblast growth factor 2 (FGF-2), and transforming growth factor beta 1 (TGF-ß1) were analysed for the corpus callosum and the cortex at 4 time points (demyelination: weeks 3.5, 4.5, and 5; remyelination: week 6). The ΔΔCt method was used to determine differences in

expression between cuprizone treated and age-matched control mice. Changes in mRNA expression level were calculated after normalisation to hypoxanthin phosphoribosyltransferase (HPRT).

4.3.6 Statistics

Statistical analysis was performed using one-way analysis of variance (ANOVA) followed by the Fisher-protected least-significant difference test for post hoc comparison if appropriate. All data are given as arithmetic means ± standard error of the mean (SEM). P-values < 0.05 were considered as statistically significant and indicated by asterisks as following: (*) $p < 0.05$, (**) $p < 0.01$, (***) $p < 0.001$.

4.4 Results

4.4.1 Accumulation of microglia during de- and remyelination in the corpus callosum and cortex

In order to determine the amount of myelin during cuprizone treatment, coronal brain sections of C57BL/6 mice were immunohistochemically stained for PLP. After 5 weeks of cuprizone diet, animals were put on normal chow for another week to allow spontaneous remyelination. We found a continuously decreasing immunoreactivity for PLP that reached a minimum at week 5 in the corpus callosum and at week 5.5 in the cortex. Re-expression of PLP took place at week 5.5 in the corpus callosum and at week 6 in the cerebral cortex. Overall, the degree of remyelination in the corpus callosum after one week of remyelination exceeds the one in the cortex where demyelination proceeded for a few days despite of cessation of curpizone feeding (Fig. 1).

The accumulation and proliferation of activated microglia during cuprizone-induced de- and remyelination was monitored by immunofluorescent double-staining for RCA-1 and Ki-67. In control animals activated and proliferating microglia/macrophages were absent in both cortex and corpus callosum. During demyelination a marked accumulation of activated microglia/macrophages could be observed with a maximum at week 4.5 in the corpus callosum and to a lesser extent in the cortex (Fig. 2B). During remyelination this number declined to the level of control mice at week 6 in the corpus callosum but remained still elevated in the cortex. Corresponding to the increasing number of microglia/macrophages during demyelination, the amount of proliferating cells was also higher (Fig. 2C). After 3 weeks of cuprizone treatment, number of double positive cells increased in the corpus callosum (437.9 ± 121.1) and cortex (25.4 ± 5.5). Thereafter, a continuous decrease occurred (corpus callosum week 5: 99.7 ± 23.9 and week 6: 3.0 ± 2.3; cortex week 5: 14.4 ± 3.5 and week 6: 12.8 ± 3.0).

4.4.2 During de- and remyelination a second population of cells arises in the corpus callosum exhibiting the expression pattern of macrophages

To differentiate between microglia and macrophages, isolated cells were stained for their expression of CD45 and CD11b. Microglia typically express $CD11b^{+}/CD45^{low}$ while macrophages express high levels of CD45 ($CD11b^{+}/CD45^{high}$). In the cell gate defined by forward and side scatter characteristics, > 95% of the gated cells were positive for these surface molecules (97.6 ± 0.6% in the cortex and 95.7 ± 1.5% in the corpus callosum in untreated mice). Remarkably, during late demyelination and remyelination in the corpus callosum a second cell population arose that showed the typical expression pattern of macrophages (Fig. 3).

4.4.3 Phagocytic receptors and TREM-2b in particular were strongly upregulated during demyelination

One of the important microglial functions in the CNS represents the phagocytosis of pathogens and apoptotic cellular material. Phagocytosis is mediated via different phagocytic receptors, e.g. Toll-like receptors (TLRs), Fc receptors (FcR), triggering receptor expressed on myeloid cells-2 (TREM-2), phosphatidylserine receptors (PSRs), scavanger receptors (SRs), complement receptors (CRs) and purine receptors (PRs). Using flow cytometry, we studied the expression of phagocytic receptors: CD11b (CRs), TREM-2b, CD36 (SRs), CD16/32 (FcγR II/III), Tim-3 (PSRs), and TLR-4 (TLRs). TLR-4 that mainly mediates the phagocytosis of pathogens remained unchanged (not shown). All receptors involved in the phagocytosis of apoptotic cells were upregulated during demyelination in both cortex and corpus callosum, except Tim-3 that was only significantly upregulated in the cortex (Fig. 4A). Interestingly, the most prominently expressed was TREM-2b. It was augmented at week 3.5 (cortex: $p = 0.003$, corpus callosum: $p = 0.019$) and remained elevated during remyelination. As mentioned above, all microglia and macrophages express CD11b. The complement receptor CD11b is not only suitable as a cell type marker for microglia/macrophages, but it is also involved in phagocytosis. Comparable to the other phagocytic receptors, the MFI of CD11b expression was also further increased during demyelination. CD11b increased from 142.7 ± 18.2 in the corpus callosum of control mice (143.6 ± 17.7 in the cortex) to 253.6 ± 15.2 at week 3.5 (243.2 ± 13 in the cortex), 291.0 ± 30.5 at week 4.5 (241.6 ± 13.8 in the cortex), and slightly decreased to 265.6 ± 21.5 at week 5 (226.8 ± 10.5 in the cortex), and 250.4 ± 30.4 at week 6 (234.1 ± 15.5 in the cortex).

The upregulation of phagocytic receptors was accompanied by an increased phagocytosis activity of microglia, as determined by the engulfment of fluorescent beads (Fig. 4B). Overall, phagocytic activity was higher in the corpus callosum as compared to the cortex.

4.4.4 Changes of microglial phenotype during cuprizone-induced de- and remyelination

To further characterise microglial phenotype, we studied the expression of MHC class II, costimulatory molecules, receptors that mediate inhibitory signals for microglia, chemokines and chemokine receptors as well as FAS-ligand, an apoptosis marker.

Expression of MHC class II was increased at the maximum of demyelination (week 5) in the corpus callosum ($p = 0.014$) and cortex ($p < 0.001$). CD80 was also upregulated during cuprizone-induced demyelination, however these changes were not statistically significant (cortex $p = 0.061$, corpus callosum $p = 0.07$; Fig. 5A). CD86 and CD40 were not expressed in either control or cuprizone treated mice (data not shown).

Receptors CD200R and SIRP-α are suggested to mediate inhibitory signals for microglia. We found an increased expression of CD200R during early demyelination (cortex and corpus callosum week 3.5: $p = 0.036$; cortex week 4.5: $p = 0.013$, corpus callosum week 4.5: $p = 0.036$) that was downregulated with increasing activation of microglia. All microglia were positive for the receptor SIRP-α and the MFI was further increased during demyelination, but not statistically significant (cortex: $p = 0.119$; corpus callosum: $p = 0.092$). Even though the number of activated microglia as well as the phagocytosis activity decreased during remyelination we found no significant upregulation of these inhibitory signals during remyelination (Fig. 5B).

The chemokine receptor CCR5 was expressed on microglia but showed no alterations during de- and remyelination. CCR7 (not shown), CCL2 and CCL3 (not shown) were not significantly changed (Fig. 5C).

FAS-ligand was expressed on all microglia and the MFI showed a trend with an upregulation at the maximum of demyelination that was not statistically significant (cortex and corpus callosum week 5: $p = 0.057$; Fig. 5D).

4.4.5 Production of TNF-α was elevated during demyelination while expression of IFN-γ, IL-12, and IL-10 remained unchanged

To investigate whether different Th1- or Th2-cytokines were produced by microglia during de- and remyelination we measured intracellular cytokine content of TNF-α,. IFN-γ, IL-12, and IL-10 by flow cytometry. We found a marked increase in TNF-α secretion in microglia (cortex: $p < 0.001$, corpus callosum: $p = 0.002$) while the other cytokines remained unchanged, particularly the expression of the anti-inflammatory cytokines IL-10 and TGF-ß (Fig. 5E and 6).

4.4.6 During demyelination microglia showed increased expression of IGF-1 and FGF-2

To determine whether microglia produce growth factors that could be relevant for the regulation of cuprizone-induced de- and remyelination, we studied the expression of IGF-1, TGF-ß, FGF-2, BDNF, and CNTF by qPCR in isolated microglia from cortex and corpus callosum. The expression of growth factors was measured in cuprizone treated and age-matched control mice after 3.5, 4.5, 5, and 6 weeks. We found a strong upregulation of IGF-1 in microglia with a maximum after 4.5 weeks of cuprizone treatment in the cortex and after 5 weeks in the corpus callosum. Furthermore, FGF-2 was increased during demyelination with a maximum after 5

weeks in both areas. During remyelination, the expression of IGF-1 as well as FGF-2 was downregulated. The expression of TGF-ß, BDNF, and CNTF was changed neither in the cortex nor in the corpus callosum (Fig. 6).

4.5 Discussion

Due to the phenotypical similarity of resident microglia and peripheral macrophages it is difficult to study primary phenotypes and functions of microglia in the CNS in inflammatory animal models for demyelinating diseases such as the experimental autoimmune encephalitis (EAE) where a break down of the blood brain barrier (BBB) leads to a massive infiltration of peripheral macrophages and T cells. Therefore we used the cuprizone model to characterise microglia in primary de- and remyelination since the BBB remains intact in this model (Bakker and Ludwin, 1987; Kondo et al., 1987; McMahon et al., 2002). Feeding with the copper chelator cuprizone leads to a reproducible demyelination in different brain regions followed by spontaneous remyelination after its withdrawal (Gudi et al., 2009; Matsushima and Morell, 2001; Skripuletz et al., 2008; Skripuletz et al., 2010).

In this study, the most prominent type of microglia during demyelination expressed $CD11b^{+}$/ $CD45^{low}$ and showed a high phagocytic activity, low expression of CD80 and MHC class II but no expression of CD40 and CD86 in the corpus callosum as well as in cortex. Overall, the accumulation of microglia and phagocytotic activity was higher in corpus callosum as compared to cortex. We found no significant changes in the expression of costimulatory molecules but expression of MHC class II was increased at the peak of demyelination in corpus callosum and cortex. Therefore, the costimulatory capacity of microglia seems not to play an

essential role in cuprizone-induced demyelination. Since demyelination is not induced by autoimmune processes in this model antingen presentation does not play a major role. However, the upregulation of MHC II at peak demyelination may be of relevance for the induction of remyelination as MCH II $^{-/-}$ mice showed delayed remyelination and regeneration of oligodendrocytes after cuprizone-induced demyelination (Arnett et al., 2003).

Only in the corpus callosum we found a second cell population of CD11b^{+} cells, particularly during remyelination. These cells exhibited the phenotype of macrophages (CD11b^{+}/CD45^{high}). It remains unclear whether these macrophages derive from the CNS itself or infiltrate from the periphery. In two recent works the recruitment of macrophages from the periphery into the CNS has been described despite an intact BBB (McMahon et al., 2002; Remington et al., 2007). Remington et al. found that most of these CD45^{high} macrophages immigrate from blood and have a high antigen-presenting capacity with increased expression of MHC I, CD86, and CD80 when compared to microglia.

In contrast to costimulatory molecules and MHC II, a distinct upregulation of the phagocytic receptor TREM-2b was evident as early as week 3. Other phagocytic receptors, e.g. CD36 and FcγR II/III, were also upregulated, but the main effect was observed for TREM-2b indicating a possible role of this molecule in microglial phagocytosis during primary demyelination. Recent data suggest that TREM-2b mediated phagocytosis is crucial for an effective debris clearance. Patients with a loss-of-function mutation of the microglial receptor TREM-2b suffer from a chronic neurodegenerative disease, named polycystic lipomembranous osteodysplasia with sclerosing leukoencephalopathy (PLOSL) or Nasu-Hakola disease (Hakola, 1972; Nasu et al., 1973), that is most probably caused by an impaired phagocytic function of

microglia (Paloneva et al., 2002). In EAE, antibody-mediated blockade of TREM-2 during the effector phase results in exacerbation of disease (Piccio et al., 2007) and the intravenous application of TREM-2-transduced myeloid cells leads to amelioration of EAE associated with increased myelin phagocytosis (Takahashi et al., 2007). Phagocytosis via TREM-2b has been suggested to promote anti-inflammatory responses in microglia (Neumann and Takahashi, 2007). However, we found a strong increase of TNF-α production in microglia during demyelination, but no upregulation of the anti-inflammatory cytokines IL-10 or TGF-ß. Consistent with these findings TNF-α as well as IL-1ß knockout mice showed a delay in remyelination associated with a reduction of proliferating oligodendrocyte progenitors (OPC) (Arnett et al., 2001; Mason et al., 2001). These data indicate that under certain conditions rather pro-inflammatory cytokines such as TNF-α and IL-1ß can promote regeneration and an anti-inflammatory cytokine milieu.

Together with phagocytic activity we detected decreasing TNF-α production in microglia during remyelination. This was not associated with an increased expression of CD200R and SIRP-α, suggested to mediate inhibitory signals in microglia. Therefore the downregulation of phagocytosis seems not to be mediated through these inhibitory molecules. Takahashi et al. (Takahashi et al., 2005) showed *in vitro* that TREM-2b overexpression in microglia reduced the level of inflammatory gene transcripts for TNF-α, IL-1ß, and NOS-2 whereas knockdown of TREM-2b in microglia resulted in the opposite. Therefore, it may be suggested that the strong upregulation of TREM-2b mediated phagocytosis is capable to counter-balance the TNF-α driven phagocytic activity of microglia as a possible mechanism to avoid an unlimited microglial activation and to allow regenerative processes.

In addition to phagocytic activity and the release of cytokines another potential microglial function to promote regeneration is the release of growth factors. Growth factors are known to influence the migration, differentiation, proliferation and survival of neuronal and glial cells and are therefore crucial for the regulation of myelination (Franklin and Ffrench-Constant, 2008). We found a vast upregulation of IGF-1 expression in microglia and to a lesser extent of FGF-2 that peaked during late demyelination between week 4.5 and 5, while BDNF and CNTF remained unchanged. IGF-1 is known to promote neurogenesis, oligodendrocyte proliferation and differentiation from precursor cells, and to protect several neuronal cells from apoptosis (Aberg et al., 2006; Hsieh et al., 2004; Perez-Martin et al., 2010). Interestingly, in cuprizone treated IL-1ß knockout mice impaired remyelination was associated with a lack of IGF-1 production and a delay in OPC differentiation into mature oligodendrocytes (Mason et al., 2001). Furthermore, transgenic mice continuously expressing IGF-1 showed a more rapid remyelination when compared to control mice after cuprizone-induced demyelination that was caused by increased survival of mature oligodendrocytes (Mason et al., 2000). These data indicate that microglial production of IGF-1 contributes to remyelination in the cuprizone model.

FGF-2 is suggested to be one of the important developmental regulator proteins involved in oligodendrocyte maturation. But disparate data have been published about the role of FGF-2 in remyelination. *In vitro* FGF-2 has been demonstrated to inhibit the differentiation but enhance the proliferation of OPC (McKinnon et al., 1990), and under certain culture conditions FGF-2 can also induce oligodendrocyte apoptosis (Muir and Compston, 1996). In contrast, inactivation of FGF-2 signalling in knockout mice does not influence proliferation and survival of oligodendrocytes but impairs OPC differentiation (Kaga et al., 2006; Oh et al., 2003). In a

chronic model of cuprizone-induced demyelination the lack of FGF-2 leads to improved remyelination of chronically demyelinated areas (Armstrong et al., 2006). But the intracranial injection of a combination of FGF with other growth factors (PDGF, NT3, IGF-1) during cuprizone treatment resulted in increased numbers of OPC and enhanced remyelination (Kumar et al., 2007). Overall, the role of FGF-2 in the regulation of regeneration remains controversial and it is most likely dependent on various factors such as the participating cell types, the surrounding environment, and the stage of development of oligodendrocytes. Our results suggest that microglial production of FGF-2 and IGF-1 could be capable to support regeneration.

In conclusion, here we demonstrate that in cuprizone-induced demyelination the main microglial population exhibits an activated phenotype with a high phagocytic activity. Microglial capacity to present antigen or to activate T cells seem to be of minor importance during primary demyelination. During remyelination the accumulation and phagocytotic activity of microglia is markedly downregulated and a small population of macrophages appears in corpus callosum indicating a dynamic change of the role of microglia/macrophages during these processes. Other conspicuous regional differences in microglial phenotype between cortex and corpus callosum were not found. Our results support the hypothesis that microglial debris clearance creates an environment that is beneficial for regeneration. In this regard regulation of phagocytosis via TREM-2b seems to play an essential role. Besides this, microglia contribute to the repair permissive environment by providing the growth factors IGF-1 and FGF-2. Thus it is likely that microglia play an essential role to allow successful remyelination and repair process in the CNS.

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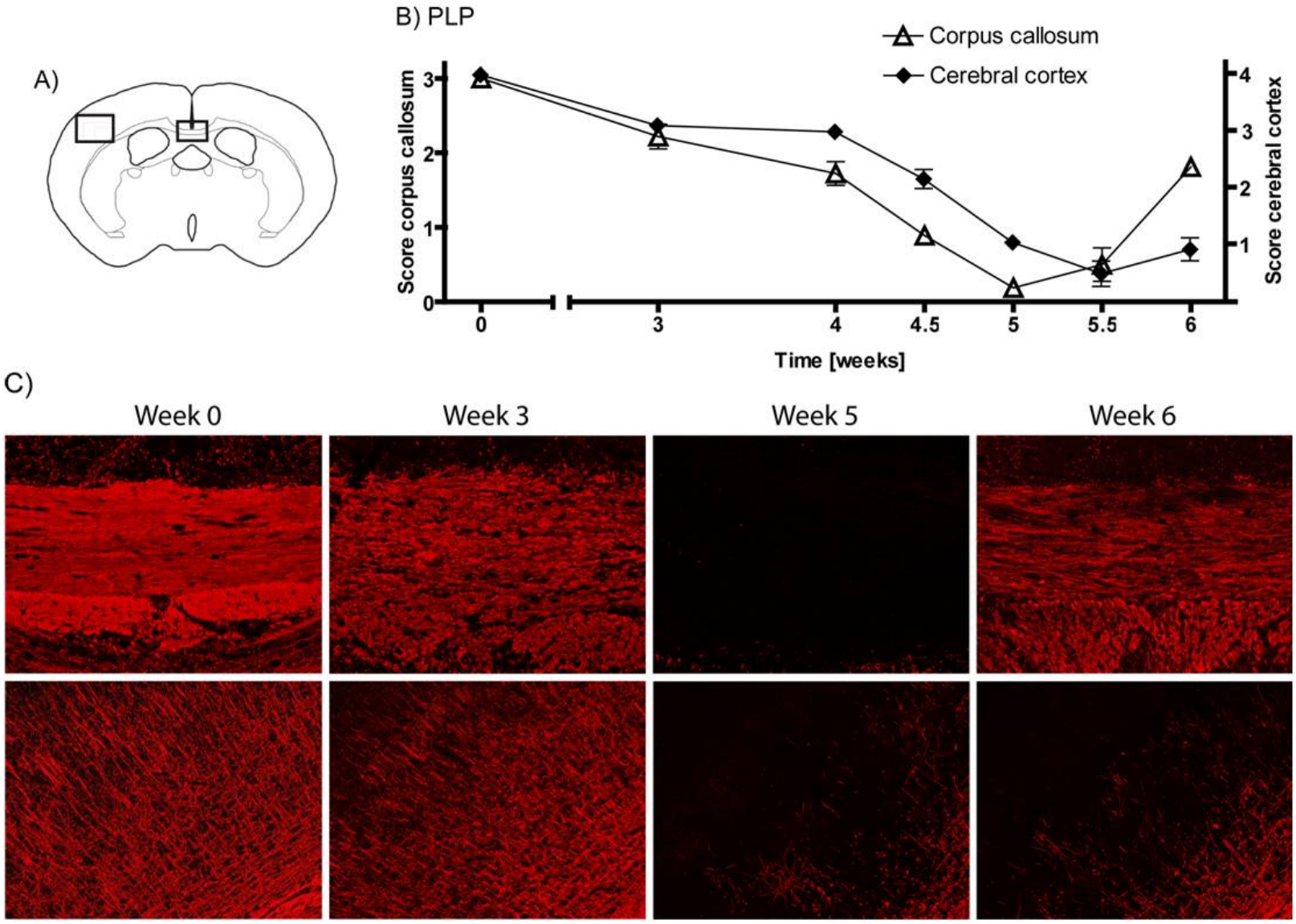

A)
B) PLP
Corpus callosum
Cerebral cortex
Score corpus callosum
Score cerebral cortex
Time [weeks]
0
3
4
4.5
5
5.5
6
C)
Week 0
Week 3
Week 5
Week 6

Figure 1: De- and remyelination of corpus callosum and cortex during cuprizone treatment. (A) Schematic diagram of the mouse brain coronal section. Boxes show medial corpus callosum and dorsolateral cortex and that were analysed. (B) Scoring of PLP expression demonstrated a complete demyelination of corpus callosum after 5 weeks of cuprizone diet. After mice had been shifted to normal chow, there was a spontaneous remyelination in the corpus callosum until week 6. A score of 0 represents complete demyelination and a score of 3 normal myelin in the corpus callosum. In the cortex, maximum demyelination was delayed (week 5.5). At week 6 remyelination was still incomplete when compared to corpus callosum. In the cortex a score of 0 corresponds to complete demyelination and a score of 4 to normal myelin. Scoring from 3 independent raters is presented as mean ± SEM. (C) Representative immunofluorescent PLP staining from one animal (out of 6) of corpus callosum (upper panel) and cortex (lower panel) in control mice (week 0) and in cuprizone treated mice after week 3, 5, and 6 are shown.

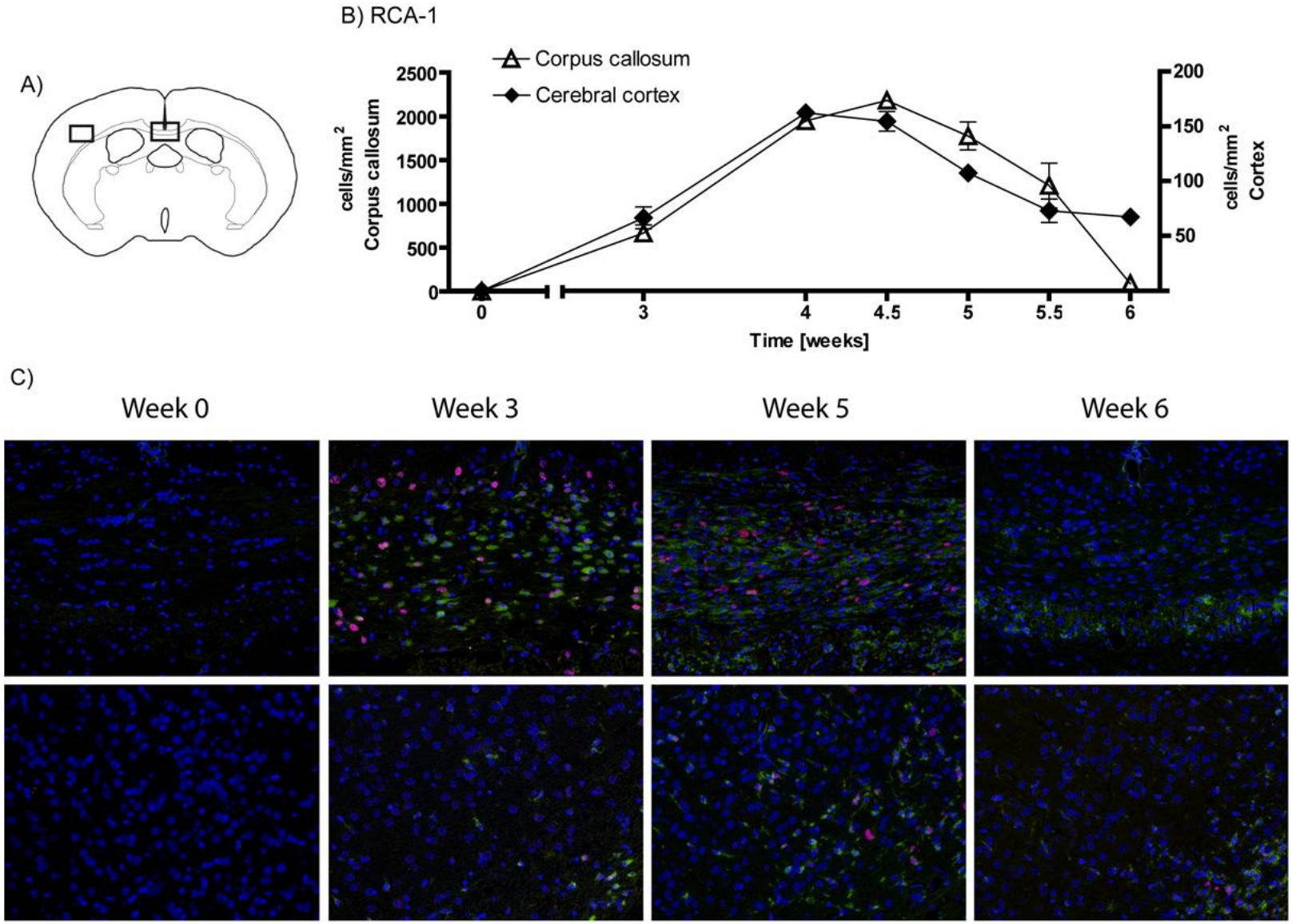

A)
B) RCA-1
Corpus callosum
Cerebral cortex
cells/mm2 Corpus callosum
cells/mm2 Cortex
2500
2000
1500
1000
500
0
200
150
100
50
0
3
4
4.5
5
5.5
6
Time [weeks]
C)
Week 0
Week 3
Week 5
Week 6

Figure 2: Accumulation of activated and proliferating microglia during cuprizone-induced demyelination in the corpus callosum and cortex. (A) Schematic diagram of the mouse brain coronal section, the boxes show the analysed areas. (B) The number of activated microglia (RCA-1 positive cells) was markedly increased during demyelination in the corpus callosum and cortex and decreased again during remyelination. Overall, accumulation of microglia was more prominent in the corpus callosum when compared to the cortex. Mean ± SEM of counted cells / mm^2 from 6 mice per timepoint and treatment group are demonstrated. (C) The immunofluorescent double-staining of brain sections with RCA-1 (green) and Ki-67 (red) showed an increasing number of proliferating microglia during demyelination. Nuclei are visualized with DAPI (blue). Immunofluorescent stainings from one representative animal (out of 6) are shown for corpus callosum (upper panel) and cortex (lower panel) in controls (week 0) and in cuprizone treated mice after week 3, 5, and 6.

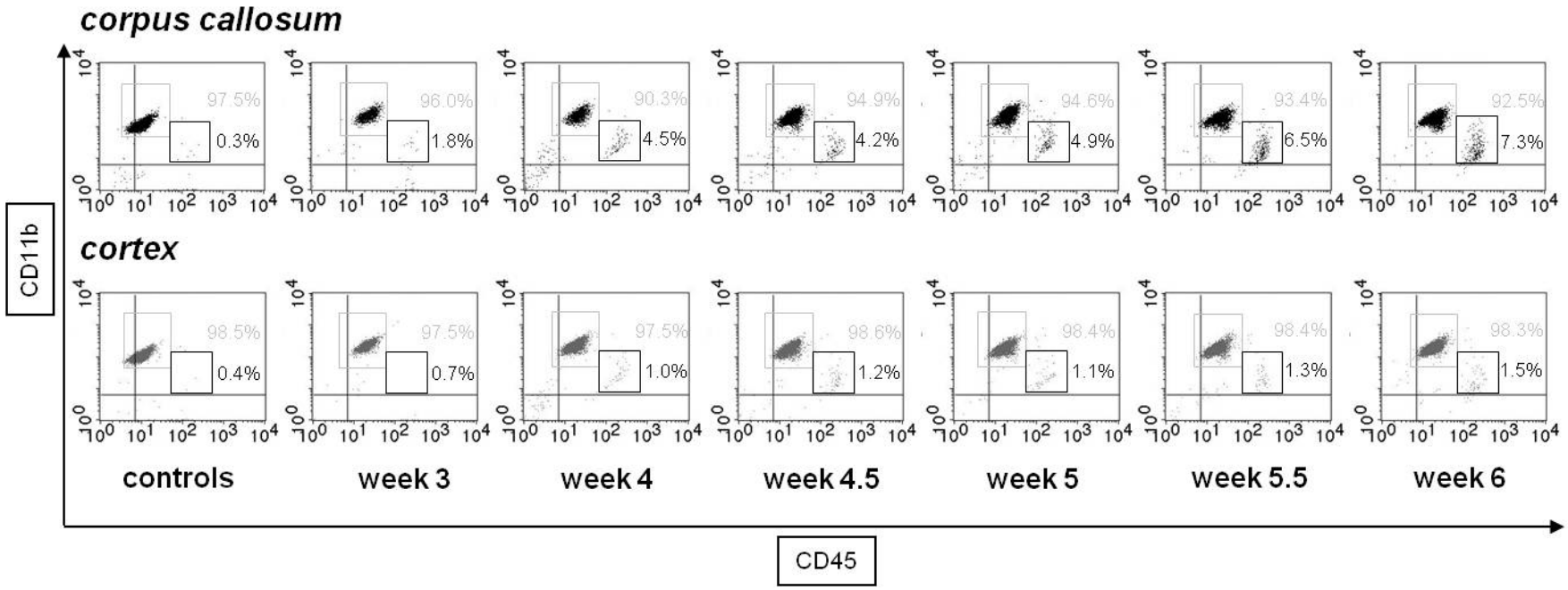
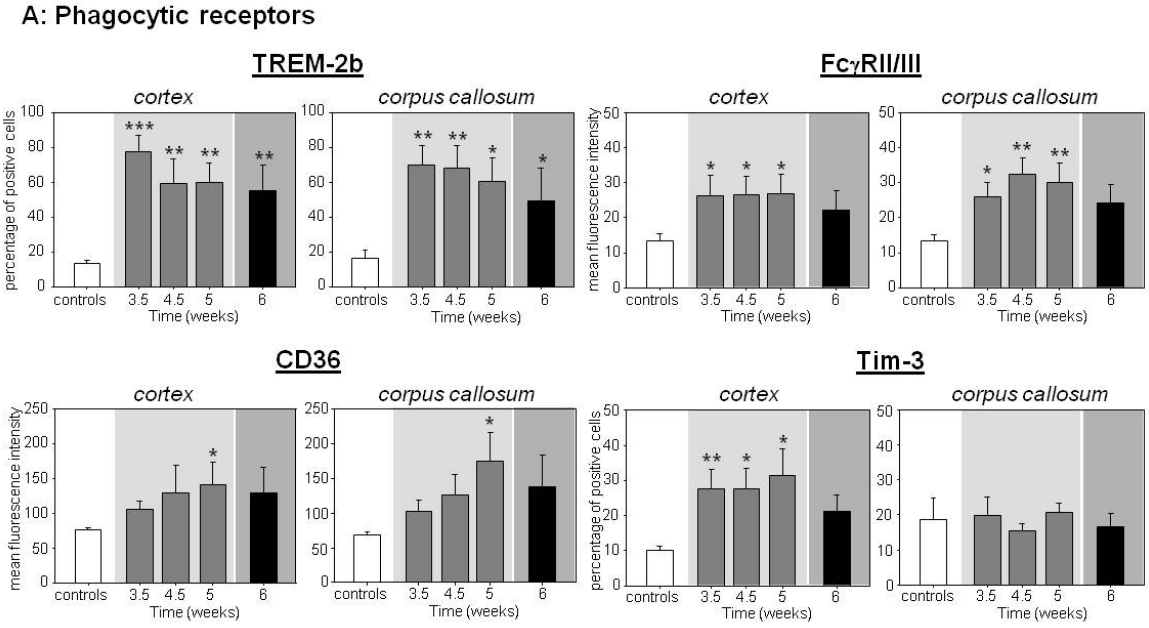

corpus callosum
cortex
CD11b
CD45
97.5%
0.3%
96.0%
1.8%
90.3%
4.5%
94.9%
4.2%
94.6%
4.9%
93.4%
6.5%
92.5%
7.3%
98.5%
0.4%
97.5%
0.7%
97.5%
1.0%
98.6%
1.2%
98.4%
1.1%
98.4%
1.3%
98.3%
1.5%
controls
week 3
week 4
week 4.5
week 5
week 5.5
week 6

Figure 3: A second population of cells showing macrophage phenotype occurs during de- and remyelination in the corpus callosum. Macrophages and microglia were differentiated due to their expression of CD11b and CD45 measured by flow cytometry. Almost all gated cells (> 95%) demonstrated the phenotype of microglia ($CD11b^{+}/CD45^{low}$: grey gate) in control mice. However, during demyelination a second population was detectable in corpus callosum (upper plots) exhibiting the phenotype of macrophages ($CD11b^{+}/CD45^{high}$: black gate). In cerebral cortex (lower plots) the percentage of $CD11b^{+}/CD45^{high}$ macrophages remained < 2%. Dot plots from one representative experiment out of 3 are shown.


A: Phagocytic receptors
TREM-2b
FcγRII/III
CD36
Tim-3
cortex
corpus callosum
percentage of positive cells
mean fluorescence intensity
controls
3.5
4.5
5
6
Time (weeks)

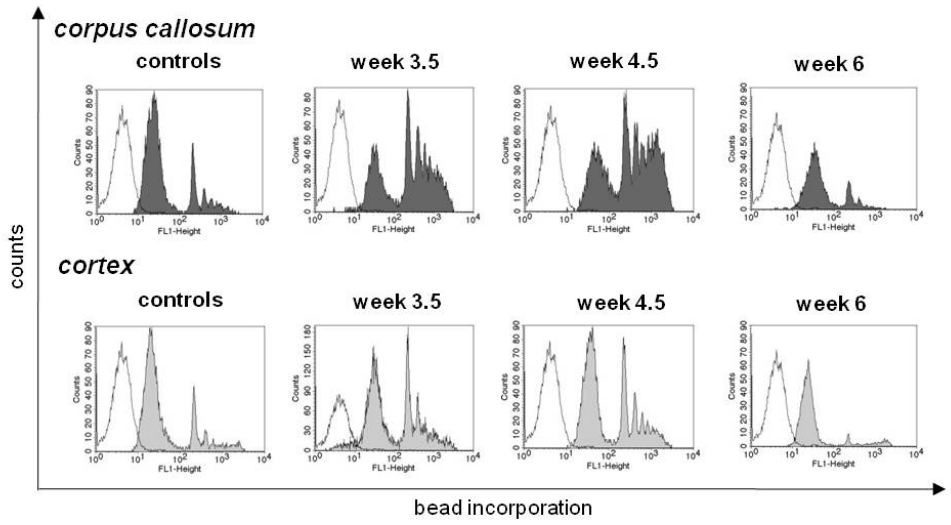

B: Phagocytosis activity
corpus callosum
cortex
controls
week 3.5
week 4.5
week 6
counts
bead incorporation
FL1-Height

Figure 4: Upregulation of phagocytic receptors and phagocytosis activity of microglia during demyelination. (A) Microglial expression of different phagocytic receptors (TREM-2b, FcγR II/III, CD36, Tim-3) was measured by flow cytometry in control mice (white bars), and cuprizone fed mice during demyelination (week 3.5, 4.5, 5: grey bars) and remyelination (week 6: black bars). The expression of TREM-2b, FcγR II/III, and CD36 was significantly increased during demyelination in both cortex and corpus callosum and of Tim-3 in the cortex only. For TREM-2b and Tim-3 mean ± SEM of percentage of positive cells are presented from 3 independent experiments. For FcγR II/III and CD36 mean fluorescence intensities are shown since all gated expressed these receptors (percentage of positive cells > 97%). (*) $p < 0.05$, (**) $p < 0.01$, (***) $p < 0.001$. (B) Phagocytic activity of microglia was assessed by their incorporation of fluorescent latex beads (FL-1). We found an increasing phagocytosis in cuprizone treated mice during demyelination with a maximum at week 4.5, which decreased during remyelination. The phagocytic activity of microglia was more pronounced in corpus callosum (dark grey histograms) when compared to cortex (light grey histograms). Black histograms: unstained negative control.

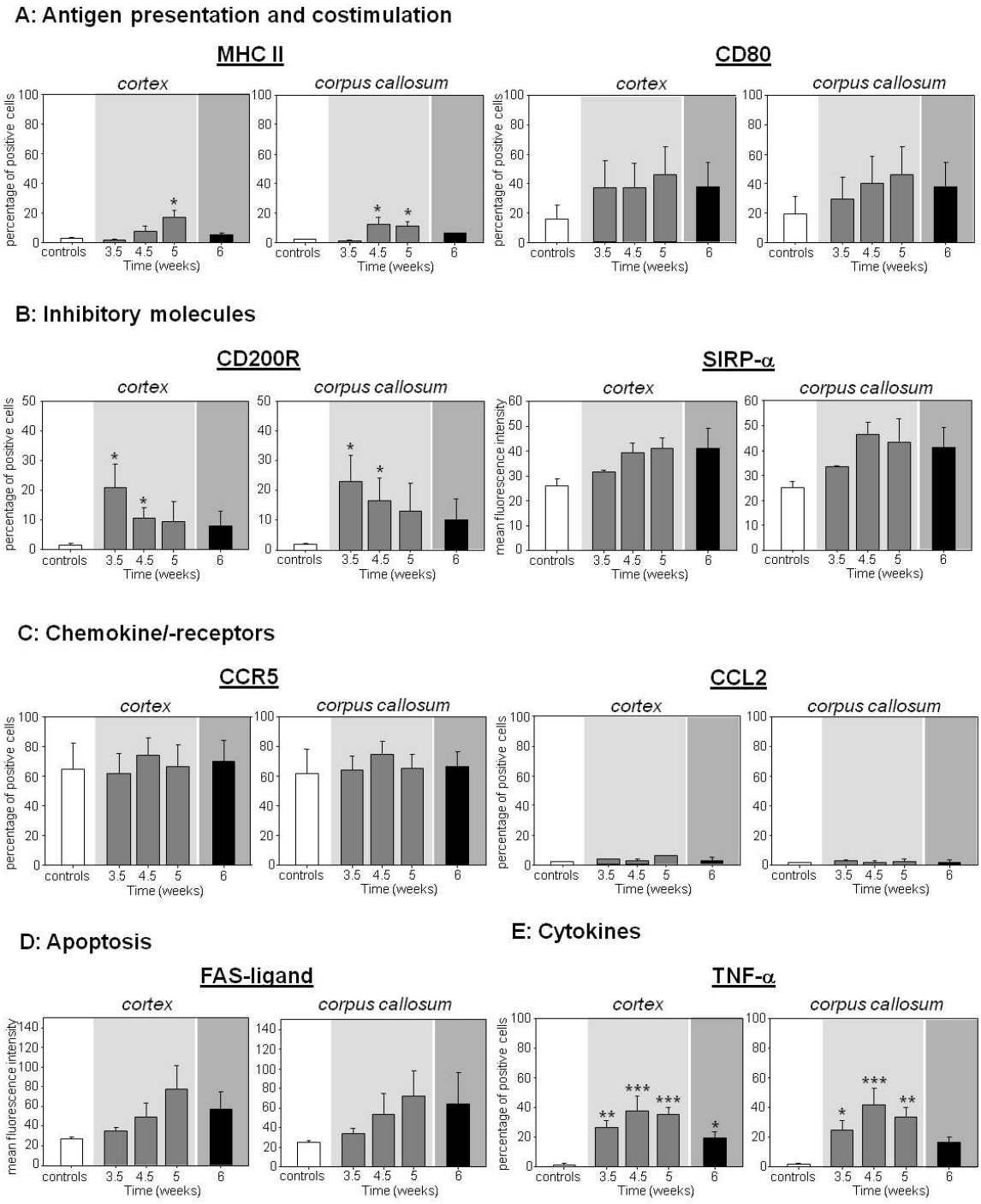

A: Antigen presentation and costimulation
MHC II
CD80
cortex
corpus callosum
percentage of positive cells
controls
3.5
4.5
5
6
Time (weeks)
B: Inhibitory molecules
CD200R
SIRP-α
mean fluorescence intensity
C: Chemokine/-receptors
CCR5
CCL2
D: Apoptosis
FAS-ligand
E: Cytokines
TNF-α

Figure 5: The expression of antigen presentation and costimulatory molecules, inhibitory molecules, chemokine, chemokine and apoptosis receptors and cytokine during cuprizone induced de- and remyelination. To evaluate changes in the phenotype of microglia during cuprizone-induced de- and remyelination we studied the expression of MHC II and the costimulatory molecule CD80 (A), CD200R and SIRPα, receptors that mediate inhibitory signals (B), the expression of CCR5 and CCL2 (C), the apoptosis receptor FAS-ligand (D) and the production of TNF-α (E). Mean ± SEM of percentage of positive cells are presented from 3 independent experiments. Because all microglia were positive for SIRP-α and FAS-L (percentage of positive cells > 97% for SIRP-α and > 95% for FAS-L) for these receptors mean fluorescence intensities are shown. (*) $p < 0.05$, (**) $p < 0.01$, (***) $p < 0.001$.

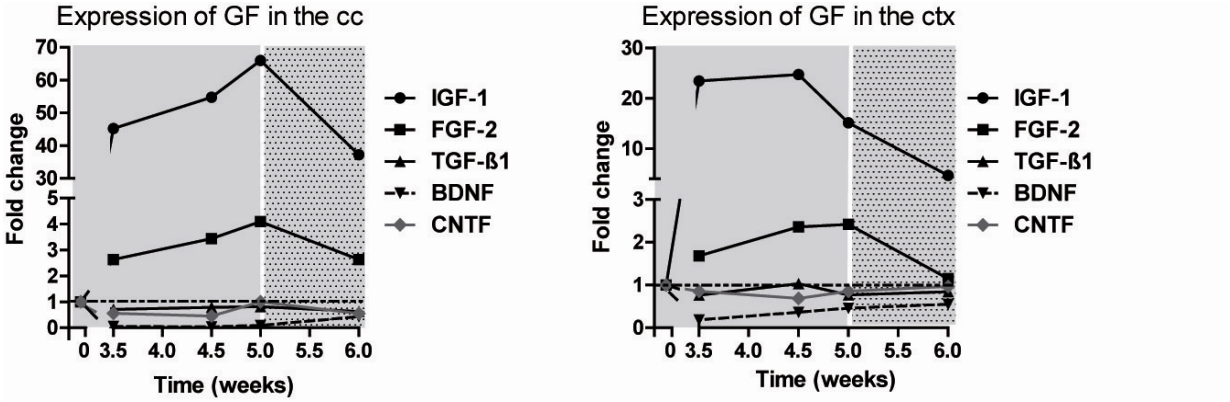


Figure 6: Microglia produce IGF-1 and FGF-2 during demyelination. The expression of the growth factors (GF) IGF-1, FGF-2, CNTF, and BDNF and the anti-inflammatory cytokine TGF-ß1 was detected by quantitative RT-PCR. Microglia were isolated from cortex (ctx) and corpus callosum (cc) and RNA concentration was measured in control mice and in cuprizone treated mice at week 3.5, 4.5, 5, and 6. We found a remarkable upregulation of IGF-1 expression during demyelination and to lesser extent of FGF-2 in both corpus callosum and cortex. Fold changes of mRNA levels of the different growth factors are presented after normalization to HPRT.

Table 1. Primer pairs used for qPCR

Growth Factor	Assay number
BDNF	Mm01334042_m1
CNTF	Mm00446373_m1
IGF-1	Mm00439560_m1
TGF-ß1	Mm00441724_m1
FGF-2	Mm00433287_m1
HPRT	Mm00446968_m1

Chapter V: General discussion

5.1 The influence of chemokines on microglia

Chemokines regulate many of the homeostatic functions in the context of CNS development and physiology (Biber et al., 2002). Moreover, in many neurodegenerative and neuroinflammatory diseases, including MS, chemokines and their receptors have been found to be upregulated (Szczucinski and Losy, 2007; Filipovic et al., 2003; Trebst et al., 2008). Besides chemotaxis, considered as the most general response to chemokine stimulation, a direct immunomodulatory effect of chemokines on monocytic cells has been proposed (Trebst et al., 2008; Rankine et al., 2006; Villalta et al., 1998). In the present study, we investigated *in vitro* the response of microglia to stimulation with CCL2, CCL3, CCL5, and CXCL1. A range of different chemokine concentrations were applied on primary rat microglia, with or without LPS co-stimulation, in order to induce cell activation or to maintain their relatively resting state. All chemokines induced chemotaxis and calcium transients, proving the functionality of chemokine receptors expressed on microglia. Of all tested chemokines, only CCL5 showed immunomodulatory effect by stimulating NO release and downregulating IL-10 and IGF-1 secretion. Nevertheless, it did not affect TNF-α production or phagocytic activity. Like most of the chemokines, CCL5 can mediate its actions through different receptors. Of these, in our experimental settings microglia expressed CCR1 and CCR5 which allows us to speculate that they might be responsible for the observed effects. However, it is not known whether chemotaxis and direct immunomodulation are mediated via the same intracellular signaling pathways. Mutational studies showed that despite the promiscuity of the chemokine receptors, the three-

dimensional structure of CCL5 is relevant for driving specific receptor conformation changes, deciding on signaling response (Wells et al., 1996). Importantly, NO stimulation and IL-10/IGF-1 downregulation is noticed only when microglia was stimulated together with CCL5 and LPS. Since CCL5 alone induced migration and an increase in intracellular calcium, it is likely that this chemokine directs migration of microglia/monocytes in physiological processes, e.g. development or surveying CNS tissue, without inducing their cytotoxic profiles. LPS has been shown to elevate basal $[Ca^{2+}]_i$ in microglia which was a prerequisite for effective release of NO and cytokines (Farber and Kettenmann, 2006). The activation step, mediated via TLR4 or another receptor, could be also critical for interfering with CCL5-induced signaling pathways in MS, increasing the pro-inflammatory outcome. Nevertheless, taken into account that the behaviour of microglia is determined largely by the nature and context of the stimuli (Schwartz et al., 2006) and that diverse stimuli are present in different pathological milieus, it is feasible that differently primed microglia obtain dissimilar responsive profiles. A genetical study of polymorphisms in MS patients revealed that the high-producer alleles for CCR5 and CCL5 were associated with worse clinical course whereas low-producer alleles for the same proteins resulted in less inflammation, less axonal loss and more remyelination (van Veen et al., 2007). However, none of the polymorphisms contributed to the development of MS, substantiating that CCL5 and CCR5 have no effect on the MS pathogenesis, but rather enhance the inflammation once it has started. Similarly, it has been shown that CCR5 is not essential for inducing EAE (Tran et al., 2000). CCR5 expression has been found on microglia/macrophages within early remyelinating MS lesions as compared to completely demyelinated lesions, whereas the number of newly infiltrating CD68-positive cells did not differ between the groups (Trebst et al., 2008). Thus, it

can be speculated that the functional meaning of microglial CCR5 upregulation in inflammatory conditions is not only a physical receptor-based regulation of migratory parameters, but also an alternative effector function at the transcriptional level, resulting in tuning the immune response parameters at the lesion site. Finally, the consequence of an altered immune profile after CCL5 stimulation is not clear.

In many pathological states, a wide spectrum of stimuli has been found to provoke changes in chemokine expression. For instance, TNF-α and IL-10 can influence the release of CCL5 in microglia (Hu et al., 1999). Even if the observed CCL5-mediated immunomodulatory effects are not high in amplitude, they could contribute to the positive feedback loop, by interacting within the complex network of inflammatory mediators. Subsequently, disequilibrium of cytokine and chemokine production might result in exacerbated damage and uncontrolled chronic inflammation. It is presumable that chemokines have different effects which could be dose- and context-dependent, although the molecular basis remains unknown. Therefore it is of particular interest to elucidate the ultimate roles and underlying pathways of CCL5 in different CNS inflammatory conditions.

5.2 Matrix metalloproteinase and tissue inhibitor of metalloproteinase gene expression during de- and remyelination of the white and gray matter

MMPs are considered as mediators of ECM remodeling. These zinc-containing enzymes maintain also non-matrix degrading functions, such as regulation of cellular survival, growth, differentiation, and apoptosis (Yong et al., 2007a). Having a high potency for degradation of different substrates, their activity has to be controlled. One level of regulation provide the natural

inhibitors of MMPs, TIMPs. In the CNS, harmful and pathological functions of MMPs, e. g. during inflammatory demyelination, have received considerable attention (Kieseier et al., 1999). However, it is increasingly appreciated that MMPs have many other important roles like promoting remyelination and tissue repair (Yong, 2005). Moreover, although MS demyelinating lesions typically appear within the white matter, recently it has been recognized that gray matter, particularly cortex, is also massively affected (Lassmann and Lucchinetti, 2008). Therefore we investigated the temporal expression pattern of 11 MMPs and 4 TIMPs in the white (corpus callosum) and grey (cortex) matter during cuprizone induced demyelination and subsequent remyelination. Even if the underlying mechanism of oligodendrocyte death is not fully understood, the cuprizone model of toxic demyelination in the corpus callosum and the cortex is well characterized (Gudi et al., 2009; Skripuletz et al., 2008). Using immunohistochemistry, after 4.5 weeks of cuprizone feeding we detected the loss of MBP signal which was complete in the corpus callosum and less prominent and slightly delayed in the cortex. This correlated with abundant accumulation of activated microglia and astrocytes as well as with depletion of Nogo-A positive oligodendrocytes. Using precise excision of corpus callosum and cortex with subsequent qPCR, we detected upregulation of MMP-3 in the corpus callosum, TIMP-4 in the cortex and MMP-12 and TIMP-3 in both areas during early demyelination. During severe demyelination we detected MMP-24 downregulation and TIMP-2 upregulation in the corpus callosum and MMP-12, -14, -15 and TIMP-1 upregulation in both areas. During remyelination we detected MMP-3, -11, -14, TIMP-3 and -4 upregulation and MMP-24 downregulation in the corpus callosum, while MMP-12 transcript numbers were still high in both areas. MMP-2, -7, -9, -10, and -13 were minimally affected throughout the cuprizone treatment. Similarly to our findings, elevated

expression of many of the MMPs and TIMPs has been found in MS and EAE during demyelination (Yong et al., 2007b; Toft-Hansen et al., 2004). As many molecules that are locally produced in the brain in low amounts have physiological functions, the net effect of acute increase in MMP production may have detrimental consequences resulting in tissue injury. Not only that aberrantly expressed MMPs may contribute to the MS pathology via direct cleavage of myelin proteins, they can also degrade vascular basement membrane resulting in BBB disruption and exacerbate inflammation by facilitating cellular invasion, releasing soluble pro-inflammatory mediators and activating other MMPs (Shiryaev et al., 2009b; McCawley and Matrisian, 2001; Sorokin, 2010). In turn, pro-inflammatory cytokines further induce MMP secretion (Crocker et al., 2006), thereby aggravating tissue destruction. High elevation of MMP-2, -7, -10, and particularly MMP-9 in serum, cerebrospinal fluid and brain parenchyma have been described in many MS and EAE studies (Yong et al., 2007b; Buhler et al., 2009; Toft-Hansen et al., 2004). However, in our experimental settings we detected minimal changes in their transcript numbers. Since cuprizone model is characterized by the intact BBB, these MMPs may be specifically involved in BBB damage.

Recently, MMP-12 was implicated in promoting OPC maturation and myelination in the CNS (Larsen and Yong, 2004; Larsen et al., 2006) and it has been proposed that MMPs are detrimental in the process of demyelination early on, while at later stages they become beneficial. This is supported by our data. During remyelination the most remarkable upregulation showed MMP-12 and -3, implicating that these MMPs are repair promoting proteins. It is also possible that MMPs facilitate CNS tissue repair by clearing tissue from myelin debris after a demyelinating episode, allowing migration of new precursor cell that will replenish the damaged

areas and releasing growth and other soluble factors that support affected tissue (Larsen et al., 2003; McCawley and Matrisian, 2001). Other beneficial aspects could include the role in angiogenesis or subtle processing of cell-cell recognition molecules such as notch or neuregulins that allow repair (Yong, 2005).

Interestingly, during severe demyelination and during remyelination we detected a downregulation of membrane type (MT)-MMP gene expression (MMP-15 and -24). A similar result has been observed in mouse models of neuroinflammation and brain injury where downregulation occured independently of the pro-inflammatory cytokine expression (Toft-Hansen et al., 2007). Similarly to secreted MMPs, MT-MMPs can cleave ECM components, chemokines, cytokines and growth factors (Stamenkovic, 2003). They are thought to play a regulatory role in activating other MMPs and molecules localized in the close proximity of the cell membrane which can then interact with cell surface receptors. There is also an increasing interest about their role in MS pathogenesis (Shiryaev et al., 2009a). However, even if the particular actions and substrate specificities of these MT-MMPs in the CNS are not yet known, their downregulation during demyelination, that we and others noticed, does not support the view of MMPs as exclusively pro-inflammatory mediators.

Regarding differences in MMP and TIMP expression patterns between corpus callosum and cortex, our data are in line with the suggested different mechanisms of tissue injury of white and gray matter in MS (Lassmann and Lucchinetti, 2008). Beside noticeable differences in TIMP mRNA expression, MMP-3 and -12 also showed dissimilar patterns. As revealed by immunohistochemistry specific for myelin protein and glia, the course of de- and remyelination in the corpus callosum and the cortex in the cuprizone model is not identical. It can be speculated

that myelin amounts as well as cellular composition and cellular quantities could account for these differences. Moreover, these variations in the molecular local environment may determine cellular phenotypes and their commitment towards certain beneficial or detrimental effects. Region-specific MMP and TIMP mRNA expression was also demonstrated in other models of CNS pathology where MMP/TIMP imbalance correlated with locally produced inflammatory cytokines (Muir et al., 2002; Khuth et al., 2001).

Ongoing research in the MS field reveal that the current therapies and clinical trials have influence on MMP and TIMP levels (Bernal et al., 2009; Shinto et al., 2009). There is also an effort in creating new approaches that utilize specific or broad spectrum MMP inhibitors (Borkakoti, 2004). Although not thoroughly established in MS, several MMPs and TIMPs are changing at different stages of the disease in animal models. This suggests that the prolonged treatment with MMP inhibitors in MS might not only prevail detrimental aspects of MMPs, but also increase the risk of inhibiting some of their repair promoting properties. Thus, differential expression of MMPs and TIMPs during de- and remyelination as well as pathophysiological differences between the white and gray matter should be taken into consideration when developing therapeutic strategies.

5.3 The function of microglia during remyelination

The aim of our third study was to examine microglial expression profiles and function during de- and remyelination in the white and grey matter. Using a density gradient method we isolated microglia *ex vivo* from the dissected corpus callosum and cortex of cuprizone treated and control young adult mice at week 3.5, 4.5, 5, and 6. Microglia were identified according to their

typical $CD11b^{+}/CD45^{low}$ expression. Living cells were used for the phagocytosis assay and staining of the surface markers/intracellular cytokines (flow cytometry), while the growth factor expression was determined from the total mRNA (qPCR).

Most of the tested phagocytic receptors (CD11b, TREM-2b, CD36, CD16/32 and Tim-3) were significantly upregulated during demyelination while TREM-2b stayed prominently upregulated also during remyelination. TREM-2b, a member of the immunoglobulin superfamily, has been suggested to be involved in the clearance of tissue debris (Neumann and Takahashi, 2007). The upregulation of phagocytic receptors was accompanied by an enhanced phagocytic activity of microglia as shown by an increased engulfment of fluorescent latex beads. Phagocytic removal of apoptotic cells and tissue debris is considered to have an important role in providing a pro-regenerative environment and tissue homeostasis in the CNS (Neumann et al., 2009). Therefore, phagocytosis mediated via TREM-2b seems to be one of the microglial repair promoting functions in the cuprizone model.

IGF-1 is known to facilitate oligodendrocyte development and survival thereby fostering myelination (D'Ercole et al., 2002). Myelin production per oligodendrocyte is enhanced in IGF-1 overexpressing mice while there is a reduction in oligodendrocyte proliferation and development in IGF-1 null mice (Carson et al., 1993; Ye et al., 2002). We detected high microglial IGF-1 mRNA expression during late demyelination and early remyelination which could reflect another beneficial aspect of microglial response.

Several studies described functional differences between microglia and acutely infiltrating inflammatory macrophages (reviewed in Carson et al., 2007). Moreover, recent analyses also revealed region-specific differences in the microglial expression pattern within the healthy and

inflamed CNS (Schmid et al., 2009; de Haas et al., 2008). Using the expression level of a cell type specific marker CD45 ($CD45^{low}$ and $CD45^{high}$ for microglia and infiltrating macrophages, respectively), during the late demyelination and remyelination we identified in the corpus callosum a second cell population that showed typical macrophage expression pattern. Interestingly, in another study intravenously transferred microglia derived from embryonic stem cells migrated across an intact BBB and selectively accumulated in the corpus callosum and hippocampus (Tsuchiya et al., 2005). However, the functional significance of the observed region-specific preference is not clear. Regarding regional differences we also found variations in the number of microglia, Tim-3 expression and phagocytic activity between the corpus callosum and cortex in the cuprizone treated animals. This indicates that the presence and immunological profile of microglia could be determined by the myelin amount and region-specific microenvironmental signals. Our Tim-3 expression data from control animals showed a similar distribution like in the human healthy brain white and grey matter tissue (Anderson et al., 2007). In MS lesions the expression of Tim-3 was notably upregulated only in microglia isolated from the border region of active lesions as compared to ones from the center of MS plaques, adjacent normal appearing white matter, or non-inflamed white matter tissue. Furthermore, levels of Tim-3 on microglia in EAE murine model peaked just before worsening of the clinical score (Anderson et al., 2007). Besides the suggested role for Tim-3 in regulating the pathogenic Th1 cell response, its elevated expression on microglia implies a functional role on resident CNS immune cells. However, it is still not clear whether Tim-3 expressing microglia ameliorate or exacerbate the disease. Although the Tim-3 ligand, galectin-9, terminates detrimental Th1 responses, it can also stimulate TNF-α production in human Tim-3 expressing monocytes

(Freeman et al., 2010). Concerning the microglial role in clearance of myelin debris and apoptotic cells and the fact that Tim-3 serves as a receptor for phosphatidylserine (DeKruyff et al., 2010), the most abundant phospholipid in myelin proteolipid complexes (Denisova, 1990), it is possible that myelin phagocytosis of microglia is mediated in part by Tim-3.

Taken together, numerous findings within the recent years have considerably altered the viewpoint about microglia in the healthy and pathological CNS. Moreover, there is a growing body of evidence that microglial functions may be tailored to both stimulus and brain region. Our data add up one more piece to the complex puzzle of microglial behavior, interactions and molecular heterogeneity which may help in developing therapies that specifically target detrimental phenotypes of microglia without altering their beneficial functions.

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The role of monocytes in remyelination and repair processes in the CNS

Summary

Due to the fragmentary knowledge about molecular mechanisms of spontaneous remyelination, up to now there is no proven approach that promotes the repair of MS lesions. *In vitro* and *in vivo* studies provide increasing evidence that mononuclear phagocytes are beneficial for remyelination. Moreover, in recent years extensive cortical demyelination in MS patients has been recognized and highlighted. The cuprizone murine model is a commonly used and well established toxic model of demyelination in the white and gray matter without disrupting the blood-brain barrier.

The aim of this project was to investigate whether CCL2, CCL3, CCL5, and CXCL1 modulate the cytokine and growth factor production, NO synthesis, and phagocytosis in non-stimulated and lipopolysaccharide (LPS)-stimulated primary rat microglia *in vitro*. We showed that CCR1, CCR5, and CXCR2 were functionally expressed on microglia. All tested chemokines stimulated chemotaxis. CCL5 and CCL2 increased NO, while only CCL5 attenuated IL-10 as well as IGF-1 production in activated microglia. None of these chemokines influenced TNF-α production or phagocytic activity.

Further goal was to investigate the time-dependent expression patterns of 11 MMPs and 4 tissue inhibitors of metalloproteinases (TIMPs) during de- and remyelination in the cuprizone model. At nine time points mRNA was extracted from the laser microdissected cortex and corpus

callosum and analyzed using quantitative PCR. In comparison with age-matched controls, MMP-12 was significantly upregulated in both areas during de- and remyelination. Interestingly, only in the white matter MMP-3, -11 and -14 were upregulated during remyelination, while MMP-24 was significantly downregulated during both de- and remyelination. Moreover, different expression patterns of TIMP-3 and TIMP-4 were observed in the corpus callosum and cortex.

In addition, in the same model we studied the expression of surface markers, cytokines and growth factors as well as the phagocytic activity of *ex vivo* isolated microglia. During demyelination we found an increase of microglial phagocytic activity, associated with an upregulation of phagocytic receptors, particularly TREM-2b. The expression of MHC II was only increased at the peak of demyelination but the costimulatory molecules showed no significant changes. Interestingly, the proinflammatory cytokine TNF-α was upregulated while the anti-inflammatory cytokines IL-10 and TGF-ß remained unchanged. The expression of growth factors IGF-1 and FGF-2 was increased during demyelination.

Based on these findings, we propose that CCL5 directly induces a pro-inflammatory phenotype of activated microglia. However, these changes were modest and should be considered as a fine-tuning of microglia. Furthermore, concerning the high expression of MMP-3 and MMP-12 during remyelination, we suggest that MMPs may also regulate CNS tissue repair. Differences in MMP and TIMP expression levels in the white and gray matter indicate different molecular mechanisms in these areas. Changes of microglial phenotypes during de- and remyelination suggest that debris clearance mediated via TREM-2b may play an important role in the regulation of these processes. Microglial phagocytosis as well as the production of TNF-α, IGF-1, and FGF-2 seem to be involved in promoting regeneration.

Jelena Škuljec

The role of monocytes in remyelination and repair processes in the CNS

Zusammenfassung

Die molekularen Mechanismen der spontanen Remyelinisierung sind nur lückenhaft bekannt und Ansätze, die zweifelsfrei Reparaturmechanismen in Multiple Sklerose (MS)-Läsionen begünstigen, liegen nicht vor. Zahlreiche in-vitro- und in-vivo-Studien belegen mittlerweile, dass mononukleäre Phagozyten für die Remyelinisierung entscheidend sind.

Wir untersuchten daher zunächst unstimulierte und lipopolysaccharid-stimulierte Ratten-Mikrogliazellen hinsichtlich direkter in-vitro Effekte der Chemokine CCL2, CCL3, CCL5 und CXCL1 auf Veränderungen der Zytokinproduktion, der NO-Synthese, der Wachstumsfaktoren und der Phagozytose. Im Vorfeld konnten wir zeigen, dass die Mikrogliazellen die Chemokin-Rezeptoren CCR1, CCR5 und CXCR2 exprimieren und die verwendeten Chemokine eine Chemotaxis fördern. Letztendlich führen die Chemokine CCL5 und CCL2 zu einem Anstieg von NO wohingegen CCL5 zusätzlich die IL-10- und IGF-1-Produktion senkt. Keines der Chemokine hat einen Einfluss auf die TNFα-Produktion oder auf die Phagozytose.

Um die Rolle der Mikrogliazelle während der De- und Remyelinisierung zu untersuchen, verwendeten wir das Cuprizone-Mausmodell, welches ein etabliertes toxisches Tiermodell der Demyelinisierung der weißen als auch der grauen Substanz darstellt, ohne die Blut-Hirn-Schranke zu beeinflussen. Anhand dieses Tiermodells untersuchten wir die Expression von 11 Matrix Metalloproteinasen (MMP) und vier ihrer Inhibitoren, die sogenannten „tissue inhibitors

of metallopreinases“ (TIMP), zu neun unterschiedlichen Zeitpunkten. Die angefertigten Lasermikroschnitte des Kortex und des Balkens wurden mittels quantitativer PCR analysiert. Hierbei stellten wir fest, dass im Vergleich zu den altersentsprechenden Kontrollen MMP-12 in beiden Hirnarealen während der Re- und Demyelinisierung signifikant erhöht ist. MMP-3, MMP-11 und MMP-14 sind lediglich in der weißen Substanz und ausschließlich während der Remyelinisierung signifikant erhöht, wohingegen MMP-24 in beiden Phasen erniedrigt ist. Bei den Inhibitoren TIMP-3 und TIMP-4 beobachteten wir ferner ein unterschiedliches Expressionsmuster im Balken und im Kortex.

In demselben Modell erfolgten zudem Untersuchungen der Expression von Oberflächenmarkern, Zytokinen, Wachstumsfaktoren und der Phagozytose an ex-vivo isolierten Mikrogliazellen. Wir konnten nachweisen, dass die Phagozytoseaktivität während der Demyelinisierung erhöht ist und mit einer Hochregulierung von Phagozytoserezeptoren, insbesondere TREM-2b, einhergeht. Die Expression des MHC-II-Moleküls ist lediglich zum Gipfel der Demyelinisierung erhöht und nicht mit einer Veränderung von kostimulatorischen Molekülen verbunden. Das pro-inflammatorische Zytokine TNF-α wird vermehrt produziert, wohingegen die anti-inflammatorischen Zytokine IL-10 und TGF-β unverändert bleiben. Die Wachstumsfaktoren IGF-1 und FGF-2 sind während der Demyelinisierung signifikant erhöht.

Die vorliegenden Ergebnisse belegen einen direkten Einfluss von CCL5 auf Mikrogliazellen, welches bei diesen Zellen einen pro-inflammatorischen Phenotyp induziert. Diese Veränderungen sind allerdings minimal i.S. eines zellulären „fine tunings“. Die erhöhte Exprimierung von MMP-3 und MMP-12 während der Remyelinisierung lässt darauf zurückschließen, dass diese Proteine Reperaturvorgänge regulieren können. Die Unterschiede in

den Proteinexpressionen in der grauen und weißen Substanz weisen ferner auf unterschiedliche molekulare Mechanismen hin.

Die phänotypische Veränderung der Mikroglia impliziert, dass das Abräumen von Debris, vermittelt durch TREM-2b, als auch die direkte Phagozytose eine bedeutende Rolle während der De- und Remyelinisierungsvorgänge spielen. Die Produktion von TNF-α, IGF-1 und FGF-2 scheinen zudem die Regeneration zu fördern.

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Declaration

I herewith declare that I autonomously carried out the PhD-thesis entitled “The role of monocytes in remyelination and repair processes in the CNS”.

I did not receive any assistance in return for payment by consulting agencies or any other person. No one received any kind of payment for direct or indirect assistance in correlation to the content of the submitted thesis. I conducted the project at the following institutions: Department of Neurology, Hannover Medical School, Germany, and Department of Pathology, University of Veterinary Medicine Hannover, Germany. The thesis has not been submitted elsewhere for an exam, as thesis or for evaluation in a similar context.

I hereby affirm the above statements to be complete and true to the best of my knowledge.

_____, Hannover, January 2011

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