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Published in:
Journal of Neuroimmunology

DOI:
10.1016/j.jneuroim.2010.04.021

Publication date:
2010

Journal of Neuroimmunology 225 (2010) 112–117

Contents lists available at ScienceDirect

Journal of Neuroimmunology

journal homepage: www.elsevier.com/locate/jneuroim

Myelin ingestion by macrophages promotes their motility and capacity to recruit myeloid cells

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ARTICLE INFO

Article history:
Received 20 December 2009
Received in revised form 23 April 2010
Accepted 29 April 2010

Keywords:
Macrophages
Cervical lymph nodes
Multiple sclerosis
Immune cell trafficking
Cerebrospinal fluid

ABSTRACT

Myelin-laden macrophages reside within the CNS, the CSF and in the CNS-draining lymph nodes during MS and EAE, suggesting migration of these macrophages between these compartments and interaction with other cells. Since chemokines and their receptors are pivotal for leukocyte trafficking, we addressed whether myelin ingestion affects chemotaxis of mouse macrophages *in vitro*. Myelin ingestion enhanced expression of CCR7 and CXCR3 on macrophages and migration towards CCL21 and CXCL10. Furthermore, myelin-laden macrophages released chemoattractants resulting in enhanced migration of myeloid cells *in vitro*. Our data demonstrate that myelin-laden macrophages have increased motility and suggest trafficking between anatomical compartments *in vivo*.

1. Introduction

Myelin-laden macrophages are abundantly present in the central nervous system (CNS) of patients with multiple sclerosis (MS) and animals with experimental autoimmune encephalomyelitis (EAE). These macrophages are derived from CNS-resident microglia and from infiltrating monocytes (Li et al., 1996) and have ingested large amounts of myelin debris. Although they were previously considered as aggressors during MS (van der Laan et al., 1996; Williams et al., 1994), there is increasing evidence that human myelin-laden macrophages have an anti-inflammatory phenotype *in situ* (Boven et al., 2006). Moreover, ingestion of myelin by both human and mouse macrophages as well as by mouse microglia *in vitro* and *ex vivo* mimics this phenotype (Boven et al., 2006; Liu et al., 2006b; van Rossum et al., 2008).

MS is characterized by complex orchestration of leukocyte movement into and out of inflamed CNS tissue (Sospedra and Martin, 2005), which is regulated by interactions between chemokines and their receptors. Myelin-laden macrophages in MS lesions and perivascular spaces can communicate with and attract other cells. Furthermore, myelin-laden macrophages also reside in the cerebrospinal fluid (CSF) (Herndon and Kasckow, 1978; Zeman et al., 2001) and in the secondary lymphoid organs of MS patients and EAE-affected animals (de Vos et al., 2002; Fabriek et al., 2005; van Zwam et al., 2009), suggesting that these cells are highly motile and apt at migrating between and within anatomical compartments.

Until now, it is unclear whether myelin-laden macrophages have the capacity to migrate and whether they are able to attract other leukocytes into the brain towards the lesions where they reside. We therefore addressed the question whether myelin ingestion by macrophages results in the release of chemokines involved in the recruitment of leukocytes into the CNS (Balashov et al., 1999; Omari et al., 2004; Quandt and Dorovini-Zis, 2004; Ubogu et al., 2006), and in altered expression of chemokine receptors which are key in regulating leukocyte trafficking and responsiveness to their corresponding ligands.

We demonstrate that myelin ingestion results in increased chemotaxis of macrophages. In addition, myelin-laden macrophages demonstrated enhanced recruitment of myeloid cells, but not lymphocytes.

2. Experimental procedures

2.1. In vitro generation of bone marrow-derived myelin-laden macrophages

Femora and tibiae from adult C57BL/6 mice (Harlan) were cleaned of muscles and tendons and ground in a mortar to obtain bone marrow. Single cell suspensions were obtained by passing the bone marrow through a 70 μm gauze. Bone marrow cells were seeded in a concentration of 1×10^7 cells/petri dish in DMEM (Cambrex) supplemented with 10% heat-inactivated FCS (Cambrex), 50 μM β-mercaptoethanol (Sigma),

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doi:10.1016/j.jneuroim.2010.04.021

100 units/ml penicillin and 100 µg/ml streptomycin (Cambrex). Bone marrow cells were differentiated into macrophages by stimulation with 10 ng/ml mouse rM-CSF (Peprotech) on day one, and 5 ng/ml mouse rM-CSF on day three. After seven days, differentiated macrophages were incubated with 25 µg/ml human myelin during 48 to 72 h. Human myelin was isolated from post-mortem white matter derived from MS patients as described (Norton and Poduslo, 1973). In short, white matter was homogenized in 0.32 M sucrose and subsequently layered on 0.85 M sucrose. After centrifugation at 75,000 *g* myelin was collected from the interface, washed in water and suspended in water for osmotic shock. Macrophages which were not fed with myelin served as control macrophages. Cells were harvested by scraping them after incubation on ice in PBS/2 mM EDTA for 30 min and used for further analysis.

2.2. Real time quantitative PCR

mRNA expression was quantified using real time quantitative reverse-transcription PCR as described (Boven et al., 2006). To compare different experiments, the expression level by control macrophages was set as 100%. The individual results from the duplicates were processed individually, after which the mean was calculated. Target gene expression levels were normalized for GAPDH mRNA levels. Sequences of PCR primers (PE Biosystems) and fluorogenic probes (Universal Probe Library, Roche) are listed in Table 1.

2.3. Flow cytometric analysis

The chemokine receptors CCR7 and CXCR3 were stained for 30 min on ice using phycoerythrin (PE)-conjugated rat–anti-mouse CCR7 (eBiosciences) and allophycocyanin (APC)-conjugated rat–anti-mouse CXCR3 (R&D). The antibodies were diluted in PBS/0.5% BSA/0.01% sodium azide. Subsequently, the cells were washed in PBS/0.5% BSA/0.01% sodium azide. Isotype-matched primary antibodies of irrelevant specificity served as negative controls. 20,000–30,000 events were measured using a FACSCalibur flow cytometer and analyzed by CellQuest software (Becton Dickinson).

2.4. Chemotaxis

Cells were washed with serum-free medium before using them in the chemotaxis assay. Chemotaxis to the indicated chemokines was determined using a 48-well chemotaxis microchamber (NeuroProbe). The lower chambers were filled with 100 nM CXCL10 or CCL21 which were diluted in serum-free DMEM. Chambers filled with DMEM alone served as a negative control. Upper and lower chambers were separated by a polycarbonate filter (8 um pore size, GE Osmonics). The upper chamber was seeded with 25,000 cells. The cells were incubated for 2 h at 37 °C and 5% CO_2. Migrated cells were fixed and stained using the Diff-Quick staining set (Merz-Dade). Cells in the upper chamber that did not migrate were removed using a wet cotton swab. Migration was determined by counting all cells within the pores using a 10× brightfield objective and the Cell Counter plugin from Image J biomedical image analysis software (Rasband, 1997–2007). Chemokine-specific migration was calculated by normalizing for basal levels of migration. All experiments were performed in triplicate within an experiment.

Table 1
Primer and probe sequences.

Gene of interest	Forward primer (5′-3′)	Reverse primer (5′-3′)	Probe (5′-3′)
CCR7	TATCTGCGTCGCCTCAAAC	TCTGATCCTGCAAGCCATC	CCACCTCC
CXCR3	GCAGCACGAGACCTGACC	GGCATCTAGCACTTGACGTTC	CAGCCACA
CCL2	CATCCACGTGTTGGCTCA	GATCATCTTGCTGGTGAATGAGT	ACCTGCTG
CCL3	TGCAACCAAGTCTTCTCAGC	GGAATCTTCCGGCTGTAGG	GCCTGCTG
CCL4	GCCCTCTCTCTCCTCTTGCT	GAGGGTCAGAGCCCATTG	GCTCCAGG
CCL5	ACTCCGGTCCTGGGAAAAT	GATTTCTTGGGTTTCGTGGTC	CTCCATCC
GAPDH	AGCTTGTCATCAACGGGAAG	TTTGATGTTAGTGGGGTCTCG	CATCACCA

2.5. Elisa

Chemokine production by macrophages was determined in supernatants using commercial capture ELISA's according to manufacturer's instructions. CCL3, CCL4, and CCL5 ELISA's were obtained from R&D.

2.6. Transwell migration assay

Migration of lymphocytes and myeloid cells towards myelin-laden macrophages was assessed using a transwell migration assay. 1×10^6 bone marrow-derived myelin-laden macrophages or control macrophages were seeded into the lower compartment of the transwell chamber (Costar) and allowed to produce chemoattractants for 24 h.

Splenocytes were harvested by passing spleens from adult C57BL/6 mice trough a 70 µm gauze. After erythrocyte lysis, spleen myeloid cells were isolated with Automacs (Miltenyi Biotec) using anti-CD11b beads (Miltenyi Biotec) and were subsequently stained with fluorescein isothiocyanate (FITC)-conjugated rat–anti-mouse CD11b (BD PharMingen). Upper compartments containing filters with pores of 8 µm were used for migration of myeloid cells. The upper chambers were coated overnight at 4 °C with 20 µg/ml fibronectin (Sigma-Aldrich). After washing, 1×10^5 myeloid cells were seeded into the upper compartments and incubated with the macrophages for 4 h at 37 °C/5% CO_2 in a humidified atmosphere. Subsequently, the myeloid cells were fixed in 4% paraformaldehyde. Myeloid cells at the upper side of the filter that did not migrate, were removed using a wet cotton swab. FITC-positive cells within the pores of the filter were counted using fluorescence microscopy.

Chemotaxis of spleen lymphocytes was performed as described above using filters with 5 µm pores. Since lymphocytes are not adherent and therefore migrate into the supernatant, migration of lymphocytes was determined as follows. After 4 h, the upper filters were removed and a fixed number of flow cytometric beads (BD Biosciences) were added to all the supernatants. Subsequently, the supernatants were run through the flow cytometer until a fixed number of beads had been counted. This way, the relative number of lymphocytes in the supernatants was counted by the flow cytometer. Data are represented as the number of lymphocytes divided by the number of beads. All transwell experiments were performed in triplicate. Migration towards medium without macrophages served as negative control.

2.7. Statistical analysis

Results were analyzed with a two-tailed Mann–Whitney *U*-test using the statistical software program SPSS, version 11.0. A significance level of 0.05 was used.

3. Results

3.1. Myelin-laden cells express increased CXCR3 and CCR7 levels and demonstrate enhanced chemotaxis towards CXCL10 and CCL21

In MS, CCR7 and CXCR3 are key chemokine receptors regulating leukocyte trafficking. The current study therefore addressed the question whether myelin ingestion by macrophages influenced the expression of the chemokine receptors CCR7 and CXCR3 *in vitro*. Bone marrow-derived mouse macrophages were incubated with human myelin and the expression of these receptors was determined before and after incubation with myelin. Although CCR7 and CXCR3 mRNA expression did not significantly differ between myelin-laden and

control macrophages (Fig. 1A), there was a pronounced increase in CCR7 and CXCR3 surface protein expressions (Fig. 1B), indicating that myelin ingestion did not result in shedding or downregulation of these chemokine receptors.

Subsequently, it was assessed whether the increase in CCR7 and CXCR3 expression after myelin ingestion enhanced the responsiveness to their ligands. Chemotaxis assays were used to determine this, and simultaneously provided information on the motility of myelin-laden cells *in vitro*. In concordance with increased chemokine receptor expression, myelin-laden macrophages demonstrated significantly increased migration towards CXCL10 and CCL21 as compared to control macrophages (Fig. 1C), indicating that the large quantities of myelin ingested by macrophages did not perturb the motility of these cells.

3.2. Myelin uptake by macrophages promotes their capacity to attract myeloid cells

Perivascular myelin-laden macrophages in CNS lesions are optimally positioned to attract other leukocytes. In order to contribute to this leukocyte recruitment, the production of inflammatory chemokines or other chemoattractants is required. Myelin-laden macrophages consistently expressed higher levels of CCL2, CCL3, and CCL4 mRNA as compared to control macrophages, albeit not statistically different (Fig. 2A). CCL5 mRNA could not be detected in myelin-laden nor in control macrophages. The release of CCL3, CCL4, and CCL5 protein in the supernatants was below the assay detection limits.

Since functional chemotaxis is not merely mediated by chemokines, but also by other chemoattractants such as complement factors and leukotrienes (Sallusto and Mackay, 2004), we assessed whether myelin ingestion by macrophages resulted in enhanced functional recruitment of leukocytes. Transwell experiments allowed macrophages to produce and release chemoattractants, and to attract leukocytes without cell-to-cell contact. Fig. 2B shows that similar numbers of lymphocytes migrated towards myelin-laden macrophages as to control macrophages. In contrast, myelin-laden macrophages recruited significantly more $CD11b^{+}$ myeloid cells than control macrophages (Fig. 2C).

4. Discussion

The current study investigated the functional effect of myelin ingestion by macrophages on chemotatic properties *in vitro*. We show that bone marrow-derived mouse macrophages show increased expression of the chemokine receptors CXCR3 and CCR7 and, in

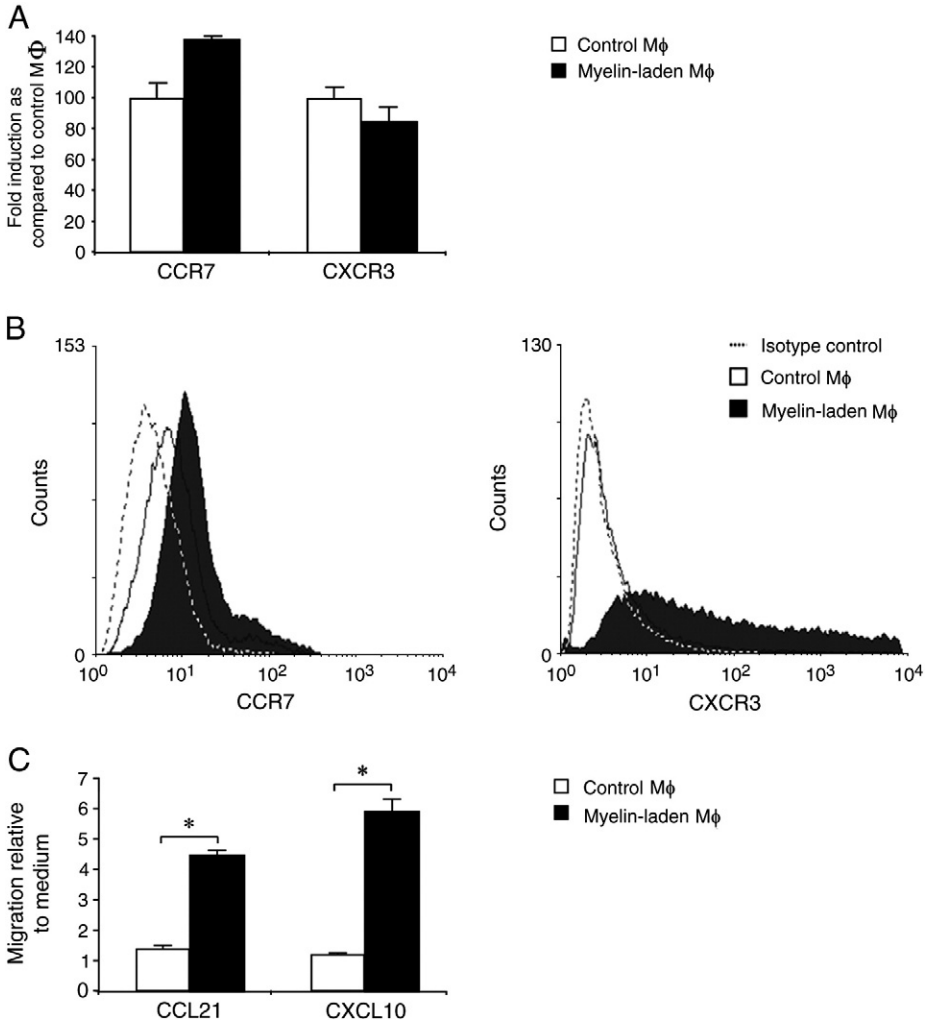

Fig. 1. Myelin ingestion enhances chemotaxis of macrophages towards CXCL10 and CCL21. Bone marrow-derived mouse macrophages (MΦ) were incubated with 25 μg/ml human myelin for 48 to 72 h. (A) CXCR3 and CCR7 mRNA expression as mean ± standard deviation of duplicates within one experiment. mRNA expression by myelin-laden macrophages was relative to control macrophages. The expression level of control macrophages was set as 100%. The data are representative for four independent experiments. (B) Myelin ingestion increased expression of CXCR3 and CCR7 surface protein by macrophages. Data represent four separate experiments. (C) Myelin-laden macrophages demonstrated enhanced chemotaxis towards CXCL10 and CCL21. The relative migration was calculated by dividing the migration towards chemokines by the basal migration towards medium. Data are presented as mean ± SEM of triplicates and are representative of three independent experiments. $^{*}p<0.05$ (two-tailed Mann–Whitney *U*-test).

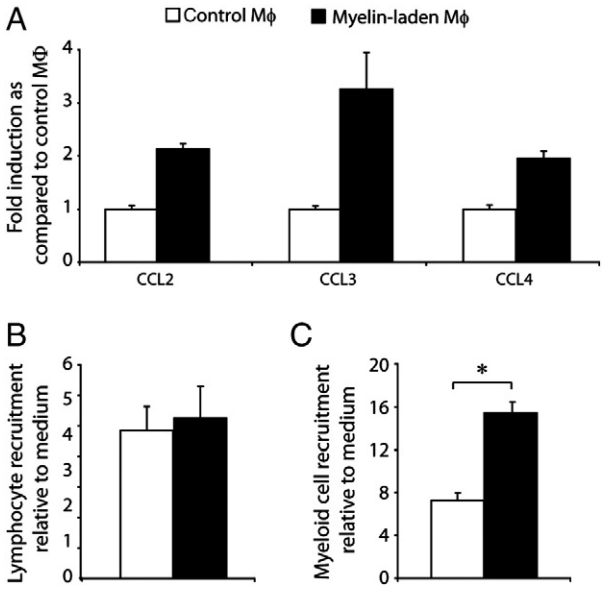

Fig. 2. Myelin-laden macrophages recruit more myeloid cells than control macrophages. Bone marrow-derived macrophages (MΦ) were incubated with 25 μg/ml human myelin for 48 to 72 h. (A) mRNA expression of CCL2, CCL3 and CCL4 by myelin-laden macrophages was slightly, but not significantly different, increased as compared to control macrophages. mRNA expression by myelin-laden macrophages was relative to control macrophages and presented as fold induction. Results are presented as mean ± standard deviation of duplicates within one experiment. Data are representative for four independent experiments. (B) Lymphocytes demonstrated similar migration towards myelin-laden macrophages as compared to control macrophages. The relative migration was determined by dividing the number of migrating cells to macrophages by the number of migrating cells to medium alone. Data are presented as mean ± standard deviation of duplicates within one experiment and represent three independent experiments. (C) Myelin-laden macrophages recruited more $CD11b^+$ myeloid cells than control macrophages. Data are presented as mean ± standard deviation of triplicates within one experiment and represent two independent experiments. $^*p<0.05$ (two-tailed Mann–Whitney *U*-test).

parallel, enhanced migration towards the corresponding chemokines CXCL10 and CCL21 after myelin ingestion *in vitro*. In addition, myelin-laden macrophages recruited significantly more $CD11b^+$ myeloid cells as compared to macrophages which did not ingest myelin.

Myelin consists for 70% of lipids (Morell and Quarles, 1999), suggesting that lipids are a key component in regulating the response seen after myelin ingestion. Nuclear lipid receptors, such as liver X receptors (LXR) and peroxisome proliferator-activated receptors (PPAR), have emerged as key regulators of inflammation and lipid homeostasis in macrophages (Valledor and Ricote, 2004). Since cholesterol metabolites are endogenous ligands for LXR and cholesterol is the main component of myelin (Morell and Quarles, 1999), it seems likely that myelin is, or contains, a ligand for LXR. As LXR agonists increased the expression of CCR7 by macrophages in a mouse model for atherosclerosis (Verschuren et al., 2009) we hypothesize that myelin may also induce CCR7 in a LXR-mediated manner.

The observation that myelin-laden macrophages show enhanced migration towards CXCL10 and CCL21 *in vitro* suggests that a similar process may take place *in vivo*. This may have several implications. These implications are visualized in a hypothetical model, shown in Fig. 3.

First, myelin-laden macrophages might emigrate from the CNS to the CNS-draining lymph nodes via a CCR7-dependent mechanism (Fig. 3A). The ligands for CCR7, CCL19 and CCL21, are constitutively produced by lymphatic endothelial cells and interdigitating dendritic cells within the lymph nodes, thereby facilitating migration of CCR7-expressing cells towards the lymph nodes (Dieu et al., 1998; Kobayashi et al., 2004; Muller and Lipp, 2003; Sallusto et al., 2000). The current study demonstrates that CCR7 surface protein expression was upregulated by myelin-laden macrophages and that these cells showed enhanced migration towards CCL21 *in vitro*. CCR7 is also expressed by myeloid cells within the CSF of MS patients and within the cervical lymph nodes of rhesus monkeys with EAE (de Vos et al., 2002; Kivisakk et al., 2004), and the CCR7 ligand CCL19 is elevated in CSF of MS patients (Krumbholz et al., 2007). Furthermore, myelin-laden macrophages are present in the CSF (Herndon and Kasckow, 1978; Zeman et al., 2001) and the CNS-draining lymph nodes (de Vos et al., 2002; Fabriek et al., 2005; van Zwam et al., 2009), supporting the hypothesis that myelin-laden macrophages emigrate the inflamed CNS to the CNS-draining lymph nodes. Nevertheless, using a CCR7 knock-out mouse model, we previously demonstrated that CCR7 is dispensable for the presence of myelin-laden cells in the CNS-draining cervical lymph nodes (van Zwam et al., 2009), implying that other or additional chemokine receptors may be involved or that myelin antigens are transferred as soluble antigens.

Second, elevated CXCR3 expression by myelin-laden macrophages may contribute to the presence of myelin-laden cells in the CSF of MS patients (Herndon and Kasckow, 1978; Zeman et al., 2001), since CXCL10 levels within the CSF are significantly elevated during active disease (Moreira et al., 2006). In addition, CXCL10 is produced by reactive astrocytes in MS lesions and in EAE (Ransohoff et al., 1993; Tanuma et al., 2006), suggesting interaction between astrocytes and myelin-laden macrophages (Fig. 3B). The function of CXCR3 is EAE is unclear, as different studies gave contradictory data (Liu et al., 2005; Liu et al., 2006a; Matsumo et al., 2005; Muller et al., 2007; Tsunoda et al., 2004). However, CXCR3 expression by macrophages was merely found in MS patients who suffered from secondary progressive MS (Tanuma et al., 2006), suggesting that expression of CXCR3 by macrophages might be involved in the worsening of disease.

Third, it has been shown that damaged neurons express and up-regulate CXCL10 and CCL21 *in vivo* after ischemia and brain injury (Biber et al., 2001; Rappert et al., 2004; Wang et al., 1998) and in cultured neurons after treatments known to induce neuronal death (Biber et al., 2001; de Jong et al., 2005; Klein et al., 2005; Sui et al., 2004). In addition, CXCR3 deficiency reduced microglial activation and neuronal loss in the entorhinal cortex lesion model (Rappert et al., 2004), strongly supporting the idea that microglia interact with neurons via CXCL10 and CCL21. Since myelin-laden macrophages reside adjacent to damaged neurons and have anti-inflammatory properties (Boven et al., 2006), it is tempting to speculate that myelin ingestion by macrophages might promote communication with damaged neurons in MS, resulting in neuroprotection (Benveniste, 1997) (Fig. 3C).

Additionally, myelin ingestion by macrophages promoted the *in vitro* recruitment of $CD11b^+$ myeloid cells, but not lymphocytes. These data indicate that myelin-laden macrophages release chemoattractants which selectively attract monocytes, macrophages and dendritic cells (Fig. 3D). As myelin-laden macrophages also reside in the perivascular spaces of inflamed CNS tissue, this might contribute to the recruitment of leukocytes into the CNS. Our results are in line with the observation that myelin-laden macrophages within the perivascular spaces express CCL2, CCL3, CCL4, and CCL5 (Boven et al., 2000; Simpson et al., 1998), suggesting that they attract leukocytes into the perivascular spaces (Balashov et al., 1999; Omari et al., 2004; Quandt and Dorovini-Zis, 2004; Ubogu et al., 2006). This concept has previously been suggested for astrocytes (Glabinski et al., 1995; Tani and Ransohoff, 1994), and is supported by the expression of the chemokine receptors CCR2 and CCR5 by myeloid cells within the perivascular cuffs of MS brain lesions (Rebenko-Moll et al., 2006; Sorensen et al., 1999).

In conclusion, the results in the present study demonstrate that *in vitro* myelin ingestion by macrophages induces CCR7 and CXCR3 expression, consistent with enhanced responsiveness to CCL21 and CXCL10. Furthermore, myelin ingestion resulted in enhanced recruitment of myeloid cells *in vitro*. These data suggest that myelin-laden macrophages migrate within and between different anatomical compartments during MS *in vivo*. Considering the anti-inflammatory

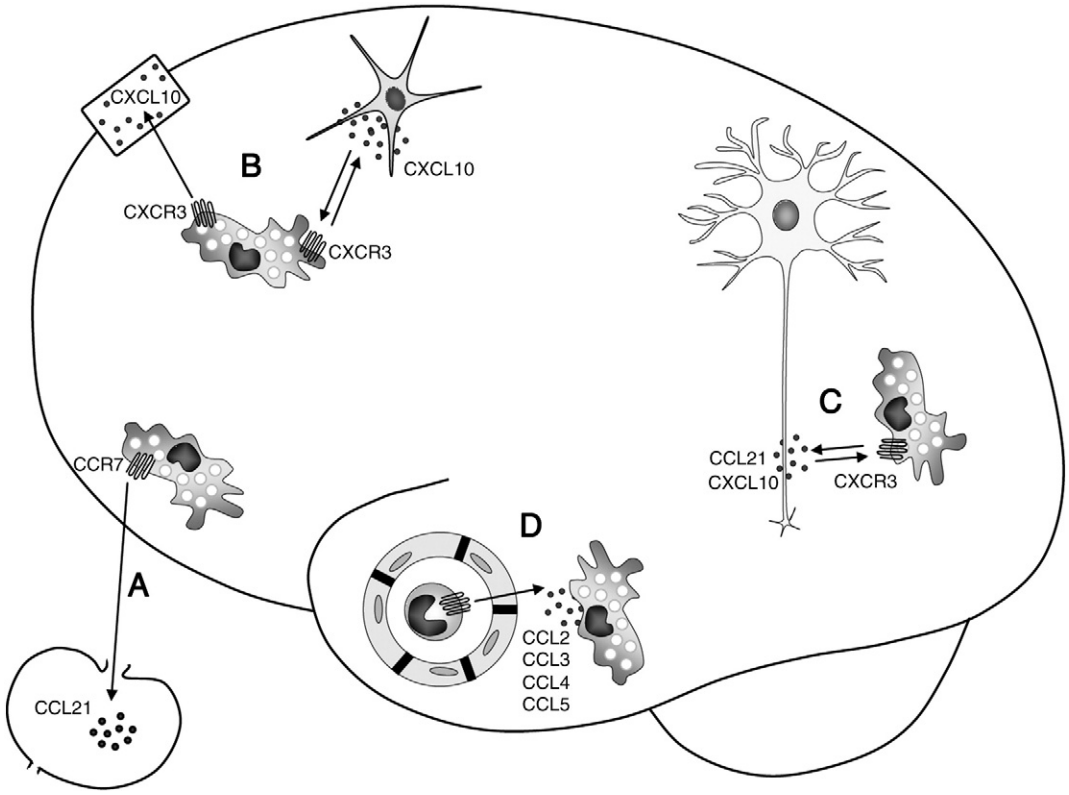

Fig. 3. Hypothetical model on the migration and interaction of myelin-laden macrophages with other cell types. (A) Myelin-laden macrophages in the CNS-draining lymph nodes and *in vitro* express CCR7, and myelin ingestion enhances migration of macrophages towards CCL21 *in vitro*. Myelin-laden macrophages *in vivo* are therefore likely to migrate towards the CCL21-expressing CNS-draining lymph nodes in a CCR7-dependent manner. (B) Myelin-laden macrophages demonstrate increased expression of CXCR3 and responsiveness to CXCL10 *in vitro*. It is therefore tempting to speculate that CXCL10, which is abundantly present in the CSF during EAE and MS, attracts CXCR3-expressing myelin-laden macrophages *in vivo*. Furthermore, reactive astrocytes secrete CXCL10, suggesting interaction between astrocytes and myelin-laden macrophages. (C) Damaged neurons produce CCL21 and CXCL10 and communicate with microglia in a CXCR3-dependent manner, implying that CXCR3-expressing myelin-laden macrophages also interact with neurons. Considering their anti-inflammatory phenotype, myelin-laden macrophages are likely to act neuroprotective. (D) Myelin-laden macrophages in perivascular spaces express the chemokines CCL2, CCL3, CCL4, and CCL5, and myelin-laden macrophages *in vitro* demonstrate enhanced expression of CCL2, CCL3, and CCL4 mRNA, which are key chemokines in the recruitment of monocytes. Moreover, myelin-laden macrophages are apt at attarcting myeloid cells *in vitro*. Myelin-laden macrophages are therefore expected to also attract infiltrating myeloid cells into the perivascular space *in vivo*.

phenotype of myelin-laden macrophages (Boven et al., 2006) and their ability to recruit myeloid cells, this suggests that myelin-laden macrophages might exert their anti-inflammatory action through interaction with other cells. The results in the present study provide further insight into the trafficking capacity of anti-inflammatory myelin-laden macrophages in MS.

Acknowledgements

We thank Dr. K. Biber for scientific input and critically reading the manuscript. This work was supported by grants of the Dutch MS Research Foundation (MS-00-417, MS-01-457, MS-01-471 and MS-02-490) and by the EC project FP6-037400 IMMUNATH. The authors further acknowledge discussions within the COST Action BM0603 Inflammation in Brain Disease Neurinfnet, and networking support from COST.

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