Immunological Function of Draining Lymph Nodes in
Multiple Sclerosis and Animal Models

Immunologische functie van drainerende lymfeklieren in
multipele sclerose en diermodellen

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"Nothing in life is to be feared, it is only to be understood.
Now is the time to understand more, so that we may fear less."

Marie Curie

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General introduction

Introduction

The current dogma in the initiation of immune responses is that pathogens as well as tissue antigens are transferred to the draining lymph nodes, where they are presented by antigen presenting cells (APC) to activate and skew naïve lymphocytes. These lymphocytes are subsequently reactivated by tissue-resident APC to exert their function. Paradoxically, tissue-draining lymph nodes are vital for generating immune responses during infections and autoimmune diseases, as well as in maintaining tolerance against tissue antigens. How drainage of central nervous system (CNS) antigens and the distinct CNS-draining secondary lymphoid organs are involved during CNS inflammation is not well understood, while these questions are crucial for understanding CNS inflammation, such as multiple sclerosis (MS). Hence, this thesis set out to address these questions by a systematic comparison of different animal models of brain inflammation and insult, MS brain tissue and by functional *in vitro* and *in vivo* assays.

The subsequent parts of this chapter provide a more extensive conceptual and theoretical background. Selected basics of MS are discussed, followed by the principles of CNS drainage and leukocyte entry into the CNS. In addition, the heterogeneous functions of macrophages in MS are introduced.

Multiple sclerosis

Clinical features

MS is one of the most common chronic and disabling disorders of the CNS. Approximately 0.1% of the Caucasian population is affected. Around 16.000 people in the Netherlands and 2.500.000 people worldwide have MS. The disease usually starts during young adulthood and affects women twice as frequently as men. MS is usually not life shortening, but disability through deficits of sensation, motor function and neurocognitive function leads to severe disease burden. This strongly affects the life quality of MS patients, and is of considerable socio-economic importance [1-4].

MS is a heterogeneous disease, with large interindividual variability in disease course and prognosis. In 80-90% of the patients, MS starts with a relapsing-remitting disease course, which is characterized by alternating periods of disease and recovery (Figure 1A). Over time, most patients develop progressive neurological deficits that occur independently of relapses, the so-called secondary progressive phase. In10-20% of the patients, MS begins with a primary progressive course, in which the symptoms gradually worsen without periods of remission [1-4]. In addition to these two major forms of MS, other subforms or variants of MS include acute disseminated encephalomyelitis, Marburg's acute MS, Balo's concentric sclerosis, Devic's neuromyelitis optica, and Schilder's myelinoclastic diffuse sclerosis [5,6].

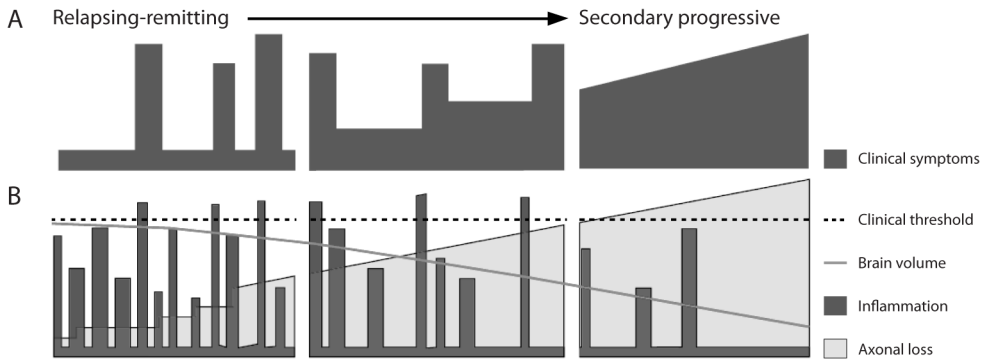

Figure 1. Association between clinical symptoms and pathogenesis in MS. (A) Relapsing-remitting patients show alternating periods of disease and recovery and can develop secondary progressive MS over time. (B) These clinical symptoms are accompanied by periods of CNS inflammation. It is thought that right from the onset of MS onwards, axonal loss gradually increases. In addition, atrophy reduces the brain volume over time. Modified after [2,4].

Pathology

Magnetic resonance imaging studies revealed that relapsing-remitting patients have acute CNS lesions with spontaneous resolution, even in the absence of clinical symptoms (Figure 1B). These lesions are often located in CNS white matter and are characterized by blood-brain barrier dysfunction, local edema and demyelination, indicative of ongoing inflammatory processes [7]. In addition, grey matter lesions have been characterized, of which the presence is associated with the progressive phases of disease. Gray matter lesions have therefore been suggested to correlate better with clinical symptoms. In contrast to white matter lesions, inflammation is not apparent in gray matter lesions [8,9].

During the relapsing-remitting phase, axonal loss gradually increases right from the onset of disease. Since the CNS has the capacity to compensate functional loss to a certain extent, axonal loss does not necessarily lead to clinical symptoms [2]. In contrast to relapsing-remitting patients, primary and secondary progressive MS patients do not often have inflammatory lesions. Instead, they show brain atrophy, which correlates with disease disability [10].

The basic histopathological feature unique to MS is the presence of multifocal demyelinated plaques scattered throughout the CNS, with a predilection for the perivascular white matter, optic nerves, brainstem, cerebellum and spinal cord [4,6]. Processes of proliferating astrocytes fill up the demyelinated areas and form multiple scars, which has led to the name multiple sclerosis [3]. In addition to demyelination, substantial axonal injury demonstrated by axonal transection, formation of axonal spheroids and axonal sprouting is abundant even in early stages of MS [11].

White matter lesions demonstrate distinct morphology and infiltrates. Based on demyelinating and inflammatory criteria [12-14], different lesion types can be distinguished (Box 1).

Box 1. Lesion types in white matter of MS patients (based on [12-14,123])

- Pre-active lesions: Abnormalities in the white matter with clusters of activated MHC class II-expressing microglia or perivascular inflammatory cells. Demyelinated areas are absent.
- Active demyelinating lesions: Inflammatory perivascular infiltrates consisting of T cells, monocytes, and B cells or plasma cells. MHC class II-expressing macrophages are most prominent in the centre of the lesion and contain myelin debris, indicating active demyelination. The composition of inflammatory cells within active demyelinating lesions varies greatly among MS patients, suggesting distinct pathogenic mechanisms.
- Chronic active lesions: Hypercellular rim with inflammatory cell infiltrates, suggesting ongoing inflammatory activity along the lesion edge. Hypocellular lesion centre containing few macrophages with residual myelin debris, and few perivascular lymphocytes.
- Chronic inactive lesions: Sharply circumscribed hypocellular lesions without active myelin breakdown. Characterized by widespread astrogliosis and widened extracellular spaces.

Immunopathogenesis

Although the pathogenesis of MS remains unclear, it is well established that CNS inflammation takes place, which is supported by the emergence of new and efficacious immunotherapies for MS (Table 1). CNS inflammation during MS involves a complex interplay between various components of the immune system (Figure 2). First, CNS antigens, pathogens or cross-reactive foreign antigens are presented by APC, such as dendritic cells or macrophages, on MHC class I or II molecules in the perivascular spaces or the CNS-draining lymphoid organs. This leads to activation of $CD8^+$ cytotoxic T cells and $CD4^+$ T helper (Th) cells, including priming, clonal expansion and acquisition of effector functions of these lymphocytes. The T cells will accumulate at sites in the CNS where they re-encounter their target antigens [1,2,15-19].

CNS antigens within the CNS are displayed on MHC class I molecules of neurons and glial cells, and are recognized by cytotoxic T cells. This will stimulate the cytotoxic T cells to cause direct damage to the expressing cells through the secretion of cytotoxic molecules such as perforin and granzymes or through direct cell contact via for instance Fas ligand [1,2,15,17-19].

In addition, target antigens are presented by CNS-resident activated microglia, macrophages, and dendritic cells on MHC class II. These APC produce cytokines such as interleukin (IL)-1, IL-6, IL-12p35, IL-23p19, and tumor growth factor (TGF)-β, giving rise to Th1 and Th17 cells, which in turn show increased production of the inflammatory cytokines IL-2, interferon (IFN)-γ, IL-17A, osteopontin and tumor necrosis factor (TNF)-α. It is thought that these pro-inflammatory cytokines direct microglia to become macrophage- and dendritic cell-like. In addition, these cytokines attract more APC and are directly toxic to oligodendrocytes and neurons [1,2,4,15-19].

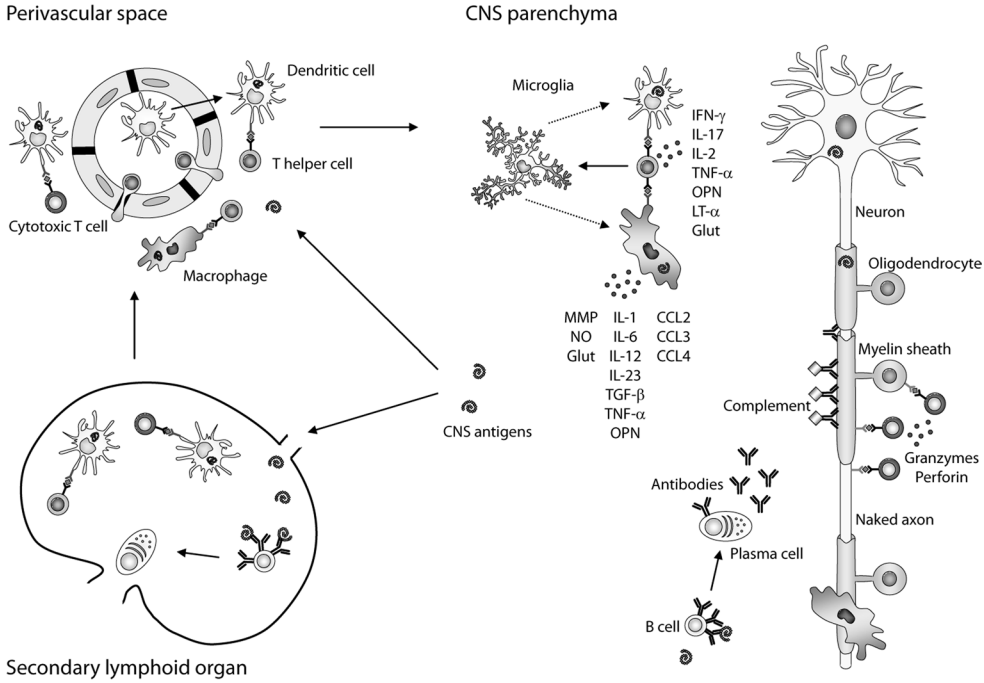

Figure 2. Hypothetical immunopathology in active demyelinating MS lesions. Neuroinflammation requires lymphocyte activation in distinct CNS compartments. It is thought that APC present CNS antigens in the secondary lymphoid organs to activate naïve lymphocytes. These lymphocytes subsequently migrate to the perivascular spaces in the CNS and the CNS parenchyma, where they recognize their cognate antigen presented by dendritic cells or macrophages. Cytotoxic T cells produce granzymes and perforin to directly damage oligodendrocytes and neurons. T helper cells produce pro-inflammatory cytokines, which activate microglia to become macrophage or dendritic cell-like. These myeloid cells in turn prod-uce cytokines involved in T helper differentiation. In addition, activated macrophages release toxic mediators which damage the blood-brain barrier, oligodendrocytes and neurons, and chemokines to recruit more leukocytes. Furthermore, antigens are recognized by B cells, which become plasma cells secreting autoreactive antibodies. These antibodies form complexes with complement leading to complement-mediated cytotoxicity. Based on [1,2,15-19,25].
Glut, glutamate; LT-α, lymphotoxin-α; MMP, matrix metalloproteinases; NO, nitric oxide; OPN, osteopontin.

APC, such as microglia, macrophages and dendritic cells, are activated by T helper cells or by endogenous ligands for innate antigen receptors. Endogenous ligands are derived from tissue debris released in lesion pathology, such as fibrin or oxidized lipids derived from myelin. APC contribute to inflammation through the release of toxic mediators, such as nitric oxide, cytotoxic cytokines, such as TNF-α and osteopontin, and matrix metalloproteinases, which facilitate breakdown of the blood-brain barrier and lead to direct injury of axons and glia. In addition, macrophages produce inflammatory chemokines to recruit more leukocytes. Furthermore, macrophages directly damage oligodendrocytes through a phagocytic attack on the myelin sheath [1,2,15,17-19].

Moreover, lymphocytes and macrophages release excessive amounts of glutamate during inflammation, which activate receptors within the glutamate receptor family, such as those for N-methyl-D-aspartate (NMDA), α-amino-3-hydroxy-5-isoxazolepropionic acid (AMPA) and kainate [20]. Activation of these receptors, which normally facilitate fast synaptic transmission, may cause necrotic damage to oligodendrocytes and neurons [21].

Recent clinical trials using B cell-depleting antibodies indicate that also B cells are important in the pathophysiology of MS [22-24]. B cells are activated through capture of soluble antigens in the draining lymph nodes and the CNS and re-encounter their specific antigen in the CNS. They mature into plasma cells which secrete vast amounts of oligoclonal immunoglobulins. These autoreactive antibodies bind soluble or membrane bound antigen to facilitate direct demyelination, possibly through the activation of complement, leading to complement-mediated cytolysis [25]. In addition, B cells may contribute to MS pathogenesis through their antigen-presenting and cytokine-secreting role. This can lead to abnormal T cell and macrophage activation, thereby perpetuating CNS inflammation and damage [24].

Once this attack has cleared the antigen from the site of the lesion, the leukocytes will undergo apoptosis or redistribute to other tissues. In parallel, repair mechanisms are initiated. Cytokines and growth promoting factors released by reactive astrocytes, microglia and macrophages as part of the inflammatory process promote endogenous remyelination. Over time, astrocytes seal the lesion and gliosis causes a physical barrier to further remyelination [1-4].

Animal models of MS

The study of MS pathogenesis is difficult, as there is limited access to brain lesion tissue during active disease. Experimental animal models are therefore indispensable for understanding the molecular mechanisms underlying tissue injury and inflammation during MS. Experimental autoimmune encephalomyelitis (EAE) is used as an animal model for MS. EAE is an inflammatory disease of the CNS with variable degrees of inflammation and demyelination, resulting in paralysis which is often transient. EAE can be induced actively or passively in susceptible rodents and monkeys. Active immunization involves the injection of CNS homogenate, myelin-derived proteins or peptides, or neuronal proteins or peptides. Frequently used antigens are derived from the myelin proteins proteolipid protein (PLP), myelin oligodendrocyte glycoprotein (MOG) and myelin basic protein (MBP). These antigens are to be emulsified in complete Freund's adjuvant (CFA). Furthermore, injections with *Bordetella pertussis* bacteria or toxin (PTX) are required to disrupt the blood-brain barrier and promote CNS inflammation by effects on autoreactive T cells [26]. Clinical symptoms typically start one to two weeks after immunization [27].

Passive induction uses adoptive transfer, in which immune cells from immunized animals are transferred to naïve animals, thereby transmitting the disease. Encephalitogenic T cells isolated from immunized animals need to be restimulated *in vitro* with the immunizing antigen, often in the presence of cytokines. Clinical symptoms usually start within one week after adoptive transfer [27].

Table 1. Examples of current and promising immunotherapies in MS.

Treatment	Target	Assumed mechanism of action	Comments
β interferons		• Promotes Th2 skewing • Reduces monocytes migration	Currently used as treatment for MS
Glatiramer acetate (Copaxone)		• Promotes Treg proliferation • Promotes Th2 skewing • Induces $CD8^+$ suppressor cells	Currently used as treatment for MS
Nataluzimab	Blocking mAb against α4 subunit of VLA-4	• Inhibits leukocyte migration into the CNS • Inhibits T cell activation	Only monotherapy due to development of progressive multifocal leukoencephalopathy
Rituximab	mAb against CD20	• Depletes circulating B cells, but not plasma cells	Phase III clinical trials ongoing
Alemtuzumab	mAb against CD52	• Induces cytolysis of T cells, B cells, and monocytes	Phase III clinical trials planned
Fingolimod (FTY720)	S1P receptor agonist	• Induces lymphocyte homing and sequestration into lymph nodes • Enhances Treg function • Downregulates pro-inflammatory signals	Phase III clinical trials planned
Daclizumab	Blocking mAb against IL-2R	• Inhibits activated T cell proliferation	Currently in phase II clinical trials

mAb, monoclonal antibody; Treg, regulatory T cell; VLA-4, very late antigen-4. Based on [124].

Demyelinating encephalomyelitis can also be induced by Theiler's murine encephalomyelitis virus [28]. Another option is to use transgenic mice, which spontaneously develop EAE and CNS inflammation. Examples are mice transgenic for the T cell receptor (TCR) against PLP [29] or MBP [30], or MOG-TCR-transgenic mice, which are crossed with MOG-specific immunoglobulin heavy chain knock-in mice, which develop disease at the age of six to seven weeks [31,32].

Many different EAE models are available, depending on species, strains and induction protocols. Each model covers specific aspects of the disease and EAE models can therefore be used to explore mechanisms of autoimmunity and neuroinflammation. However, there is no single EAE model which mimics MS as a whole. For these reasons, proper selection of the best suited EAE model to study specific questions of MS pathology and pathogenesis is essential [27,33,34].

In this thesis, we have used tissues derived from two distinct monkey EAE models. Rhesus monkeys (*Macaca Mulatta*) develop hyperacute neurological symptoms and progress rapidly to death after EAE induction. Brain lesions are necrotic and characterized by hemorrhage, and dramatic destruction of myelin and axons [35]. EAE in common marmoset monkeys (*Callithrix jacchus*) is chronic progressive and the CNS contains sharply-edged lesions characterized by primary demyelination and axonal damage [35].

In addition, we used three distinct EAE models in different mouse strains (Figure 3). EAE in these models was induced by active immunization with myelin peptides. EAE in Biozzi ABH mice (H-2^{dq1}) was induced by MOG_{8-21} in CFA. The animals show chronic-relapsing EAE, which is characterized by an acute phase of disease, after which the mice go into remission. Subsequently, the mice suffer from one to two relapses. Demyelination in the CNS is little in the acute phase of the disease, but apparent during relapses. Furthermore, axonal damage is present in every phase of the disease [36]. SJL/J mice show acute EAE after immunization with $PLP_{139-151}$ in CFA. The clinical symptoms start approximately ten days after immunization and last for around one week. Chronic EAE in C57BL/6 mice was induced by MOG_{35-55} in CFA. These mice suffer from clinical EAE symptoms for approximately four weeks. Both SJL/J mice and C57BL/6 mice demonstrate demyelination and axonal damage in the CNS [37,38].

Immunogenicity of antigens in the CNS

In the healthy CNS, inflammation is under strict control of multiple anatomical and immunoregulatory processes to minimize tissue damage and loss of function in such a vital organ [39,40]. Despite the immune privileged status of the CNS, destructive inflammation in the CNS occurs, and contributes to diseases such as MS [3]. Immunogenicity of CNS antigens seems to depend on the location of origin within the CNS. The CNS is organized into different compartments:

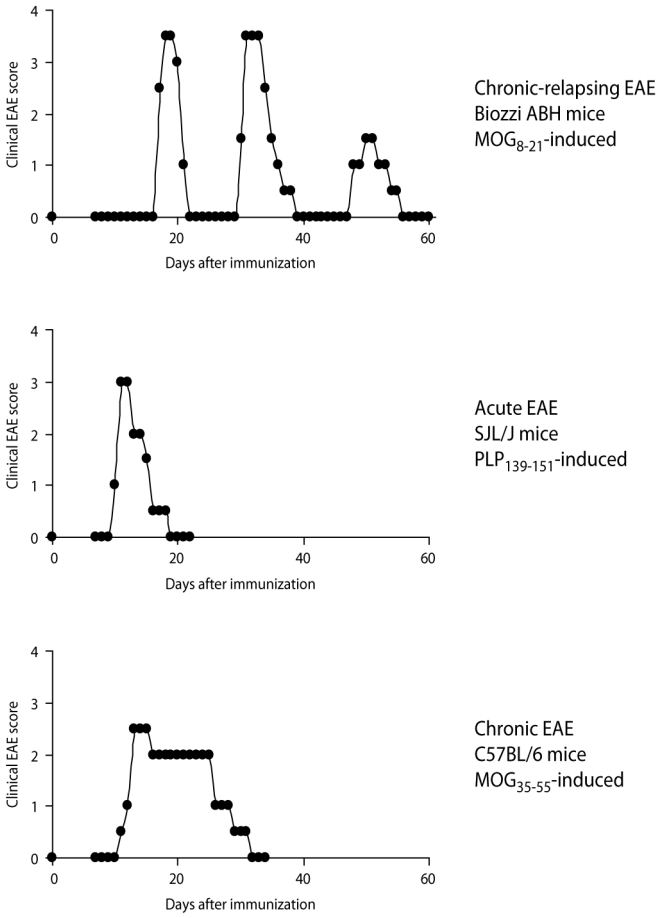

Figure 3. EAE course in the three mouse models used in this thesis. Three distinct EAE mouse models are used in this thesis to explore drainage of CNS antigens to the CNS-draining lymph nodes, T cell proliferation within the CNS-draining lymph nodes and the effect of lymphadenectomy on clinical EAE in different phases of the disease.

the CNS parenchyma, the ventricles and the CSF, and the meninges [39]. Intracerebral injection of the pathogens *Bacille Calmette-Guerin* or influenza virus is ignored by the immune system [41,42], whereas intraventricular injection of the same pathogens evokes delayed type hypersensitivity lesions against *Bacille Calmette-Guerin* [43], and B and T cell responses against influenza [41]. Similarly, intracerebral injection of non-pathogenic ovalbumin results in low serum antibody titers, whereas intraventricular injection gives a strong antibody response [44]. It therefore seems that CNS antigens are more immunogenic when they are present in the ventricles and the CSF than when they are present in the parenchyma.

Immune responses within the CNS require presentation of CNS antigens by APC to naïve T cells, resulting in priming and activation. In most tissues, antigens are transported by APC or as soluble proteins to the draining lymph nodes or the spleen to evoke immune reactions against the antigen. A similar mechanism may be operational in MS and EAE. This is supported by the observation that myelin-containing cells are present in the cervical lymph nodes of deceased MS patients and EAE-affected animals [45]. Using ultrasound-guided fine needle aspiration cytology, myelin antigens were also observed in living MS patients [46]. Importantly, myelin antigens are present in APC with macrophage and dendritic cell markers [45,46], implying that myelin antigens are presented to lymphocytes. Indeed, myelin antigen-specific proliferation was found in the cervical lymph nodes of EAE-affected animals [45,47,48]. Furthermore, lymphadenectomy reduced brain lesion expansion during EAE in rats [49]. In addition to the draining lymph nodes, lymphocytes may also be activated in the CNS itself, e.g. in the perivascular spaces, the parenchyma and the meninges [50-52]. There is an ongoing debate on how, and to what extent the different anatomical compartments contribute to disease by initiating CNS antigen-specific immune responses.

CNS drainage

For most tissues, antigen transport to the draining secondary lymphoid organs is critical in generating primary immune responses. APC in the periphery are positioned as sentinels, where they frequently encounter foreign antigens. After such an encounter, they readily relocate to secondary lymphoid organs where they take optimal position to interact with naïve and memory T cells. The mobilization of APC from the inflamed tissue to the lymph nodes is regulated by the lymph node homing molecule CCR7, which is upregulated by APC upon maturation. The CCR7 ligands CCL19 and CCL21 are produced by the endothelial cells of the lymphatic vessels and by interdigitating dendritic cells within the lymph nodes to create a chemotactic gradient along which the APC migrate [53-57]. In addition to this cellular route, antigens are transferred as soluble proteins through the lymphatic vessels and are subsequently ingested by local APC in the lymphoid organs [57].

Although the CNS parenchyma lacks lymphatic vessels as seen in the rest of the body, CNS antigens from the parenchyma and the CSF do reach the periphery. CNS drainage pathways have been studied in various animals, including rodents, sheep, and primates, using various tracers that are injected both intracerebrally as well as intraventricularly [58]. These studies revealed that tracers are transported along the two extracellular fluids of the CNS: interstitial fluid and CSF. These drainage pathways are discussed in detail in the sections below (Figure 4).

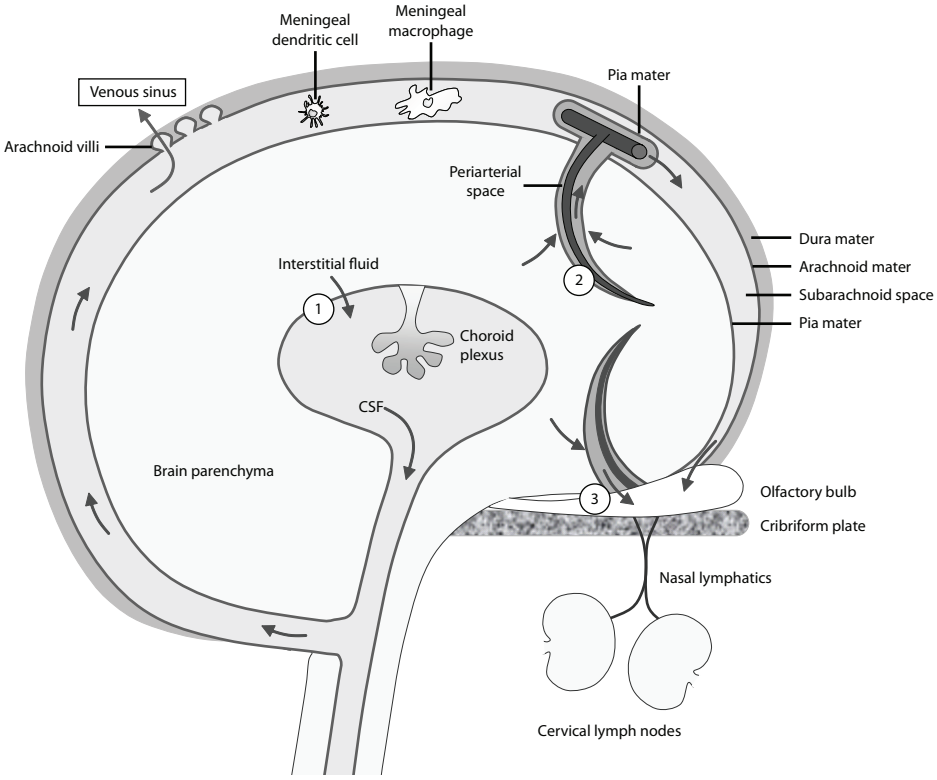

Figure 4. Schematic depiction of CNS drainage pathways. Antigens in brain parenchyma flow along the interstitial fluid. Interstitial fluid enters the CSF through exchange across the ependyma lining the cerebral ventricles (1). Antigens within the CSF drain along the ventricular system and subarachnoid space. From this compartment, antigens can either enter the venous sinuses through arachnoid villi and reach the blood, or drain to cervical lymph nodes through the olfactory bulb and cribriform plate. Antigens present in the interstitial fluid also drain into periarterial pathways (2). Since the arteries within the CNS are lined by the pia mater, they are separated from the CSF. However, some interstitial fluid may reach the CSF through the pia mater. The subarachnoid space and the periarterial space of the nasal-olfactory artery connect directly with nasal lymphatics and cervical lymph nodes (3). Dendritic cells and macrophages reside in the meninges, stroma of the choroid plexuses, and in the perivascular spaces, where they can take up antigens. This figure combines elements from [125-127].

Drainage along the flow of interstitial fluid

Thin-walled perivascular channels, resembling lymphatic capillaries, are observed in CNS lesions of MS patients. These defined channels contained macrophages, suggesting uptake of myelin debris which has drained through the interstitial fluid [59].

Interstitial fluid is partly derived from plasma at the capillary wall and partly from recycled CSF, and flows through the extracellular spaces in CNS parenchyma with an estimated drainage rate of 0.1-0.3 μl/min/gram of brain [60,61]. Intracerebrally injected tracers are diffusely distributed through the narrow extracellular spaces of the white matter, and then spread more selectively along periarterial spaces and white matter fiber bundles through the bulk flow of interstitial fluid [62,63]. Soluble tracers, such as dextran (3 kDa) and ovalbumin (40 kDa), drain along the basement membrane of the capillary walls and along basement membranes between the smooth muscle cells in the tunica media of the arteries. In contrast, particulate tracers seem to split the capillary basement membranes and create a perivascular space between the wall of the artery and the surrounding glia limitans. Indian ink-derived carbon particles, and particulate fluospheres (0.02 μm and 1.0 μm in diameter) are ingested by perivascular macrophages, or by meningeal macrophages and dendritic cells surrounding the larger vessels [63,64]. The motive force for perivascular drainage of interstitial fluid may be pulsations in the artery walls and not passive diffusion through the tissue [65], which is supported by the fact that perivascular drainage is absent following cardiac arrest in mice [63].

Intracerebrally injected tracers are present in the cervical lymph nodes, indicating that they drain along the interstitial fluid to the periphery. It has been hypothesized that these tracers drain along the walls of the intracranial arteries and the internal carotid artery to the cervical lymph nodes. However, intracerebrally injected tracers were absent in the wall of the internal carotid artery in the neck [60], suggesting that they reached the cervical lymph nodes at the base of the skull. In addition, approximately 15% of interstitial fluid enters the CSF in rats [60], providing another route for interstitial fluid to leave the CNS parenchyma.

Drainage of cerebrospinal fluid

The adult human brain contains 150 ml CSF, which is produced by the choroid plexus in the ventricles with a production rate of 600 ml/day [66]. CSF flows into the subarachnoid space and drains into the blood via the arachnoid granulations and villi within the walls of venous sinuses [61,67,68]. Qualitative CSF tracer studies, using tracers and artificial pressure, revealed that CSF also drains along the olfactory nerves through the olfactory bulb and the cribriform plate of the nasal bone into the nasal lymphatics in the nasal mucosa and the cervical lymph nodes of rodents, sheep, monkeys and humans [69-71].

The olfactory route was confirmed in more detail in rats (Figure 5) [72]. The subarachnoid space surrounding the olfactory nerves decreases in size while the dura mater encloses the olfactory nerves tightly to the cribriform plate. This suggests that the subarachnoid space is not directly open to the

space surrounding the olfactory nerves. The olfactory nerves in the cribriform plate are surrounded by dense connective tissue, forming a network of channels around the nerves through which CSF passes. At the nasal mucosa, CSF drains along the olfactory nerves through a space limited by the perineural and epineural layers around the olfactory nerves. The nasal lymphatics are close to the olfactory nerves, but they are not directly connected to each other. This results in diffusion of CSF into the mucosal connective tissue after which it is rapidly absorbed by the nasal lymphatics to reach the cervical lymph nodes [72]. In contrast, the nasal lymphatics seem to encircle the olfactory nerves in sheep and may therefore provide a barrier that separates the perineural space from the surrounding mucosal tissue [73].

In addition to the CNS antigens derived from the brain, spinal cord-derived antigens drain via the spinal CSF to the lumbar or para-aortical lymph nodes and to the cervical lymph nodes [74-77].

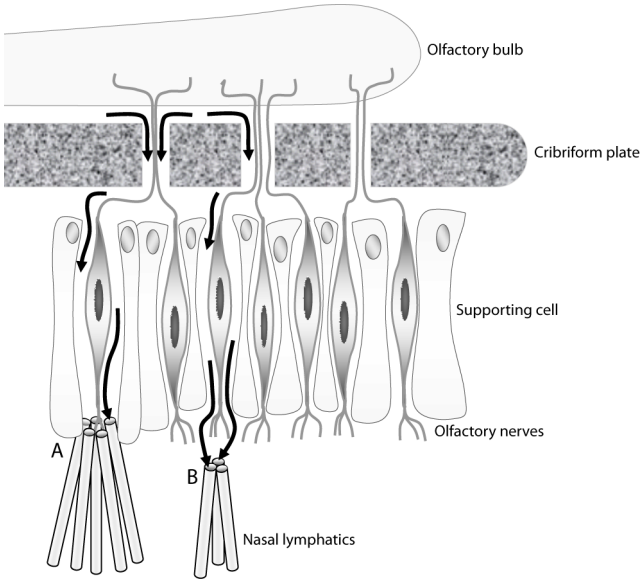

Figure 5. CSF drainage to the cervical lymph nodes. CSF flows through the foramina in the cribriform plate along the olfactory nerves through a network of channels formed by dense connective tissue. In the nasal mucosa, the olfactory nerves are surrounded by a layer of perineural and epineural supporting cells. CSF drains in between these cells to the nasal connective tissue, after which it is rapidly absorbed by the nasal lymphatics to reach the cervical lymph nodes. The cervical lymphatics may encircle the olfactory nerves to directly absorb CSF (A), or CSF may first enter the nasal mucusa and then enter the nasal lymphatics (B). The flow of CSF is depicted by the black arrows. Based on [72,87].

A tolerogenic microenvironment in the cervical lymph nodes

Mucosal tolerance is of major importance to prevent allergy and to maintain tolerance against harmless foreign dietary and inhaled antigens. The deep and superficial cervical lymph nodes are the principal draining sites for the nasal mucosa. As such, they are critically involved in maintaining mucosal tolerance. This is demonstrated by the fact that surgical excision of the cervical lymph nodes abrogated tolerance against inhaled antigens [78]. Interestingly, tolerance induction could be restored by the transplantation of cervical lymph nodes, but not by the transplantation of peripheral lymph nodes [78]. This indicates that the cervical lymph nodes constitute a unique microenvironment that favors the induction of immunological tolerance. Further analysis of this microenvironment revealed that the cervical lymph nodes preferentially express IgG2b, which suppresses IL-12p40 production by dendritic cells through the Fc gamma receptor IIB [79,80]. In addition, dendritic cells within the cervical lymph nodes showed enhanced expression of indoleamine 2,3-dioxygenase (IDO) [81] and secretory leukoprotease inhibitor [82], which are involved in regulatory T cell activation and suppression of inflammatory responses.

The specific microenvironment of the cervical lymph nodes may also be involved in immune responses against CNS antigens. Intracerebral injection of T cell-dependent antigens ovalbumin and human serum albumin without adjuvant in mice elicited production of IgG1 in the cervical lymph nodes, but failed to induce a peripheral delayed type hypersensitivity response [83]. Furthermore, injection of tumor cells in the CNS parenchyma of mice resulted in pronounced drainage of tumor cells to the deep cervical lymph nodes. There was a substantial number of myeloid suppressor cells, but only few cytotoxic cells to destroy the tumor [84]. Together, these findings support the notion that immune response in the cervical lymph nodes against brain antigens are tolerogenic or Th2-biased.

Leukocyte entry into the CNS

Naïve and activated lymphocytes enter the CNS through distinct routes, resulting in the presence of leukocytes in the CSF, perivascular spaces and the CNS parenchyma [85,86]. First, blood-derived leukocytes enter the CSF by crossing the endothelium of the choroid plexus stroma, the stromal core, and the epithelial cells of the villi in the choroid plexus. This pathway seems to be involved in surveillance of the healthy brain by memory T cells, which patrol the subarachnoid space in search of antigens and then return to the blood or lymph nodes [85,87].

Second, leukocytes within arteries reach the capillaries and the postcapillary venules where they extravasate into the perivascular space through the pial layer. Here, they encounter APC and the perivascular spaces are therefore considered as sites where CNS antigens are presented to infiltrating lymphocytes during CNS inflammation [85]. Leukocytes enter the CNS parenchyma

through the basement membrane of the glia limitans and the glia limitans itself [40].

Alternatively, leukocytes may enter the CNS parenchyma directly through the capillaries [85]. However, this route does not seem to be the main route through which leukocytes enter the CNS parenchyma, since leukocytes preferentially enter the CNS through the postcapillary venules [88].

The traffic of leukocytes into the CNS is a highly regulated process in which a complex orchestration of adhesion molecules and chemokines is involved [86,89]. Under inflammatory conditions of the CNS, the endothelium of the blood vessels in the CNS secretes the inflammatory chemokines CCL2, CCL3, CCL4, and CCL5 [90-93]. Infiltrating monocytes express CCR1 and CCR5 and also phagocytic macrophages express CCR5, suggesting that chemokines regulate the migration of monocytes and macrophages into the CNS [87,94]. Furthermore, the expression of the adhesion molecules vascular adhesion molecule-1 (VCAM-1) and intercellular adhesion molecule-1 (ICAM-1) is induced on the endothelium of the blood-brain barrier and the choroid plexus. This provides signals for the circulating leukocytes, which express α4-integrins and lymphocyte function-associated antigen-1 (LFA-1) as ligands for the adhesion molecules, to penetrate the vessel wall [86,87]. The importance of interactions between the vessel wall and penetrating leukocytes is underscored by the clinical benefit that blocking antibodies directed against α4-integrins provide for MS patients and EAE animals [95,96].

Most lymphocytes in the CNS express CCR7 and CXCR3. CXCR3-expressing T cells are often observed in CNS lesions near parenchymal vessels and in the CSF, and the CXCR3 ligand CXCL10 is widely expressed in the CNS of MS patients and EAE-affected animals [97-100]. The CCR7 ligand CCL19 is elevated in CSF of MS patients [101] and CCR7 is important for the guidance of T cell to the lymphoid organs. These chemokine receptors therefore seem imperative for CNS infiltration and the return to the secondary lymphoid organs and the blood [87].

In addition to chemokines and adhesion molecules, antigen presentation by CNS resident cells seems to be important for the entry of $CD8^+$ cells [102]. By contrast, $CD4^+$ T cells do not seem to need antigen-specificity to enter the CNS [103].

Macrophages

Macrophage heterogeneity

Macrophages exhibit remarkable plasticity, and may be able to change from one phenotype to another, depending on the microenvironment [104]. *In vitro* studies, using diverse stimuli, have revealed distinct macrophage populations. These seem to be part of a continuum of macrophage phenotypes with classically activated macrophages, alternatively activated macrophages, and regulatory macrophages as hallmarks of macrophage activation (Figure 6) [105].

Classically activated, or M1 macrophages, are induced by pro-inflammatory stimuli, such as IFN-γ, TNF-α and lipopolysaccharide, leading to the production of TNF-α, IL-1, IL-6, IL-12, IL-23, IL-27 and NO, and to the upregulation of MHC class II and costimulatory molecules. They are typically involved in Th1 and Th17 responses, the killing of micro-organisms and tumor cells, thereby often causing tissue damage [104-109].

Development of alternatively activated, or M2a macrophages, is driven *in vitro* by the cytokines IL-4 and IL-13. These cytokines are produced by cells from the innate and adaptive immune system, such as basophils, mast cells, and Th2 cells. Alternatively activated macrophages express anti-inflammatory molecules, such as IL-1 receptor antagonist, IL-10, TGF-β, and CCL18, and are associated with scavenging of debris, promotion of tissue repair and angiogenesis. They are therefore also called wound-healing macrophages [104,105,107,109].

Regulatory macrophages, or M2b/c are activated by a number of stimuli, including immune complexes, prostaglandins, glucocorticoids, apoptotic cells or IL-10, and in addition a second stimulus, such as ligands for toll-like receptors. Regulatory macrophages are involved in the suppression of immune responses through the production of IL-10, promoting development of regulatory T cells, and they bias T cell skewing towards a Th2 phenotype [105,109,110].

Hence, functional differentiation of macrophages is driven by the presence of microbial and endogenous ligands, and the cytokine environment, resulting in a heterogenic population with distinct functional properties The *in vitro* studies indicate that macrophages are directed to kill microbes, promote wound healing, or secrete anti-inflammatory cytokines to terminate inflammation as regulatory macrophages. These functions are likely hallmarks within a continuum of phenotypes, since macrophages may acquire intermediate or hybrid phenotypes upon different combinations of stimuli [105].

Macrophages in MS

In MS lesions, macrophages and activated microglia constitute the majority of the immune cells [111]. They can contribute to inflammation by a direct attack on the myelin sheath and by the release of toxic mediators. Specifically, they secrete pro-inflammatory cytokines to direct Th1 and Th17 differentiation. Furthermore, macrophages release other pro-inflammatory cytokines, such as TNF-α and osteopontin, free radicals, matrix metalloproteinases, and glutamate, which directly damage oligodendrocytes and neurons and which facilitate breakdown of the blood-brain barrier. In addition, macrophages produce inflammatory chemokines to recruit more myeloid cells [1,2,15-19].

The importance of macrophages in the pathology of EAE is emphasized by the fact that blockade of macrophage activation or inhibition of their migration into the CNS ameliorates clinical EAE and tissue injury [18], and depletion of macrophages blocks T cell infiltration into the CNS and suppresses EAE [112-114].

Besides being involved in inflammation-mediated toxicity, macrophages are also crucial for remyelination through the production of trophic factors and the clearance of myelin debris [115-119]. Recently it became clear that macrophages and microglia acquire an anti-inflammatory phenotype after myelin ingestion *in vitro* [120-122]. Importantly, human myelin-laden macrophages express various anti-inflammatory molecules also *in situ*, including TGF-β, IL-1 receptor antagonist, IL-10, and CCL18, which strongly suggests a local immunosuppressive mode of action. Furthermore, myelin-laden macrophages upregulate the anti-inflammatory molecules CCL18 and prostaglandin E_2 synthase *in vitro* and do not respond to pro-inflammatory stimuli such as LPS [120]. *In vitro*, IFN-γ and LPS-stimulated mouse microglia demonstrated increased production of TNF-α, IL-1β, IL-10 and CXCL10 after 6 h of myelin ingestion. This was, however, followed by a rapid and marked decrease after 24 h of myelin ingestion, indicating a biphasic response to myelin ingestion shifting from a pro-inflammatory to an anti-inflammatory response [121]. Furthermore, myelin phagocytosis has been described in a co-culture model of macrophages with mouse central (optic) or peripheral (sciatic) nerve preparations. This coculture system produced a cytokine and chemokine environment that promotes Th2 skewing, i.e. increased production of IL-6, IL-10, TGF-β, CCL2, CCL11, CCL22, and CXCL1 [122].

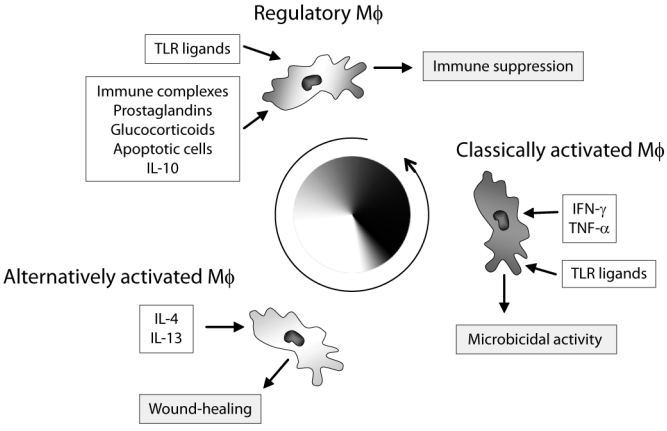

Figure 6. Diverse stimuli give rise to a spectrum of macrophages with distinct physiologies. Macrophages show remarkable phenotypic and functional plasticity, allowing them to efficiently respond to various stimuli. Three hallmark phenotypes are distinguished. Classically activated macrophages arise in response to bacterial products, such as LPS, and the pro-inflammatory cytokines IFN-γ and TNF-α, and are involved in microbicidal activity. Alternatively activated macrophages are driven by the Th2 cytokines IL-4 and IL-13, and promote tissue repair by secreting components of the extracellular matrix, scavenging debris and supporting angiogenesis. Regulatory macrophages are generated in response to various stimuli, including immune complexes, prostaglandins, glucocorticoids, apoptotic cells, or IL-10. They suppress immune responses through the production of IL-10. Depending on the stimuli present in the local microenvironment, macrophages switch between the phenotypes, or acquire intermediate or hybrid phenotypes as depicted by the circular arrow and the gradient wheel. Based on [105,109].

In conclusion, there is ample evidence that macrophages can act as aggressors in EAE and likely also in MS pathogenesis. In contrast, macrophages are also required for remyelination and the anti-inflammatory phenotype of myelin-laden macrophages implies that they can contribute to the resolution of inflammation. It seems that myelin ingestion is a trigger to induce this anti-inflammatory phenotype. However, whether and how myelin-laden macrophages exert anti-inflammatory action during MS and EAE remains unexplored.

Outline of this thesis

Autoreactive lymphocytes directed against myelin and likely also neuronal antigens are recruited into the CNS [3]. However, the exact locations of lymphocyte activation and expansion for both encephalitogenic and regulatory responses are still unclear. This is a critical issue, in view of understanding disease mechanisms and possible therapeutic interventions aimed at limiting activation of autoreactive lymphocytes. The main question in the studies described in this thesis is whether transfer of CNS antigens to the CNS-draining lymph nodes during MS and EAE results in the initiation of CNS antigen-specific lymphocyte activation and whether this interferes with the EAE disease course.

Since tracers injected into the brain readily drain to the CNS draining lymph nodes, we assessed in **chapter 2** whether also CNS damage results in drainage of CNS antigens, and whether the type and extent of CNS damage correlated with the frequencies of CNS antigens in the CNS draining lymph nodes. Drainage of myelin antigens results in myelin-specific immune responses in the cervical lymph nodes [45,47,48]. A similar process may be involved in initiating or modulating neuron-specific immune responses. Hence, the phenotype of CNS antigen-containing cells was determined to address potential interactions with and functional skewing of encephalitogenic lymphocytes. Finally, the role of CCR7 in drainage of CNS antigens was addressed using *in vitro* assays and CCR7 knock-out mice.

Antigens, which are injected into the CNS induce Th2-biased and tolerogenic responses in the CNS-draining lymph nodes. Furthermore, the cervical lymph nodes constitute a specific tolerogenic microenvironment for the induction of mucosal tolerance. However, it is unclear whether this tolerance induction or Th2 skewing also holds for CNS antigens resulting from inflammatory brain damage. In **chapter 3** we functionally addressed the role of the CNS-draining lymph nodes in the pathogenesis of EAE by surgical excision of the deep cervical, the superficial cervical and the lumbar lymph nodes prior to EAE induction. Three EAE mouse models were used demonstrating distinct EAE courses to assess the effect of lymphadenectomy in the different stages of EAE. In addition, we determined immune responses against CNS antigens in the distinct CNS-draining lymph nodes of EAE-affected mice to gain more insight into potential functional specialization of the lymph nodes.

The presence of myelin-laden myeloid cells in the CNS parenchyma, perivascular spaces, CSF, and secondary lymphoid organs of MS patients and EAE-affected animals suggests that these cells are highly motile and apt at migrating between and within these anatomical compartments. In addition, myelin-laden cells are optimally positioned for cell-cell interactions. Since chemokines and chemokine receptors are pivotal for migration and the interplay between cells, **chapter 4** addressed the question whether myelin ingestion regulates chemokine receptor expression and chemokine production by macrophages *in vitro*. Chemotaxis experiments were subsequently performed to functionally assess the capacity to migrate and to attract other leukocytes.

Anti-inflammatory myelin-containing myeloid cells in the CNS parenchyma, perivascular spaces and in the CNS-draining lymphoid organs are in close proximity to T cells. However, it is unclear whether myelin-laden macrophages have the capacity to present antigens to T cells and if so, whether this affects differentiation of functional T cell subsets. Considering the anti-inflammatory phenotype of myelin-laden macrophages, we hypothesized that myelin-laden macrophages modulate T cell function, resulting in protection against EAE. This hypothesis is tested *in vitro* and *in vivo* in **chapter 5**.

Chapter 6 provides an overview of the studies in this thesis. The results are discussed in view of our hypothesis that CNS antigens drain to the CNS-draining lymph nodes and elicit CNS antigen-specific immune responses that interfere with EAE. Furthermore, implications for MS patients are discussed and suggestions for future research are given.

Part II

Functional analysis of CNS-draining lymph nodes

Part II

Functional analysis of CNS-draining lymph nodes

Chapter | 2

Brain antigens in functionally distinct antigen-presenting cell populations in cervical lymph nodes in MS and EAE

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Abstract

Drainage of CNS antigens to the brain-draining cervical lymph nodes (CLN) is likely crucial in the initiation and control of autoimmune responses during MS. We demonstrate neuronal antigens within CLN of MS patients. In monkeys and mice with EAE, and in mouse models with non-inflammatory CNS damage, the type and extent of CNS damage was associated with the frequencies of CNS antigens within the cervical lymph nodes. In addition, CNS-antigens drained to the spinal cord-draining lumbar lymph nodes. In human MS CLN, neuronal antigens were present in pro-inflammatory APC, whereas the majority of myelin-containing cells were anti-inflammatory. This may reflect a different origin of the cells or different drainage mechanisms. Indeed, neuronal antigen-containing cells in human CLN did not express the lymph node homing receptor CCR7, whereas myelin antigen-containing cells *in situ* and *in vitro* did. Nevertheless, CLN from EAE-affected CCR7-deficient mice contained equal amounts of myelin and neuronal antigens as wild type mice. We conclude that the type and frequencies of CNS antigens within the CLN are determined by the type and extent of CNS damage. Furthermore, the presence of myelin and neuronal antigens in functionally distinct APC populations within MS CLN suggests that differential immune responses can be evoked.

Introduction

Irreversible neuronal damage is a major pathological feature of MS and ranges from mild pathology to complete axonal transection [3,128]. The cause of neuronal damage is not yet elucidated, but autoreactive B and T cells directed against neuronal antigens could conceivably be instrumental [129]. Indeed, MS patients have increased circulating antibody levels against the neuronal proteins neurofilament light (NF-L) and neurofilament heavy (NF-H) [130-132] in serum and against the medium subunit of neurofilament in the CSF [133]. In addition, T cells from MS patients proliferate in response to the neuronal antigens synapsin and neuron specific enolase (NSE) [134,135]. In mice, T cell-mediated autoimmunity against neuronal antigens leads to CNS inflammation and EAE symptoms [136-140].

Autoreactive lymphocytes against myelin proteins and likely also against neuronal antigens are recruited into the central nervous system (CNS) [3]. Where in the body these lymphocytes initially are activated is still unclear. This is a critical issue, in view of possible therapeutic interventions aiming to limit activation of autoreactive T cells. Under experimental conditions, dendritic cells activate naïve $CD4^+$ transgenic T cells directed against $PLP_{139-151}$ within the CNS [50]. However, the classical view on the initiation of naïve T cell activation holds that antigens or antigen-containing APC must be transferred to the brain-draining CLN to effectively activate naïve T cells [141,142]. CNS-resident APC subsequently reactivate these antigen-experienced T cells and allow them to exert their effector functions within the target organ [143,144]. A crucial role of the CLN in CNS inflammation is supported by the observation that surgical removal of the CLN reduced the number of brain lesions in cryolesion-enhanced EAE in the rat [49]. Furthermore, MBP, PLP and neutral lipid-containing APC are present in the CLN of MS patients and EAE-affected marmoset and rhesus monkeys [45,46]. These APC stimulated myelin-specific T cell proliferation [45], demonstrating that myelin drainage to the CLN during demyelinating disease results in the activation of autoreactive T cells.

We hypothesize that drainage of neuronal antigens from the target organ to the CLN may be similarly involved in initiating or modulating neuron-specific immune responses. We have analyzed whether brain-derived neuronal antigens are present in CLN of MS patients and of selected animal models with different degrees of CNS damage. In addition, we determined whether these antigens are present in distinct APC subsets, which may influence T cell activation.

Results

Presence of neuronal antigens within the CLN of MS patients

To determine the presence of neuronal antigens in CLN of MS patients with active disease, cryosections were stained with antibodies specific for three neuronal antigens: microtubule associated protein-2 (MAP-2), NSE and NF-H. Cells containing each of the neuronal antigens were detectable in CLN from all MS patients, which were predominantly large cells located in the medulla of the lymph node with a morphology resembling macrophages. Neuronal antigen-containing cells were also found in the paracortex, where dendritic cells and T cells are located (Figure 1A-C).

As previously described, neuronal antigen-containing cells were also present in CLN of control subjects (Figure 1D-F) [145,146]. To investigate whether the presence of neuronal antigens in CLN of MS patients was the result of neuronal damage in the CNS or the result of expression by cells in the CLN, neuronal antigen-containing cells were quantified and compared with the number of neuronal antigen-containing cells in CLN of control subjects without neurological disease. As the numbers of MAP-2, NSE, and NF-H positive cells in CLN of MS patients and control subjects were not statistically different (Figure 1D-F), we could not definitively conclude that increased neuronal damage within the CNS resulted in increased neuronal antigen drainage to CLN. Hence, we performed an elaborate analysis in a variety of EAE models and in animal models of non-inflammatory CNS damage (Table 1).

Presence of neuronal antigen-containing cells in CLN of EAE-affected animals reflects the intensity of neuronal damage

It has been well established that the recombinant human myelin oligodendrocyte glycoprotein (rhMOG)-induced EAE model in common marmoset monkeys is characterized by chronic progressive disease with limited axonal destruction, whereas the same EAE model in rhesus monkeys follows a short-lasting acute disease course with extensive axonal destruction [35]. In CLN of EAE-affected marmoset monkeys, NSE and NF-H-containing cells were observed (Figure 2A, B). Quantification revealed a higher, but not significantly different, number of neuronal antigen-containing cells in CLN of EAE-affected marmosets as compared to CFA-controls (Figure 2C, D). In contrast, the CLN of EAE-affected rhesus monkeys contained significantly more MAP-2 and NF-H-containing cells (Figure 2E, F), than the CLN of complete Freund's adjuvant (CFA)-controls ($p<0.05$; Figure 2G, H).

The neuronal antigen NF-L was detected in deep CLN from EAE-affected Biozzi ABH mice with clinical EAE, but only rarely in superficial CLN (Figure 2I). This EAE model is characterized by a first peak of acute disease with inflammation and axonal injury, followed by one or more relapses. As an example, Figure 2K shows the typical relapsing-remitting disease course in this model. CLN isolated during acute EAE as well as during the first relapse had significantly more NF-L-containing cells than CFA-immunized control mice ($p<0.05$; Figure 2J). We observed similar numbers of NF-L-containing cells in the first EAE episode and the subsequent relapse.

As in CLN of MS patients, neuronal antigen-containing cells in the CLN of EAE-affected mice and monkeys were large macrophage-like cells which were located in the medulla of the lymph node, and, although in lower numbers, in the paracortex.

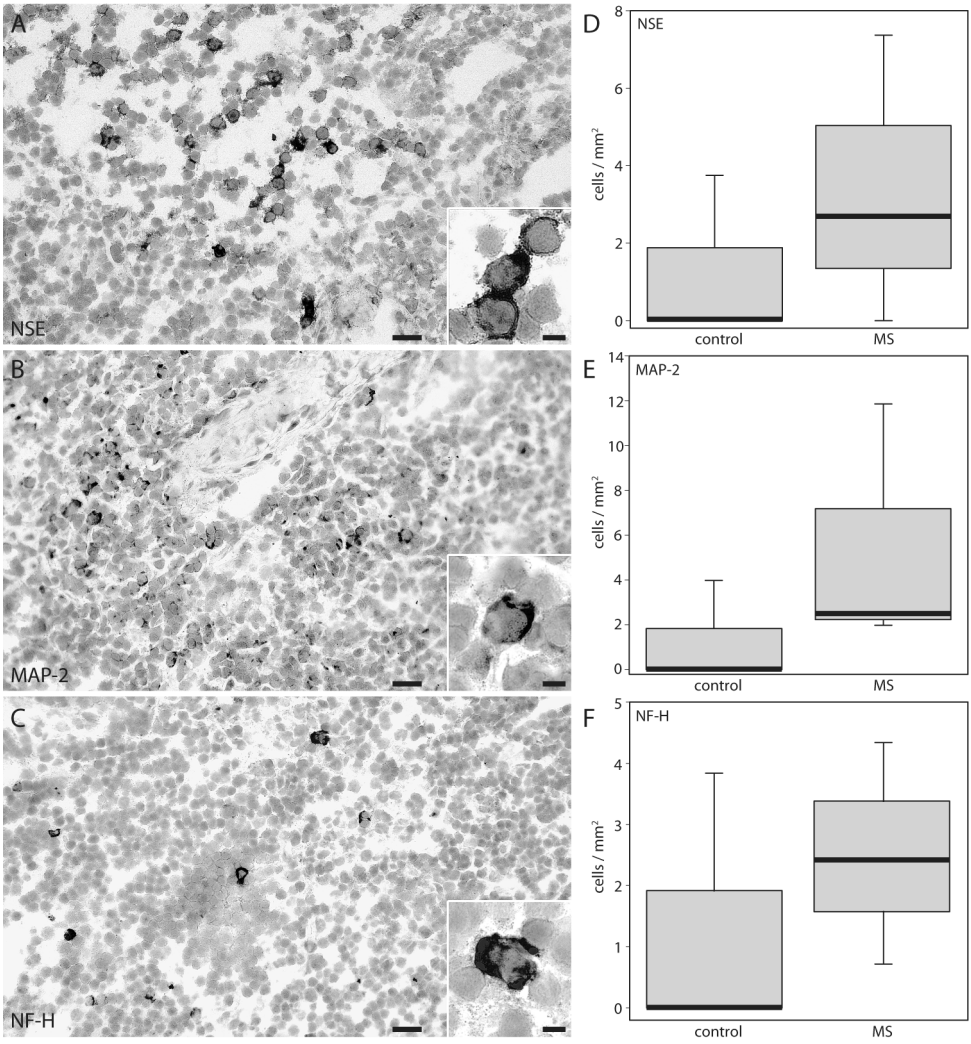

Figure 1. Neuronal antigens drain to the CLN in MS. CLN of MS patients contain cells that are positive for the neuronal antigens NSE (A), MAP-2 (B), and NF-H (C). Scale bars: 20 μm; insert 50 μm. Quantification of the number of MAP-2 (D), NSE (E), and NF-H (F) positive cells demonstrated a higher, but not significantly different, number of neuronal antigen-containing cells in CLN of three MS patients than in CLN of three controls without neurological disease. Three sections from CLN of each patient were quantified for each neuronal antigen. Results are shown as box plots with medians, twenty-fifth and seventy-fifth percentiles as boxes, tenth and ninetieth percentiles as whiskers.

Drainage of CNS antigens to the CNS-draining lymph nodes is reflected by the extent and type of CNS damage

To further assess whether the extent and also the type of CNS insult determines drainage of CNS compounds, and to investigate drainage routes of CNS antigens in mice, we used different mouse models with non-inflammatory CNS damage. The middle cerebral artery occlusion (MCAO) model is characterized by massive ischemic lesions in the cortex, striatum and the hippocampus [147]. Superficial as well as deep CLN isolated 24 h after MCAO contained numerous PLP and NF-L-containing cells (Figure 3B, C). This number was reduced after 72 h (Figure 3B, C), indicating rapid and transient drainage following CNS damage. In CLN isolated from mice with entorhinal cortex lesion (ECL) and facial nerve axotomy (FNA), few PLP and NF-L-containing cells were observed in the CLN (Figure 3B, C), reflecting the mild CNS damage in these models [148,149]. The cuprizone model is characterized by extensive CNS demyelination induced by cuprizone, which selectively kills oligodendrocytes [150]. Little drainage of the myelin antigen PLP to the superficial CLN was noticed (Figure 3A, B). In contrast, the deep CLN of both SJL/J mice (Figure 3A, C) as well as C57BL/6 mice (data not shown) contained significantly higher numbers of PLP-containing cells ($p<0.001$). We also detected myelin antigens in the lumbar lymph nodes (LLN) (Figure 3A, C), reflecting drainage ofantigens from the spinal cord. Only few NF-L-containing cells were observed in the CLN of cuprizone treated mice (Fig 3B, C), which is in line with the limited neuronal damage in this model [151].

Neuronal antigen-containing cells have an APC phenotype

Although the majority of neuronal antigen-containing cells in MS CLN were located in the medulla of the lymph node, such cells were also found in the paracortex (Figure 1A-C), where APC interact with naïve T lymphocytes. We therefore investigated whether neuronal antigen-containing cells in MS CLN express APC markers. As expected in a secondary lymphoid organ, numerous cells in the CLN expressed MHC class II and CD40 of which only a fraction contained the neuronal antigen MAP-2. However, almost all (90%-100%) MAP-2 positive cells expressed MHC class II (Figure 4A) and the costimulatory molecule CD40 (Figure 4B). This indicates that neuronal antigen-containing cells are APC, such as dendritic cells or macrophages, which may be involved in the induction of autoimmunity against neuronal antigens, or conversely in the control of autoreactivity.

T cells in CLN proliferate to MOG peptide, without evident epitope spreading to NF-L

The localization of neuronal antigens in APC within the CLN may activate autoreactive T cells. This was tested in three independent experiments. Two experiments were using C57BL/6 mice in which EAE was induced by injection with MOG_{35-55} (n=5-10 per experiment). One additional experiment was using Biozzi ABH mice (n=10) in which EAE was induced by injection with MOG_{8-21}. Figure 5 shows a representative experiment, in which we observed dose-dependent

T cell proliferation against the immunizing peptide MOG_{35-55} in deep CLN, superficial CLN as well as in LLN. CLN and LLN from mice immunized with MOG_{8-21} demonstrated quantitatively similar proliferation against MOG_{8-21} (data not shown). No proliferation against the irrelevant control antigen ovalbumin (OVA) was seen. We did not detect NF-L specific T cell proliferation in the two EAE models used for these experiments.

Table 1. Characteristics of MS and the animal models used in this study.

Disease	Selected characteristics for this study	Demyelination	Neuronal damage
Human MS	Myelin antigens in CLN (neutral lipids, MBP, PLP) [45]	Present [3]	Present [3]
Marmoset EAE	Chronic EAE Myelin antigens in CLN (neutral lipids, MBP, PLP) [45]	Present [35]	Limited [35]
Rhesus EAE	Acute EAE Myelin antigens in CLN (MBP, PLP) [45]	Dramatic destruction of myelin [35]	Dramatic destruction of axons [35]
Biozzi ABH EAE	Chronic relapsing EAE	Little in acute phase, abundant in relapse [36]	Present in every phase of disease [36]
C57BL/6 EAE	Chronic EAE	Present [38]	Present [38]
Cuprizone	Chemically-induced demyelination without clinical disease	Extensive demyelination [150]	Little neuronal damage [151]
MCAO	Ischemic lesions in the cortex, striatum and hippocampus	Massive myelin loss [147]	Massive neuronal loss [147]
ECL	Perforant pathway is stereotactically lesioned, leading to anterograde axonal degeneration within dentate gyrus of the hippocampus [173]	Limited, since the perforant pathway is not myelinated	Present in hippocampus [149]
FNA	Blood brain barrier remains intact. Retraction of the motoneurons in the brainstem [148]	Limited, since the facial nucleus is not myelinated	Limited [174]

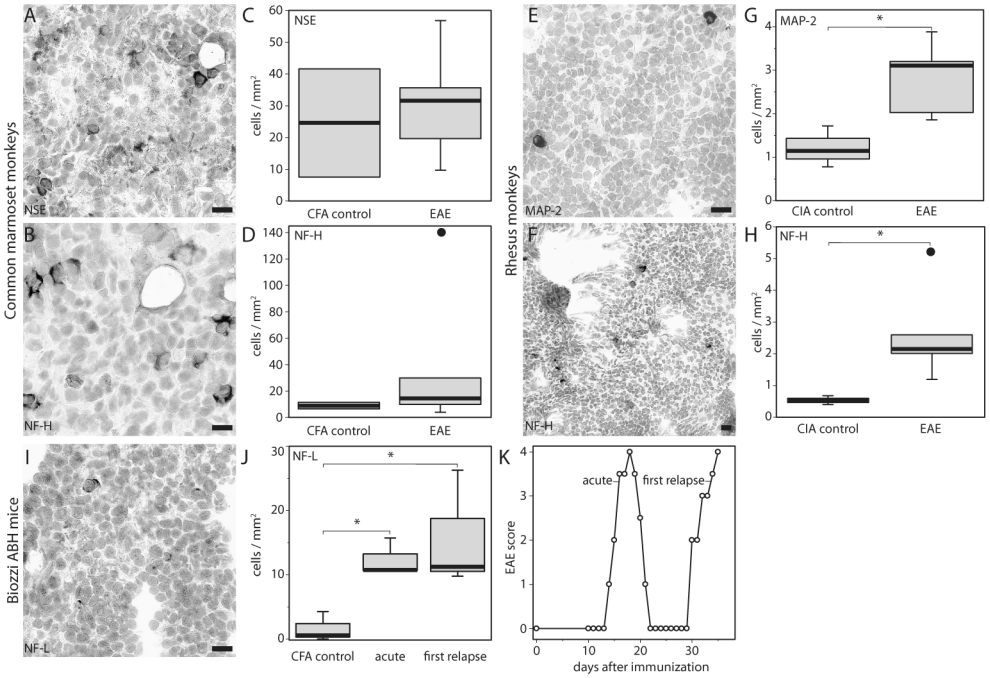

Figure 2. The presence of neuronal antigen-containing cells during EAE reflects the extent of neuronal damage within the CNS. CLN of EAE-affected common marmoset monkeys contain NSE (A) and NF-H (B) positive cells. More NSE (C) and NF-H (D) positive cells were observed in CLN of five marmosets with EAE as compared to two CFA-immunized control marmosets, although this difference was not significant. In CLN of EAE-affected rhesus monkeys MAP-2 (E) and NF-H (F) positive cells were found. Quantification of MAP-2 (G) and NF-H (H) positive cells revealed a significantly higher number of cells in CLN of five rhesus monkeys with EAE than in CLN of three collagen-induced arthritis (CIA) control monkeys (*p<0.05). NF-L-positive cells were present in the deep CLN of EAE Biozzi ABH mice (I). Deep CLN from three animals with acute disease as well as from three mice in the first relapse contained significantly more NF-L positive cells than CLN from three CFA-immunized control mice (J; *p<0.05). EAE scores from one representative animal out of three are shown (K). Three sections from CLN of each monkey and four sections from CLN of each mouse were quantified for each neuronal antigen. Results are given as box plots as described in Figure 1 with extreme values as filled circles. Scale bars: 10 μm.

Differential expression of pro- and anti-inflammatory molecules by myelin-containing versus neuronal antigen-containing cells

Myelin-laden myeloid cells in MS brain with foamy appearance demonstrate a strong anti-inflammatory phenotype *in situ* and *in vitro*, implying a possible role in the resolution of local inflammation during MS [120]. To assess whether this also holds true for myelin antigen-containing cells and neuronal antigen-containing cells in CLN of MS patients, we determined the expression of pro- and anti-inflammatory molecules. The majority of MOG-containing cells (90-100%) co-expressed the anti-inflammatory molecules IL-1 receptor antagonist (IL-1ra) (Figure 6A) and

TGF-β (Figure 6B), whereas only a minority of the cells (2-30%) expressed the pro-inflammatory molecule IL-12p40/p70, indicating that most MOG-containing cells display an anti-inflammatory phenotype (Table 2). In contrast, none of the MAP-2-containing cells expressed IL-1ra and TGF-β, while the majority of the cells (86-100%) expressed IL-12p40/p70 (Figure 6C) and TNF-α (Figure 6D), indicating a pro-inflammatory phenotype (Table 2). These data were paralleled in the CLN of EAE-affected rhesus monkeys, where 50-95% of the MOG-containing cells expressed IL-1ra and 13-47% expressed IL-12p40/p70, whereas 75-94% of the MAP-2-containing cells expressed IL-12p40/p70 and none expressed IL-1ra.

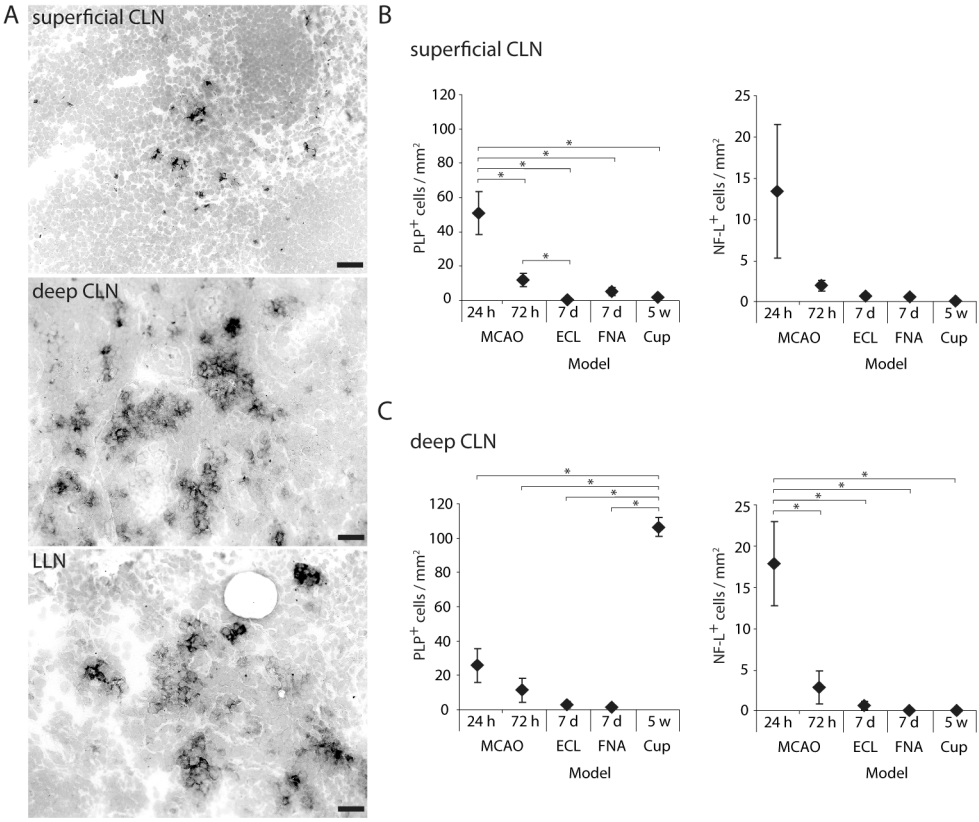

Figure 3. The type and extent of non-inflammatory CNS damage correlates with the frequencies of CNS antigens in the CNS-draining lymph nodes. PLP-containing cells in the superficial CLN, deep CLN, and the spinal cord-draining LLN of cuprizone-treated SJL/J mice. Scale bars: 50 μm (A). Quantification of the number of PLP- and NF-L-containing cells in the superficial CLN (B) and deep CLN (C). Tissues were isolated 24 h (*n*=5) and 72 h (*n*=5) after MCAO, seven days after ECL (*n*=5), seven days after FNA (*n*=3), and after six weeks of cuprizone treatment (Cup; *n*=3). Cells were quantified in at least two sections of each tissue. Data represent the mean number of positive cells ± SEM. *$p<0.05$.

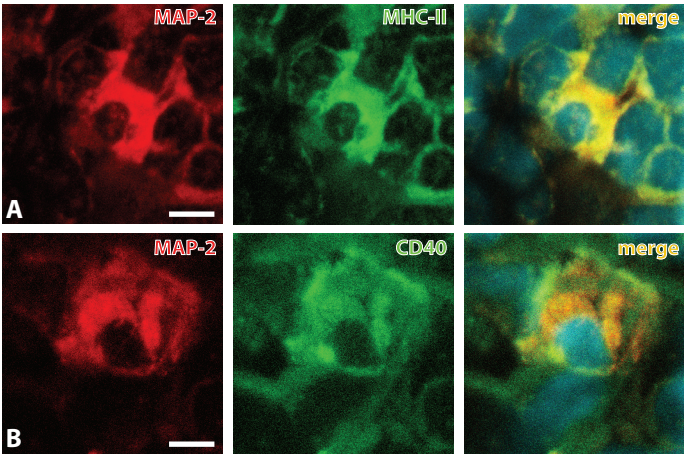

Figure 4. MAP-2 containing cells in human MS CLN are immunocompetent APC. Immunofluorescent MAP-2 staining in human MS CLN (red) and staining for the APC markers MHC class II (green) (A) and CD40 (green) (B). Overlay shows co-expression of MAP-2 with MHC class II and CD40, which was observed in 90 to 100% of the MAP-2 positive cells. Nuclei are stained with DAPI (blue). Stainings were performed on two sections of CLN from three different MS patients. Scale bars: 5 μm.

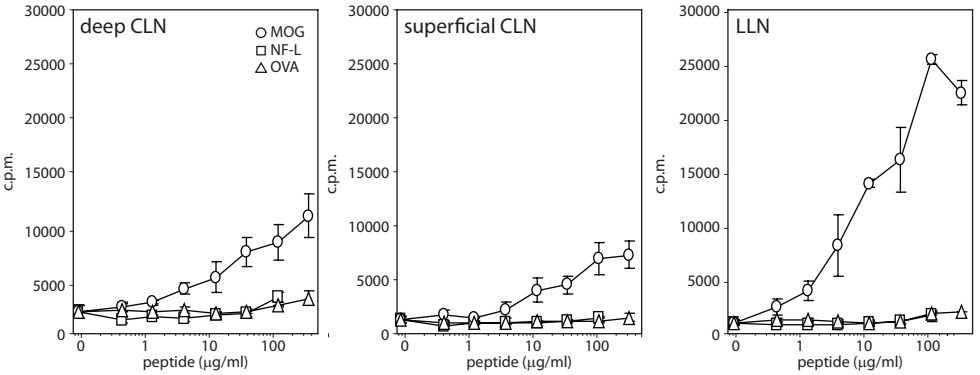

Figure 5. Drainage of neuronal antigens to CLN in MOG peptide-induced EAE does not elicit a detectable NF-L-specific T cell response. Stimulation of lymph node cells from deep CLN, superficial CLN and LLN from EAE-affected C57BL/6 and Biozzi ABH mice with rmNF-L, MOG_{35-55} and ovalbumin demonstrates a dose-dependent proliferation against the myelin-derived peptide MOG, whereas no proliferation was observed against NF-L and ovalbumin. Lymph nodes of five to ten EAE-affected mice were pooled per experiment to obtain enough cells for restimulation. Results are presented as mean with the standard deviation of triplicates and are representative for three independent experiments using two different MOG peptide-induced EAE models.

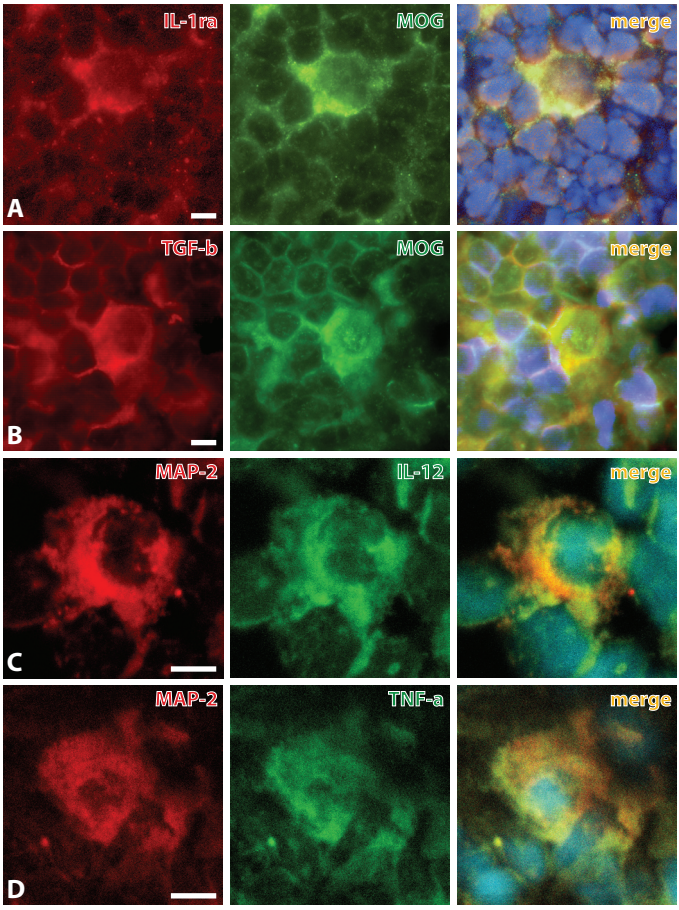

Figure 6. Differential expression of pro- and anti-inflammatory molecules by MAP-2 and MOG-containing cells in human MS CLN. Immunofluorescent labeling of MOG (green) and the anti-inflammatory molecules IL-1ra (red) (A) and TGF-β (red) (B) and immunofluorescent labeling of MAP-2 (red) and of the pro-inflammatory molecules IL-12p40/p70 (green) (C), and TNF-α (green) (D) in human MS CLN. The overlays show co-localization of MOG and the anti-inflammatory molecules IL-1ra and TGF-β, which was observed in 90 to 100% of the MOG positive cells, and co-localization of MAP-2 and the pro-inflammatory molecules IL-12p40/p70 and TNF-α, which was observed in 86 to 100% of the MAP-2 positive cells. Nuclei are stained with DAPI (blue). Results are representative for two sections of CLN from an MS patient for double stainings of MOG with TGF-β and TNF-α and of MAP-2 with TGF-β and for at least two sections of CLN from three different MS patients for double stainings of MAP-2 with IL-12p40/p70, TNF-α and IL-1ra and double stainings of MOG with IL-1ra and IL-12p40/p70. Scale bars: 5 μm.

Part of MOG-containing cells in CLN of MS patients express CCR7, whereas MAP-2-containing cells do not express CCR7

Drainage of CNS antigens to the CLN may either occur as soluble proteins or within phagocytes [67,141]. We have previously shown that myelin antigen-containing cells in rhesus monkey CLN express the chemokine receptor CCR7 [45], which mediates leukocyte homing to draining lymph nodes. Similarly, we observed that 20-42% of MOG-containing cells in human MS CLN expressed CCR7, whereas none of the MAP-2-containing cells expressed this marker (Table 2). These data were paralleled in the CLN of EAE-affected rhesus monkeys, in which 20-46% of MOG-containing cells expressed CCR7, and MAP-2-containing cells did not express CCR7.

To investigate whether CCR7 expression is induced by the uptake of myelin antigens, we incubated human monocyte-derived macrophages from healthy donors *in vitro* ith human myelin and determined CCR7 mRNA expression and CCR7 surface protein expression. We observed a time-dependent increase of CCR7 mRNA expression after myelin ingestion. As compared to control macrophages, myelin-laden macrophages demonstrated a 38.4±4.2 fold increase in CCR7 mRNA expression after 24 h of myelin ingestion, which was reduced to a 6.3±0.9 fold increase after 7 days of myelin ingestion. In addition to mRNA, CCR7 surface protein expression was also increased after myelin ingestion (data not shown).

Finally, we assessed whether CCR7 expression affects the drainage of CNS antigens to CLN. To this end we compared the number of CNS antigen-containing cells in the deep and superficial CLN of EAE-affected CCR7-deficient and wild type mice. In our hands, CCR7-deficient mice developed mild EAE and CLN of these mice were therefore compared to CLN from wild type mice with comparable EAE symptoms. Unexpectedly, the numbers of both myelin and neuronal antigen-containing cells in CLN of EAE-affected CCR7-deficient mice were not statistically different as compared to wild type mice (Table 3).

Table 2. Contrasting functional phenotype of MAP-2 versus MOG-containing cells in human MS CLN.

Antigen	Pro-inflammatory		Anti-inflammatory		Lymph node homing potential
	IL-12p40/p70	TNF-α	IL-1ra	TGF-β	CCR7
MAP-2	++++	++++	-	-	-
MOG	MOG	+	+/-	++++	+++

Quantification of double-labeling of cells in CLN sections of MS patients. – 0%, +/- 1-20%, + 21-40%, ++ 41-60%, +++ 61-80%, ++++ 81-100% of the cells co-expressing both molecules. Results are representative for two sections of CLN from an MS patient for double stainings of MOG with TGF-β and TNF-α and of MAP-2 with TGF-β and for at least two sections of CLN from three different MS patients for double stainings of MAP-2 with IL-12p40/p70, TNF-α, IL-1ra and CCR7 and double stainings of MOG with IL-1ra, IL-12p40/p70 and CCR7.

Table 3. CCR7-deficiency does not affect CNS antigen load in CLN.

	MOG	PLP	MAP-2	NF-L
WT	10.3±10.6	0.8±1.1	3.3±4.6	1.5±2.1
CCR7-deficient	29.7±15.7	8.6±6.1	4.7±4.2	4.8±7.1

Quantification of CNS antigen-containing cells in sections of deep CLN from four EAE-affected wild type mice and four EAE-affected CCR7-deficient mice. Numbers represent mean number of cells per mm^2 ± standard deviation of four sections per animal.

Discussion

Interaction between the brain and the secondary lymphoid organs allows regulation of immune responses in the brain [152]. This is exemplified by the crucial role of CLN in brain lesion expansion in cryolesion-enhanced EAE in rats [49], and by the presence of myelin antigens in APC in CLN of MS patients and EAE-affected rhesus monkeys and common marmoset monkeys [45,46]. The current study assessed whether neuronal antigens also drain to the CLN after neuronal damage in the CNS.

MS is both clinically and pathologically a heterogeneous disease demonstrating various degrees of neuronal damage. The obtained CLN tissues were therefore from patients at various disease stages. To further determine drainage in a more controlled setting with defined disease stages, we used a variety of EAE models and models of non-inflammatory CNS insult, representing different degrees of neuronal damage. We report here that neuronal antigens are present in CLN of MS patients and of animals after induction of EAE, MCAO, ECL and FNA. In rhesus monkeys and Biozzi ABH mice, animals in which EAE results in significant neuronal damage within the CNS, the quantity of neuronal antigen-containing cells in the CLN reflected the intensity of neuronal damage in the CNS. Furthermore, qualitative and quantitative drainage of CNS antigens followed the type and extent of CNS damage in mice after MCAO, ECL, FNA, and cuprizone treatment. Interestingly, the frequency of CNS antigens in the CLN was high 24 h after MCAO and reduced 72 h after MCAO, indicating that drainage occurs rapidly but transient following CNS damage. This transient CNS drainage might also cause the little CNS antigen-containing cells in the CLN of the ECL and FNA model, which were isolated 7 days after CNS damage induction.These data extend our previous data and demonstrate that, following CNS damage, both myelin and neuronal antigens drain to CLN.

The presence of neuronal antigen-containing cells in CLN of control subjects was not unexpected, and may be due to innervation of the CLN [153], expression of neuronal antigens by the cells themselves [145,146], or to ageing. The frequency of neuronal antigen-containing cells in CLN from control subjects seems to differ between species, which might be due to differential neuronal turnover rates between species or differential expression levels of neuronal antigens by the cells.

To investigate drainage routes from the brain to the CLN we used different non-inflammatory CNS damage models [147-151]. In cuprizone-treated animals, numerous cells containing myelin antigens were observed in the deep CLN, whereas such cells were observed only occasionally in the superficial CLN. We have found the same drainage route in EAE-affected mice, where we observed high numbers of neuronal antigen-containing cells in the deep CLN, but hardly in the superficial CLN. These data indicate that CNS antigens preferentially drain to the deep CLN. In contrast, both the deep CLN as well as the superficial CLN of MCAO-treated mice contained numerous CNS antigens. This may be caused by a different drainage route as the result of the massive CNS damage, or by the fact that this massive CNS damage has destroyed (part) of the drainage route. Myelin antigens were also observed in the LLN of cuprizone-treated mice, which are likely derived from the spinal cord, in which demyelination also takes place as is the case in MS and EAE. In fact, whereas CLN are crucial in brain lesion expansion in cryolesion-induced EAE in rats, they have no effect on lesion expansion in the lumbar part of the spinal cord [49], suggesting distinct functional relationships between CNS compartments and their local draining lymph nodes.

The nature of immune responses against CNS antigens in the CLN might be dictated by the functional phenotype of the CNS antigen-containing cells. We therefore determined the phenotype of myelin and neuronal antigen-containing cells in human MS CLN. Neuronal antigen-containing cells in MS CLN expressed the APC markers MHC class II and CD40, indicating that these cells are equipped for antigen presentation to T lymphocytes. Furthermore, neuronal antigen-containing cells were observed in the paracortex of the lymph node, where APC encounter and activate naïve T cells. Indeed, T cell responses against neuronal antigens have been demonstrated in MS patients and EAE-affected animals [134-136], and T cell-mediated EAE symptoms can be induced by immunization with neuronal antigens [137,139]. Despite this, we did not detect T cell proliferation against NF-L in CLN from EAE mice immunized with MOG_{35-55} or MOG_{8-21}. The lack of T cell proliferation we consistently found might be due to the absence of intermolecular spreading or to the fact that the draining antigens may act immunosuppressively on proliferation within the draining lymph nodes.

The current study shows that phagocytes containing the neuronal antigen MAP-2 in CLN of MS patients and EAE-affected rhesus monkeys had a pro-inflammatory phenotype. In contrast, the majority of phagocytes containing the myelin antigen MOG demonstrated an anti-inflammatory phenotype, suggesting a relation with the anti-inflammatory myelin-laden foamy macrophages present within MS lesions [120]. This differential expression of pro- and anti-inflammatory molecules likely influences the type of immune response against these antigens. The difference in functional phenotype between MAP-2 and MOG-containing cells may be the consequence of the inflammatory status of the micro-environment in which the cells have taken up their antigens. Alternatively, the nature of the phagocytozed antigen may direct the immunophenotype of the cell into a pro- or anti-inflammatory mode of action.

As both myelin and neuronal antigens are found in CLN the question arises how these antigens reach these lymph nodes. The two likely mechanisms are either as soluble antigen or by active transport within phagocytozing cells [141,142], such as the anti-inflammatory foamy macrophages within MS lesions [120]. Since anti-inflammatory macrophages express the lymph node homing receptor CCR7 [154], we determined whether a CCR7-dependent mechanism could be involved. This study shows that, similar to APC in MS brain as well as MOG-containing cells in EAE-affected rhesus CLN [45,155], myelin-containing cells in human MS CLN and *in vitro* express CCR7, but MAP-2-containing cells do not. CLN from EAE-affected CCR7-deficient mice contain slightly more myelin and neuronal antigens as compared to CLN from EAE-affected wild type mice, indicating that CCR7 is not necessarily involved. This strongly suggests that either other chemokine receptors are able to guide cell migration to the CLN, or that CNS antigens drain as soluble antigens through CSF to the CLN. This hypothesis is supported by increased free neuronal proteins in CSF of MS patients and EAE mice as compared to healthy controls [156,157].

In conclusion, we here report that neuronal antigen-containing cells are present in CLN during MS and in various animal models for CNS damage. The frequencies of these cells correlated with the extent of neuronal damage. In addition, neuronal antigen-containing cells in human MS CLN are present in functionally different APC subsets as compared to the majority of myelin antigen-containing APC containing. The presence of neuronal antigens in APC with a pro-inflammatory phenotype and of myelin antigens in APC with an anti-inflammatory phenotype points at a different potential to activate functionally distinct T cell subsets.

Experimental procedures

MS tissues

Human jugular (deep) CLN and supraclavicular CLN were taken from MS patients with active disease at autopsy. Chronic inactive and active plaques were present in the cerebrum of these patients. The MS patients died of non-MS-related causes. In addition, CLN were taken from controls without neurological disease at autopsy by the Netherlands Brain Bank (coordinator Dr. R. Ravid). CLN were snap-frozen in liquid nitrogen and stored at –80°C until use.

EAE tissues

All animal studies in the current study followed the principles of animal care and were approved by local ethical committees based on national legislation. Tissues were obtained from animals that were used for studies designed for other purposes, thus avoiding the sacrifice of animals for the present study only.

Deep CLN were isolated from rhesus monkeys (*Macaca mulatta*) with acute EAE (*n*=5) which was induced as described [158] and from rhesus monkeys with collagen-induced arthritis (*n*=3) [159]. Deep CLN were also isolated from common marmoset monkeys (*Callithrix jacchus*) during chronic EAE (*n*=5) [160,161]. Control CLN were from marmosets (*n*=2) immunized with ovalbumin in CFA [45]. The monkeys were housed at the Biomedical Primate Research Centre (BPRC, Rijswijk, the Netherlands).

Deep and superficial CLN were isolated from Biozzi ABH (H-2^{dq1}) mice, obtained from stock bred at the BPRC, at the acute phase of disease (*n*=3) and during the first relapse (*n*=3). EAE was induced by immunization with spinal cord homogenate or MOG_{8-21} in CFA as described [162,163]. Deep and superficial CLN were also isolated from CCR7-deficient (*n*=4) and wild type mice (*n*=4) [164] with chronic EAE, obtained from stock bred at the animal facility of the Max Delbrück Centre for Molecular Medicine (Berlin, Germany). Chronic EAE was induced by subcutaneous injections of 200 μg MOG_{35-55} emulsified in CFA (Difco Laboratories) containing 400 μg of *Mycobacterium tuberculosis*. Mice received intravenous injections with 200 ng *Bordetella pertussis* toxin (Sigma) on day 0 and 2 postimmunization. The CCR7-deficient mice used in these experiments had been backcrossed to C57BL/6 mice for eight generations.

All animals were examined daily for clinical symptoms of EAE as described [45,160,163,165]. Tissues were snap-frozen in liquid nitrogen and stored at –80°C.

Non-inflammatory CNS damage models

Ischemia was induced in C57BL/6 mice (BgVV) by occlusion of the left middle cerebral artery [147]. The MCAO did not exceed 60 min. CLN were isolated 24 h (*n*=5) and 72 h (*n*=5) after MCAO. Entorhinal cortex lesion and facial nerve axotomy were performed as described [166] in C57BL/6 mice (*n*=5 and *n*=3, respectively; Charles River). CLN were isolated seven days later.

Demyelination was induced chemically by cuprizone (Sigma-Aldrich) in SJL/J mice (*n*=3; Janvier) and C57BL/6 mice (*n*=3; Harlan) as described [150]. These animals were representative animals within a larger experiment designed for other purposes, consisting of 18 SJL/J mice and 18 C57BL/6 mice. Five to six weeks after start of treatment with cuprizone, superficial CLN, deep CLN and LLN were isolated. All tissues were snap-frozen and stored at –80°C.

Immunohistochemistry

Immunohistochemistry was performed as described [167,168]. Primary antibodies were polyclonal rabbit antibodies against TGF-β (Santa Cruz) and NF-L (Abcam), and monoclonal mouse-antibodies against NSE (MIG M3; Abcam), MAP-2 (HSM 5; Pierce Biotechnology), NF-H (SMI-32; Sternberger Monoclonals), PLP (J1/06 [169]), HLA-DP/DQ/DR (CR3/43; DAKO), CD40 (5D12; dr. M. de Boer), IL-1ra (A71B6D11; Bioscource), TNF-α (61E71; U Cytech), IL-12p40/p70 (C8.6; BD) and CCR7 (2H4; BD).

Primary antibodies were detected by biotinylated donkey-anti-rabbit Ig (Amersham) or rabbit-anti-mouse Ig (DAKO), and HRP-conjugated avidin-biotin complex (DAKO). HRP activity was revealed with 3-amino-9-ethylcarbazole (Sigma-Aldrich), which resulted in a red precipitate. Brain or spinal cord sections from the same species were used as positive control tissue. As negative controls, sections were incubated with isotype-matched primary antibodies of irrelevant specificity, or the primary antibody was omitted.

Immunofluorescence

Double-labeling was performed using immunofluorescence as described [170]. Sections were incubated with primary antibodies for 1 h at RT, followed by FITC- or TRITC-labeled rabbit-anti-mouse Ig (DAKO) or TRITC-labeled swine-anti-rabbit Ig (DAKO) for 30 min at RT. Subsequently, sections were incubated for 1 h at RT with Alexa594-labeled anti-MAP-2 (IgG1 Zenon labeling kit, Molecular Probes) or with anti-MOG for 1 h at RT, followed by biotinylated anti-IgG2a (SBA) for 30 min at RT and FITC-labeled streptavidin (DAKO) for 1 h at RT. Sections were mounted in glycerol/tris/DAPI (50 μg/ml; Molecular Probes).

As controls, single stainings were performed following the procedure described above but with omission of one of the primary antibodies used in the double-staining. Human reactive tonsil or brain was used as positive control tissue. Negative controls are described above.

CCR7 expression by myelin-laden human monocyte-derived macrophages

Monocytes from healthy donors were routinely purified and cultured [120]. For the current study, experiments were performed with cells from two individual donors that responded representatively for at least 50 donors assessed in the preceding years. Monocyte-derived macrophages were seeded into 24-well tissue culture plates at $2x10^5$ cells/well. After 24 h, non-adherent cells were removed and remaining cells were incubated with human myelin [171] for the indicated times. Control macrophages were obtained from the same donor.

Total mRNA was extracted from cell cultures as described [120]. CCR7 mRNA expression was analyzed by real-time PCR using the iCycler (Bio-rad) and the iQ SYBR Green supermix (Bio-rad). The housekeeping gene HPRT1 was used for normalization. The following primers were used: 5′-TGGTCGTGGTCTTCATAGTC-3′ and 5′-CAGGTGCTACTGGTGATGTT-3′ for CCR7, and 5′-TGACACTGGCAAAACAATGCA-3′ and 5′-AGCTTGCTGGTGAAAAGGACC-3′ for HPRT1.

CCR7 surface expression by human monocyte-derived macrophages was determined using flow cytometry. Fc-receptors were blocked using 20% Fc-block (Miltenyi Biotec) and CCR7 was stained using phycoerythrine-labeled mouse-anti CCR7 (150503; R&D Systems) for 30 min on ice. An isotype-matched antibody of irrelevant specificity was used as negative control. Samples were analyzed on a FACSCalibur flow cytometer using CellQuest analysis software (Becton Dickinson).

T cell proliferation assay

Deep CLN, superficial CLN, and LLN were isolated from C57BL/6 mice with chronic EAE and from Biozzi ABH mice with relapsing-remitting EAE. EAE in C57BL/6 mice was induced by immunization with 200 μg MOG_{35-55} in CFA [165] and in Biozzi ABH mice as described above. Lymph nodes from five to ten EAE-affected animals were pooled per experiment to obtain enough cells for restimulation. A single cell suspension was obtained by passing the lymph nodes through a 70 μm gauze. $2x10^5$ lymph node cells were seeded into 96-well round bottom plates (Nunc) and stimulated with the indicated concentrations of MOG_{8-21}, MOG_{35-55} (both from Advanced Biotechnology Centre, Imperial College London), recombinant mouse NF-L (rmNF-L) [172] or OVA (Worthington). After 4 days, T cell proliferation was determined by incorporation of [^{3}H]-thymidine for 18 h (Amersham Biosciences) as described [165].

Image analysis

The area of the sections was measured using a VIDAS-RT image analysis system (Kontron Elektronic/Carl Zeiss) to obtain the number of antigen-positive cells per square millimeter. Differences in number of neuronal antigen-containing cells in CLN between treatment groups were determined by a two-tailed Mann-Whitney *U*-test using the statistical software program SPSS, version 11.0. A significance level of 0.05 was used.

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Chapter | 3

Surgical excision of CNS-draining lymph nodes reduces relapse severity in chronic-relapsing experimental autoimmune encephalomyelitis

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Abstract

Despite lack of classical lymphatic vessels in the CNS, cells and antigens do reach the CNS-draining lymph nodes. These lymph nodes are specialized to mediate mucosal immune tolerance, but can also generate T and B cell immunity. Their role in multiple sclerosis and EAE therefore remains elusive. We hypothesized that drainage of CNS antigens to the CNS-draining lymph nodes is vital for the recurrent episodes of CNS inflammation. To test this, we surgically removed the superficial cervical lymph nodes, deep cervical lymph nodes and the lumbar lymph nodes prior to disease induction in three mouse EAE models, representing acute, chronic, and chronic-relapsing EAE. Excision of the CNS-draining lymph nodes in chronic-relapsing EAE reduced and delayed the relapse burden and EAE pathology within the spinal cord, which suggest initiation of CNS antigen-specific immune responses within the CNS-draining lymph nodes. Indeed, superficial cervical lymph nodes from EAE-affected mice demonstrated proliferation against the immunizing peptide, and the deep cervical lymph nodes, lumbar lymph nodes, and spleen demonstrated additional proliferation against other myelin antigen epitopes. This indicates that intermolecular epitope spreading occurs and that CNS antigen-specific immune responses are differentially generated within the different CNS-draining lymphoid organs. Proliferation of splenocytes from lymphadenectomized and sham-operated mice against the immunizing peptide was similar. These data suggest a role for CNS-draining lymph nodes in the induction of detrimental immune responses in EAE relapses, and conclusively demonstrate that the tolerance-inducing capability of cervical lymph nodes is not involved in EAE.

Introduction

Tissue-draining lymph nodes are vital for generating immune responses during infections and autoimmune disease, as well as in maintaining tolerance against tissue antigens [175-177]. In most tissues, antigens reach the local draining lymph nodes through the lymphatics [178]. However, classical lymphatic vessels are lacking in the central nervous system (CNS) parenchyma [59,68,152]. Despite this unique anatomical feature, CNS antigens and cells do reach secondary lymphoid organs through alternative pathways [68,74,126,141,142,152,179-181]. Brain antigens in interstitial fluids leak into the CSF along the artery walls in the meninges [63]. Subsequently, CSF diffuses along the ventricular system and the arachnoid space where it enters the venous sinuses through the arachnoid villi and finally reaches the spleen. In addition, CSF passes in channels through the cribriform plate into the nasal lymphatics to the cervical lymph nodes [68,74,126]. Spinal cord-derived antigens drain to the cervical lymph nodes as well as to the lumbar lymph nodes [74-77].

Inflammation within the CNS is under strict control of multiple anatomical and immunoregulatory processes to minimize tissue damage and loss of function in such a vital organ [39,40]. Yet, destructive inflammation in the CNS occurs if this control falters, and critically contributes to diseases such as MS [3]. In both MS patients as well as in primate and mouse EAE models, CNS antigens are present in the cervical lymph nodes [45,46,182]. In addition, transfer of CNS-derived antigens to the cervical lymph nodes leads to antibody production [183] and T cell proliferation [45,47,48,184] within these lymph nodes. Furthermore, the cervical lymph nodes are pivotal for brain lesion expansion in cryolesion-enhanced EAE in rats [49].

In addition to the drainage site of the CNS, the cervical lymph nodes are the draining lymph nodes for the nasal mucosa. As such, these lymph nodes are critical to maintaining tolerance against nasal antigens [78,79], which is partly due to a specialized tolerogenic environment within the cervical lymph nodes [79].

The CNS-draining lymph nodes are thus likely essential for the balancing of protective or tolerogenic versus detrimental responses in the CNS. However, surprisingly little is known about the function of CNS-draining lymph nodes in diseases of the CNS. We hypothesized that CNS-draining lymph nodes are essential in the induction and severity of EAE, and that their tolerance-inducing capacity is not involved in this model. To test this hypothesis, we surgically removed the superficial cervical lymph nodes, deep cervical lymph nodes and lumbar lymph nodes prior to EAE induction and determined the EAE course and severity. To assess the function of the differential CNS-draining lymphoid organs in generating CNS-specific immune responses during EAE, we also determined proliferation against various myelin epitopes in the superficial cervical lymph nodes, deep cervical lymph nodes, lumbar lymph nodes and spleen of EAE-affected mice.

Results

Excision of CNS-draining lymph nodes reduces disease incidence, relapse severity and EAE pathology

Biozzi ABH mice with chronic-relapsing EAE demonstrate an acute phase of disease, after which the mice go into remission. Subsequently, the mice suffer from one to two relapses. To assess whether CNS-draining lymph nodes influence disease severity, we compared the mean clinical score of EAE-affected lymphadenectomized mice with the mean clinical score of EAE-affected mice in the sham-group. Lymphadenectomized mice showed a similar disease course and severity during the acute phase of disease, but demonstrated a lower relapse burden (as reflected by the area under the curve) of EAE relapses as compared to sham-operated mice. Although this difference did not reach statistical significance, the declining trend from acute disease to second relapse is very clear (Figure 1A). In concordance with this, the maximum clinical score of lymphadenectomized mice as compared to sham-operated mice was reduced in the first and even more so in the second relapse (Figure 1B). Furthermore, lymphadenectomized mice demonstrated a delay of 9 days in onset of the second relapse as compared to sham-operated mice (Table 1).

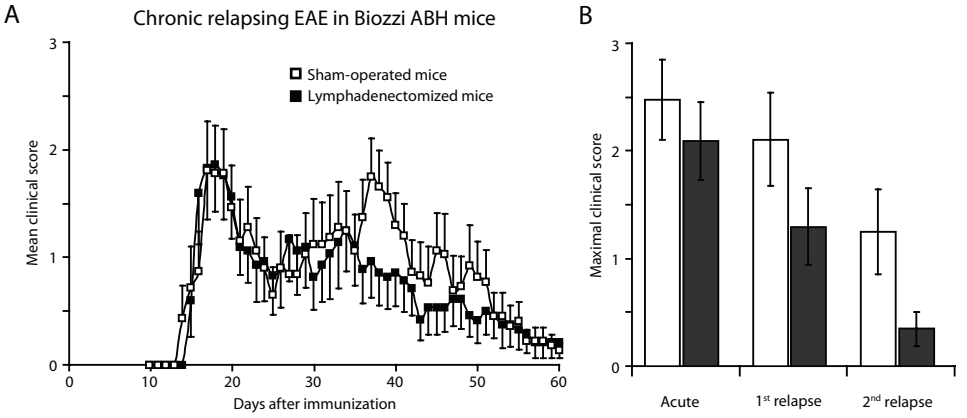

Figure 1. Excision of CNS-draining lymph nodes limits relapse severity in Biozzi ABH mice. (A) Superficial cervical lymph nodes, deep cervical lymph nodes and lumbar lymph nodes were surgically removed from Biozzi ABH mice (*n*=22) prior to induction of chronic-relapsing EAE induced by immunization with MOG_{8-21}. The acute phase of disease in lymphadenectomized mice was similar as compared to sham-operated mice (*n*=19), but relapse severity was reduced in lymphadenectomized mice. Mean group scores from diseased mice are indicated ± SEM. (B) The maximum EAE score of lymphadenectomized mice demonstrated a modest decrease as compared to sham-operated mice during the first relapse, but a more pronounced decrease in the second relapse. Differences did not reach statistical significance (exact two-tailed Mann-Whitney *U*-test and variance analysis for repeated measurements).

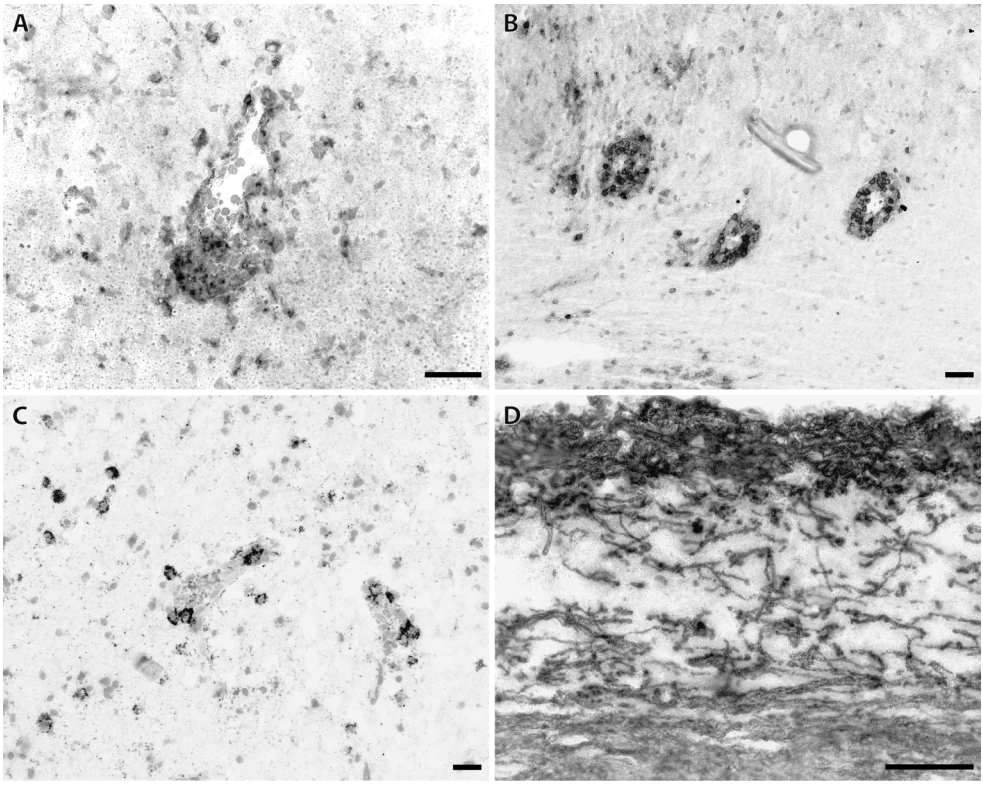

Figure 2. Diminished EAE pathology within the spinal cord of lymphadenectomized Biozzi ABH mice. The spinal cords of Biozzi ABH mice were isolated during the first or second relapse. Infiltrates containing (A) acid phosphatase$^+$ macrophages, (B) CD3$^+$ T cells, and (C) B220$^+$ B cells were present in the spinal cords of all sham-operated mice (*n*=4), but in only 25% of the lymphadenectomized mice (*n*=4). (D) In addition, loss of myelin was observed in the spinal cords of all Biozzi ABH mice in remission. Scale bars: 10 μm.

Table 1. Clinical features of lymphadenectomized and sham-operated mice.

Clinical feature	Chronic-relapsing EAE		Acute EAE		Chronic EAE	
	Sham	LN	Sham	LN	Sham	LN
Incidence	16/19 (84%)	15/22 (68%)	5/6 (84%)	5/6 (84%)	9/10 (90%)	6/10 (60%)
Day of onset acute disease	19.7±1.6	20.1±2.0	12.0±1.3	12.6±1.4	12.0±0.8	11.2±1.3
Day of onset 1st relapse	32.3±2.5	33.0±2.5				
Day of onset 2nd relapse	40.5±2.5	49.2±3.7				

Results are presented as mean ± SEM.
LN; lymphadenectomized mice.

In addition to the clinical symptoms, the spinal cords of Biozzi ABH mice were assessed for typical EAE pathology, i.e. infiltrates containing macrophages, T cells and B cells [36]. Careful examination revealed that the spinal cords of all sham-operated mice with a first or second relapse contained infiltrates with macrophages, T cells, and few B cells (Figure 2). In contrast, the spinal cord of only one out of four lymphadenectomized mice had infiltrates, indicating that EAE pathology after lymph node excision is less severe. The few animals which were in remission during isolation had minimal infiltrates, although loss of myelin was observed (Figure 2).

To further corroborate the findings in Biozzi ABH mice, we induced acute EAE in SJL/J mice, which demonstrate a relatively short disease period of approximately ten days, as well as chronic EAE in C57BL/6 mice, which show clinical EAE symptoms for approximately four weeks. EAE-affected lymphadenectomized mice demonstrated similar disease severity during acute (SJL/J) and chronic EAE (C57BL/6) as the EAE-affected sham-operated mice (Figure 3A and B).

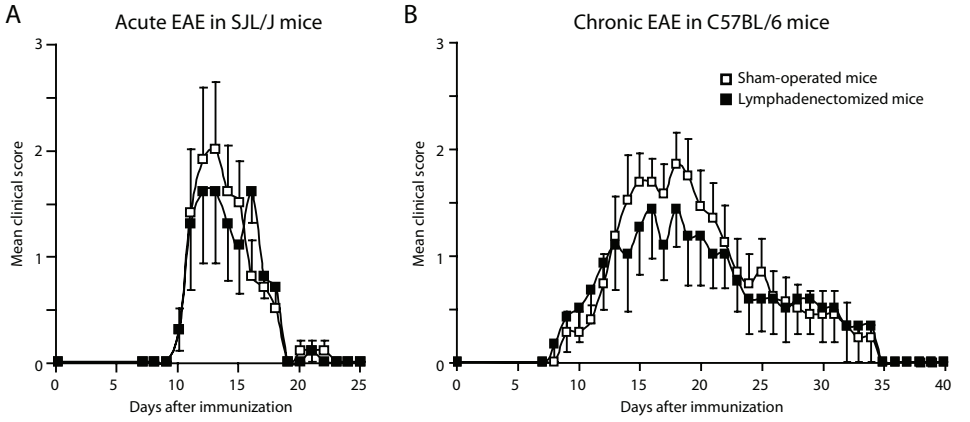

Figure 3. Similar disease severity after lymphadenectomy in acute and chronic EAE models. (A) Superficial and deep cervical lymph nodes were surgically removed from SJL/J mice (n=6) prior to EAE induction by immunization with $PLP_{139-151}$ to induce acute EAE. Disease severity was similar as compared to sham-operated mice (n=6). Results are representative for two independent experiments. (B) Superficial cervical lymph nodes, deep cervical lymph nodes and lumbar lymph nodes were surgically removed from C57BL/6 mice (n=10) prior to EAE induction with MOG_{35-55} to induce chronic EAE. Lymphadenectomized mice demonstrate a similar to slightly reduced disease severity as compared to sham-operated mice (n=10). Mean group scores from diseased mice are indicated ± SEM.

Epitope spreading against different myelin antigens in the deep cervical lymph nodes as well as the lumbar lymph nodes

The clinical and pathological data suggest that CNS antigen-specific immune responses are at least partly initiated within the CNS-draining lymph nodes. We therefore assessed proliferation against myelin peptides within deep cervical lymph nodes, superficial cervical lymph nodes, lumbar lymph nodes and spleens from C57BL/6 mice with chronic EAE. As expected, all CNS-draining secondary lymphoid organs showed proliferation towards the immunizing peptide MOG_{35-55} (Table 2). Furthermore, proliferative responses towards PLP_{57-70}, myelin associated glycoprotein $(MAG)_{97-112}$ and MBP_{12-26} peptides were observed in the deep cervical lymph nodes, as well as in the lumbar lymph nodes and the spleen, indicating that intermolecular epitope spreading took place within these CNS-draining secondary lymphoid organs. Interestingly, the lumbar lymph nodes demonstrated the highest stimulation indices, which is in line with the fact that in this EAE model mainly the spinal cord is affected.

The axillary and inguinal lymph nodes showed merely proliferation against the immunizing peptide, indicating that these lymph nodes drained the immunization site and not the CNS. In parallel, the axillary and inguinal lymph nodes of EAE-affected Biozzi ABH mice proliferated against the immunizing peptide MOG_{8-21} (SI 7.4±2.3), and not against PLP_{56-70}, MAG_{97-112}, and MBP_{12-26}. The gut-draining mesenteric lymph nodes, which were used as reference lymph nodes did not show a response against any of the myelin antigens tested, indicating that CNS antigens only evoke specific T cell responses in the CNS-draining lymphoid organs.

Table 2. CNS-draining lymph node cells proliferate against multiple myelin epitopes.

		Superficial CLN	Deep CLN	LLN	Spleen	ALN/ILN	MLN
Immunizing peptide	MOG_{35-55}	2.4±0.4	3.5±0.1	35.0±0.8	3.5±0.1	5.7±0.4	-
Intramolecular spreading	rmMOG	-	-	19.6±5.3	-	nd	-
	MOG_{8-21}	-	-	-	-	nd	-
Intermolecular spreading	PLP_{56-70}	-	-	3.9±0.4	2.5±0.1	nd	-
	$PLP_{139-151}$	-	2.5±0.1	5.9±1.6	-	-	-
	MAG_{97-112}	-	2.4±0.0	5.0±0.2	2.7±0.1	nd	-
	MBP_{12-26}	-	-	2.3±0.3	-	nd	-

Superficial cervical lymph nodes (CLN), deep CLN, lumbar lymph nodes (LLN), spleen, axillary and inguinal lymph nodes (ALN/ILN) and mesenteric lymph nodes (MLN) were isolated from EAE-affected C57BL/6 mice with chronic EAE (n=10) and restimulated with 50 μg/ml of the indicated peptides and protein. Results represent mean stimulation indices (SI) ± SEM of triplicate cultures. SI were calculated by dividing the mean counts per minute from antigen-stimulated cells by the mean counts per minute from untreated cells. An SI > 2 was considered significant. An SI ≤ 2 is indicated as "–". The gut-draining MLN were used as negative control lymph nodes not draining the CNS. nd: not determined.

Similar MOG_{8-21} response in spleens from lymphadenectomized mice and sham-operated mice

Finally, we assessed whether lymphadenectomy affected the proliferative response to the immunizing peptide MOG_{8-21} in the spleens of Biozzi ABH mice with chronic-relapsing EAE, since the spleen would be the obvious candidate organ to take over immune responses from the removed lymph nodes. Figure 4 shows that proliferation against MOG_{8-21} was quantitatively similar in lymphadenectomized mice as compared to sham-operated mice. This demonstrates that the spleen generated a robust T cell response in the absence of the CNS-draining lymph nodes and that the spleen did not compensate for the removed lymph nodes by generating higher T cell proliferative responses.

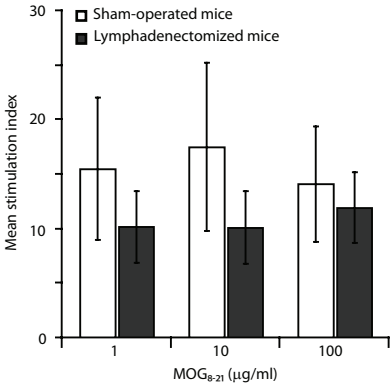

Figure 4. Similar splenic immune reactivity despite lymphadenectomy in chronic-relapsing EAE. Splenocytes from lymphadenectomized Biozzi ABH mice (*n*=5) and sham-operated mice (*n*=6) were restimulated with 1, 10, and 100 μg/ml MOG_{8-21}. Results represent the mean stimulation indices ± SEM from triplicate cultures as compared to unstimulated splenocytes.

Discussion

In the current study, we hypothesized that the CNS-draining lymph nodes contribute to the induction and severity of clinical EAE and that their tolerance-inducing potential is not operational in this model for MS. To test this, we surgically removed the cervical and lumbar lymph nodes prior to EAE induction. This study comprised three different EAE models induced with distinct encephalitogens representing acute, chronic, and chronic-relapsing EAE which allowed us to assess the effect of the lymphadenectomy in different stages of EAE. We demonstrate that excision of CNS-draining lymph nodes modestly reduced and delayed the relapse burden during chronic-relapsing EAE and the severity of EAE pathology within the spinal cord during the relapsing stage. The effect of lymphadenectomy on clinical EAE was reflected by the presence of intermolecular epitope spreading observed in the deep cervical and lumbar lymph nodes from EAE-affected mice, suggesting that CNS antigen-specific immune responses within these lymph nodes contribute to disease.

Our results are in line with previous results from Phillips and colleagues, demonstrating that excision of the deep and superficial cervical lymph nodes reduced the number of brain lesions in a cryolesion-enhanced rat EAE model [49]. This study underscored the role of the cervical lymph nodes in brain lesion expansion, and we now confirm and extend these findings by showing that lymphadenectomy reduced clinical disease as well as pathology within the spinal cord. In addition, our study confirms previous findings, showing that myelin antigen-specific T cell responses are detectable in the cervical lymph nodes of EAE-affected animals [45,47,48,184]. Moreover, we extend these findings to the spinal cord-draining lumbar lymph nodes. Interestingly, these lymph nodes demonstrated higher proliferative responses as compared to the cervical lymph nodes and spleen, reflecting the high lesional burden in the spinal cord of EAE-affected mice. The proliferation data indicate that responses against CNS antigens differ between the CNS-draining lymph nodes from different anatomical sites. Whereas the superficial cervical lymph nodes just showed a response against the immunizing peptide, the deep cervical lymph nodes, the lumbar lymph nodes and the spleen also reacted against additional myelin-derived peptides. Within the spleen, proliferation may be induced by antigen-presenting cells within the spleen that contain antigens drained from the CNS [45], or from recirculating T cells that were activated in the lymph nodes or the CNS. These results strongly suggest that intermolecular epitope spreading occurs and that CNS antigens drain preferably to the deep cervical and lumbar lymph nodes.

Immune responses within the CNS require efferent and afferent pathways. The efferent limb involves traffic of encephalitogenic lymphocytes from the periphery to the CNS. The afferent limb is provided by the transit of antigens from the CNS to the CNS-draining lymph nodes to generate encephalitogenic immune responses [39]. The current study clearly interfered with the afferent pathway and supports the classical view of T cell activation, which state that naïve T cells are primed in the secondary lymphoid organs and subsequently migrate to the CNS to re-encounter

myelin antigens presented by APC within the CNS [75,83,141-144,180,185,186]. However, T cells against the immunizing peptide are also continuously activated by the antigen depot in the axillary and inguinal lymph nodes draining the immunization sites [187]. This may explain the fact that lymphadenectomy does only modestly and not significantly reduce relapse severity. In addition, naïve T cells are also activated within the CNS itself, at least in a transgenic system where all T cells are directed against a PLP epitope [50]. Using this transgenic system, naïve T cell proliferation was not observed the CNS-draining lymph nodes [50], which is in contrast to our results.

Despite the fact that cervical lymph nodes have unique features allowing induction of mucosal tolerance [78,79], our data conclusively show that tolerance against CNS antigens is not controlled by these lymph nodes during EAE. In all three EAE models used, excision of the cervical lymph nodes did not result in higher disease burden, but instead reduced clinical disease in the relapsing phase of EAE.

The current findings support the concept that the CNS-draining lymph nodes are an anatomical target for two reasons. First, targeting the CNS-draining lymph nodes, for instance via the intranasal or oral mucosa routes, provides a non-invasive and convenient therapeutic strategy, which bypasses the blood-brain barrier [188]. These data do not suggest that surgical removal of the CNS-draining lymph nodes would benefit MS patients. Instead, systemic administration of novel therapeutics (e.g. blocking antibodies against cytokines or costimulatory molecules, drugs promoting regulatory T cells) allows direct access to the CNS-draining lymph nodes, thereby allowing application of medication that cannot reach the CNS due to the blood-brain barrier. Second, the cervical lymph nodes provide a special opportunity in view of their specialized microenvironment driving tolerogenic responses upon intranasal delivery [78,79]. Different animal studies tried to induce immunological tolerance against brain-derived peptides by intranasal administration. However, the results were inconsistent and effectiveness therefore remains unclear [189-191]. Understanding the mechanisms operational in the cervical lymph nodes leading to either antigen-specific tolerance or to immune reactivity provides new insights to treat autoimmune diseases such as MS. Such insights are also critical to understand efficacy versus unexpected adverse encephalitogenic events in vaccination strategies for Alzheimer disease [192].

The results of the present study prove that tolerance-generating potential of CNS-draining lymph nodes is not operational in these three distinct EAE models. Instead, our results may suggest that CNS-draining lymph nodes contribute to CNS autoimmunity. Furthermore, we show generation of differential immune responses within the distinct CNS-draining lymphoid organs. In addition to a role for the brain-draining deep and superficial cervical lymph nodes we also show functional relevance of the spinal cord-draining lumbar lymph nodes in EAE.

Experimental procedures

Mice

SJL/J mice were purchased from Janvier (France) and C57BL/6 mice from Harlan (UK). Biozzi ABH (H-2^{dq1}) mice were obtained from stock bred at the Biomedical Primate Research Centre (Rijswijk, the Netherlands). Mice were housed under specified pathogen-free conditions and received water and food *ad libitum*. All animal experiments were performed with approval of the local committees for animal ethics according to national legislation.

Surgical procedures

Mice were anaesthetized using 3-4% isoflurane in O_2. Deep and superficial cervical lymph nodes were removed from SJL/J mice, C57BL/6 mice, and Biozzi ABH mice as previously described [78]. A small incision was made into the skin overlying the salivary gland and the skin was separated from the underlying fascia. With the aid of a surgery microscope, the eight superficial cervical lymph nodes anterior to the salivary gland were removed (Figure 5). Subsequently, the salivary gland was lifted to gain access to the deep cervical lymph nodes, which are located para-tracheally (Figure 5). The two deep cervical lymph nodes were also removed. For the lumbar lymph nodes, we developed the following surgical procedure. An incision was made into the skin and peritoneum. Then, the intestines were retracted to gain access to the lumbar lymph nodes, which are located para-aortically where the abdominal aorta bifurcates into the iliac arteries (Figure 5). All three lumbar lymph nodes were removed from C57BL/6 and Biozzi ABH mice. Subsequently, the intestines were replaced and the incisions were closed using four to six stitches. Mice received 0.1 mg/kg buprenorphine (Temgesic®; Schering-Plough B.V.) subcutaneously as analgesia immediately before surgery. Sham-operated mice were exposed to the exact same procedures as the lymphadenectomized mice, but without removal of the lymph nodes. Mice were allowed at least one week of recovery before EAE was induced. At the end of the experiments, mice were carefully examined for regenerated lymph nodes, or lymph nodes possibly left behind. Although the removed CNS-draining lymph nodes were counted during the surgery and the relevant regions were carefully surveyed to remove all thirteen lymph nodes, one or two CNS-draining lymph nodes could be found at the end of the experiment.

EAE induction

Acute EAE in female SJL/J mice (8-12 weeks old) was induced by subcutaneous injection of 200 μg $PLP_{139-151}$ in complete Freund's adjuvant (CFA; Difco) at four ventral sites in the axillary and inguinal regions as described [193]. Chronic EAE was induced in female C57BL/6 mice (8-12 weeks old) by subcutaneous injection of 200 μg MOG_{35-55} emulsified in CFA at four ventral sites in the axillary and inguinal regions as described [194]. In male and female Biozzi ABH mice (8-12 weeks old), chronic-relapsing EAE was induced by subcutaneous injection of 200 μg MOG_{8-21} emulsified in CFA

at two sites in the flanks, followed by another subcutaneous injection of 200 μg MOG_{8-21} emulsified in CFA at two sites in the flanks seven days after the first injection as described [195].

Mice were weighed and scored for clinical signs daily. For acute EAE in SJL/J mice and chronic EAE in C57BL/6 mice, EAE scores were as follows: 0, no disease; 0.5, partial tail paralysis; 1, complete tail paralysis; 1.5, limb weakness without tail paralysis; 2, limb weakness and tail paralysis; 2.5, partial limb and tail paralysis; 3, complete hind or front limb paralysis; 3.5, paraplegia; 4, quadriplegia; 5, death due to EAE.

For chronic-relapsing EAE in Biozzi ABH mice, EAE scores were as follows: 0, no disease; 1, tail paralysis; 2, impaired righting reflex; 3, paralysis of one hind limb; 4, paralysis of two hind limbs; 5, moribund. Animals exhibiting signs that were less severe than typically observed were scored 0.5 less than the indicated grade. Paralyzed mice with EAE scores above 2 were offered easier access to food and water.

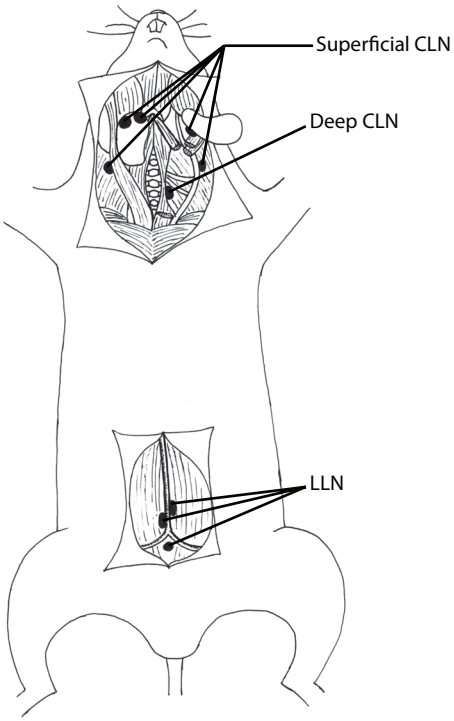

Figure 5. Schematic presentation of CNS-draining lymph nodes. Schematic representation of the cervical lymph nodes (CLN) in the neck region and the lumbar lymph nodes (LLN) in the lower abdominal region. A total of eight superficial cervical lymph nodes (also called submandibular, facial and jugular lymph nodes), the two deep cervical lymph nodes (also called internal jugular lymph nodes), and the three lumbar lymph nodes (also called caudal or sacral lymph nodes) were surgically removed. Hence, a total of thirteen lymph nodes draining the CNS were removed. Adapted from [78,200].

Immuno- and enzyme-histochemistry

Spinal cords of Biozzi ABH mice were snap frozen in liquid nitrogen and stored at -80°C. Immunohistochemistry was performed as described [167,196]. Primary antibodies were rat-anti-B220 (RA-3-6B2; BD Pharmingen), rabbit-anti-CD3 (DAKO) and mouse-anti-MOG IgG2a (Z12 [197]). The primary antibodies were detected by horseradish peroxidase (HRP)-conjugated rabbit-anti-rat Ig (DAKO), biotinylated donkey-anti-rabbit Ig (Amersham) and goat-anti-mouse IgG2a (Santa Cruz), and HRP-conjugated avidin-biotin complex (ABC-HRP; DAKO). HRP activity was revealed with 3-amino-9-ethylcarbazole (Sigma-Aldrich), which resulted in a red precipitate. Spleen sections from the same species were used as positive control tissue. Isotype-matched primary antibodies of irrelevant specificity served as negative controls.

Acid phosphatase, a lysosomal marker of macrophages was detected as described [198] using naphthol-ASBI-phosphate (Sigma) as the substrate, yielding a bright red color.

Proliferation assays

To assess antigen-specific proliferation in lymphoid organs from C57BL/6 mice with chronic EAE, spleen, CNS-draining lymph nodes, inguinal and axillary lymph nodes, and mesenteric lymph nodes were aseptically isolated four to five weeks after EAE induction. Single cell suspensions were obtained by passing the tissue through a 70 μm gauze. After red blood cell lysis with Gey's lysing buffer, cells were resuspended in RPMI 1640 (Lonza) supplemented with 5% fetal bovine serum (Lonza), 100 units/ml penicillin, and 100 μg/ml streptomycin (Lonza) and 50 μM β-mercaptoethanol (Gibco). To assess intramolecular and intermolecular epitope spreading, $2x10^5$ cells were plated in 96-well roundbottom plates (Nunc) and stimulated with 50 μg/ml recombinant mouse MOG (rmMOG, residues 1-116) [199], $PLP_{139-151}$ (Isogen Lifescience), PLP_{56-70}, MOG_{8-21}, MOG_{35-55}, MBP_{12-26}, and MAG_{97-112} (all from ABC Biotechnology). Cells were stimulated with 10 μg/ml phytohaemagglutinin (PHA; Sigma) as a positive control for T cell responsiveness. Proliferation in the absence of peptide served as a negative control. After four days, T cell proliferation was determined by incorporation of [^{3}H]-thymidine (Amersham Biosciences) for 18 h as described [165].

To assess the effect of lymphadenectomy on proliferation against the immunizing peptide MOG_{8-21} in spleens, the spleens from lymphadenectomized and sham-operated Biozzi ABH mice with chronic-relapsing EAE were isolated. Spleen cells were obtained as described above and cultured in RPMI 1640 supplemented with 2% normal mouse serum, 2 mmol/L glutamine (Gibco), 100 units/ml penicillin, and 100 μg/mL streptomycin and 50 μM β-mercaptoethanol. Cells were stimulated as described above with MOG_{8-21}.

Stimulation indices were calculated by dividing the mean counts per minute from antigen-stimulated cells by the mean counts per minute from untreated cells. A stimulation index (SI) > 2 was considered significant. Cultures were performed in triplicate.

Statistical analysis

Differences between groups were statistically analyzed by an exact two-tailed Mann-Whitney *U*-test and a Chi square test using the statistical software program SPSS, version 11.0 and with a variance analysis for repeated measurements using the statistical analysis software SAS. A significance level of $p<0.05$ was used.

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Part III

Myelin-laden macrophages in MS and EAE

Part III

Myelin-laden macrophages in MS and EAE

Chapter | 4

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

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Submitted for publication

Abstract

Myelin-laden macrophages are abundant in the CNS and in the CNS-draining lymph nodes of patients with MS, and acquire anti-inflammatory features by ingesting myelin. However, the exact function of these cells remains elusive. Since chemokines and chemokine receptors are pivotal for the interplay between leukocytes and their trafficking during MS and EAE, the question was addressed whether myelin ingestion regulates chemokine receptor expression and chemokine production by macrophages *in vitro*. We demonstrate that uptake of myelin by mouse bone marrow-derived macrophages enhanced surface protein expression of CCR7 and CXCR3, two critical chemokine receptors involved in leukocyte trafficking into and out of the CNS. Concordantly, myelin-laden macrophages showed increased migration towards the corresponding ligands CCL21 and CXCL10. Myelin ingestion also resulted in a slightly higher mRNA expression of the inflammatory chemokines CCL2, CCL3 and CCL4, which are key chemokines in the recruitment of leukocytes into the CNS. In parallel, myelin-laden macrophages attracted significantly more myeloid cells, but not lymphocytes, as compared to control macrophages. Our *in vitro* data suggest that myelin-laden macrophages migrate within the CNS and to the CNS-draining lymph nodes, and contribute to myeloid cell recruitment into the CNS *in vivo*, thereby contributing to the regulation of CNS inflammation.

Introduction

Myelin-laden macrophages are abundantly present in CNS lesions of patients with MS and animals with EAE. They are derived from CNS-resident microglia and infiltrating monocytes [111] and have ingested large amounts of myelin debris. Although they were previously considered as aggressors during MS [201,202], there is increasing evidence that both human and mouse myelin-laden macrophages have an anti-inflammatory phenotype *in situ* [120]. Moreover, ingestion of myelin by macrophages and microglia *in vitro* and *ex vivo* mimics this phenotype [120-122]. In addition to the CNS, myelin-laden macrophages are present in the CSF of MS patients [203,204] and secondary lymphoid organs of MS patients and EAE-affected animals [45,46], suggesting that these cells are highly motile and apt at migrating between and within anatomical compartments. However, 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. Therefore, we here addressed the question whether myelin ingestion by macrophages results in altered expression of chemokines, chemokine receptors and responsiveness to the corresponding ligands.

MS is characterized by complex orchestration of leukocyte movement into and out of inflamed CNS tissue [3], which is regulated by interactions between chemokines and their receptors. Two chemokine receptors are of main interest, namely CCR7 and CXCR3. In the periphery, CCR7 is upregulated by maturing antigen-presenting cells. The ligands CCL19 and CCL21 are produced by lymphatic endothelial cells and interdigitating dendritic cells within the lymph nodes, thereby facilitating migration of antigen-presenting cells towards the lymph nodes [53-56]. CCR7 is also expressed by myeloid cells within the CSF of MS patients and within the cervical lymph nodes of rhesus monkeys with EAE [45,155], and the CCR7 ligand CCL19 is elevated in CSF of MS patients [101]. Taken that myelin-laden macrophages are present in the CSF [203,204] and the CNS-draining lymph nodes [45,46], these data suggest that myelin-laden macrophages may emigrate from the inflamed CNS.

The chemokine receptor CXCR3 is widely expressed in MS and EAE by infiltrating T cells and macrophages [97-100]. CXCR3 knock out mice suffer from increased EAE severity [205,206], which is characterized by enhanced demyelination and axonal degeneration, inflammatory lesions throughout the CNS, augmented regulatory T cell recruitment to the CNS [206], worsened blood-brain barrier disruption and increased IFN-γ production by T cells [205]. In contrast, CXCR3 is critical to EAE relapses in chronic-relapsing EAE in rats [99], and blocking the CXCR3 ligand CXCL10 improves clinical signs and histopathology [207].

Leukocytes are recruited by inflammatory chemokines, such as CCL2, CCL3, CCL4, and CCL5 [90-93]. These chemokines are produced by brain endothelial cells [91,92,208], facilitating recruitment to the blood-brain barrier. In addition, myelin-laden macrophages within the perivascular spaces also express these chemokines [208,209], suggesting that these macrophages attract leukocytes into the perivascular spaces.

Since myelin-laden macrophages have an anti-inflammatory phenotype, we have proposed that they are involved in the resolution of inflammation in MS [120]. However, the exact function of these macrophages remains elusive. We here demonstrate that myelin ingestion results in increased expression of CCR7 and CXCR3, and, consequently, increased migration of macrophages towards CCL21 and CXCL10. In addition, myelin-laden macrophages demonstrated enhanced recruitment of myeloid cells, but not lymphocytes.

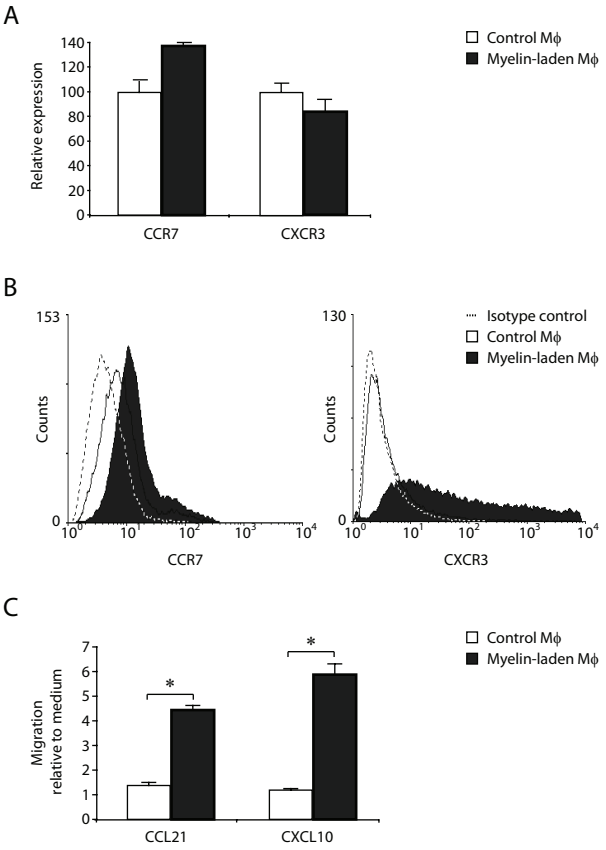

Figure 1. Myelin ingestion enhances chemotaxis of macrophages towards CXCL10 and CCL21. Bone marrow-derived mouse macrophages (Mϕ) were incubated with 25 μg/ml human myelin for 48 to 72 h. (A) CXCR3 and CCR7 mRNA expression as mean ± standard deviation of duplicates within one experiment. mRNA expression 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).

Results

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. Since myelin-laden macrophages are most abundant in CNS lesions of MS patients and EAE-affected animals, the current study 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 therefore 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 (Figure 1A), there was a pronounced increase in CCR7 and CXCR3 surface protein expression (Figure 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 also enhanced the responsiveness to their ligands. Chemotaxis assays were used to determine this responsiveness, which 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 (Figure 1C), indicating that the large quantities of myelin ingested by macrophages did not perturb the motility of these cells

Myelin uptake by macrophages promotes their capacity to attract myeloid cells

Myelin-laden macrophages in CNS lesions are optimally positioned to attract other leukocytes. In order to contribute to this leukocyte recruitment, the production of chemoattractants is required. It was therefore next assessed whether myelin-laden macrophages secrete inflammatory chemokines to attract other leukocytes. We determined mRNA expression and secretion of the chemokines CCL2, CCL3, CCL4, and CCL5, since it was previously shown that human myelin-laden macrophages in MS brain lesions express these chemokines [208,209], and since these chemokines attract leukocytes into the CNS [90-93]. As shown in Figure 2A, mRNA expression of CCL2, CCL3, and CCL4 is somewhat elevated in myelin-laden macrophages, but not statistically different as compared to control macrophages. 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 also 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 [210], we assessed whether myelin ingestion by macrophages resulted in enhanced functional recruitment of leukocytes. Transwell experiments allowed macrophages to produce and release chemoattractants to attract

leukocytes without cell-to-cell contact. Figure 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 (Figure 2C).

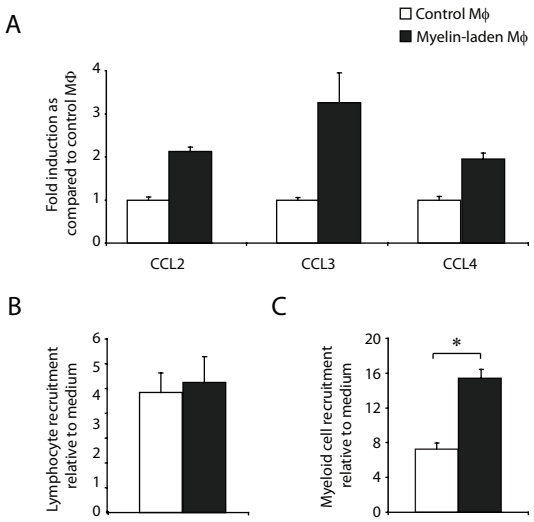

Figure 2. Myelin-laden macrophages recruit more myeloid cells than control macrophages. Bone marrow-derived macrophages (Mϕ) were incubated with 25 μg/ml human myelin for 48 to 72 h. (A) mRNA expression of CCL2, CCL3 and CCL4 by myelin-laden macrophages was slightly increased, but not significantly different, 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 duplicates within one experiment and represent two independent experiments. *p<0.05 (two-tailed Mann Whitney U-test).

Discussion

Although blood-derived macrophages do not reside in the healthy CNS, they are abundantly present in the CNS of MS patients, where they ingest large quantities of myelin debris. Besides the CNS, these myelin-laden macrophages also occur in the secondary lymphoid organs of MS patients, suggesting that these cells are highly motile and apt at migrating between and within anatomical compartments. In the current study, bone marrow-derived myelin-laden mouse macrophages show increased expression of the chemokine receptors CXCR3 and CCR7 and, in parallel, enhanced migration towards the corresponding chemokines CXCL10 and CCL21 *in vitro*. CCR7 is key to direct antigen-presenting cells from inflamed tissue to lymphoid organs [53-56]. CXCR3 was assessed since this receptor is widely expressed in MS lesions and plays an important role CNS inflammation during EAE [97-100,206].

The fact that myelin ingestion by macrophages promotes their responsiveness to CXCL10 and CCL21 may have several implications. First, myelin-laden macrophages might emigrate from the CNS to the CNS-draining lymph nodes via a CCR7-dependent mechanism. CCL21, the ligand for CCR7, is constitutively expressed by secondary lymphoid organs and by endothelial cells of afferent lymph vessels, controlling the homing of antigen-presenting cells and of naïve T cells [54,55]. 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*, supporting the hypothesis that myelin-laden macrophages migrate from the CNS to the CNS-draining lymph nodes. Our data are in line with the observations that myeloid cells within the cerebrospinal fluid and myelin-laden macrophages in the CNS-draining lymph nodes express CCR7 [45, 155].

Second, elevated CXCR3 expression by myelin-laden macrophages may contribute to the presence of myelin-laden cells in the CSF of MS patients [204], since CXCL10 levels within the CSF are significantly elevated during active disease [211]. In addition, CXCL10 is produced by reactive astrocytes in MS lesions and in EAE [100,212], suggesting interaction between astrocytes and myelin-laden macrophages. The function of CXCR3 in EAE is unclear, as different studies gave contradictory results [99,205-207,213]. However, CXCR3 expression by macrophages was merely found in MS patients who suffered from secondary progressive MS [100], suggesting that expression of CXCR3 by macrophages might be involved in the worsening of disease.

Third, distressed neurons express and upregulate CXCL10 and CCL21 *in vivo* [166,214,215] and *in vitro* [215-218], although this has not yet been found in MS lesions. Since microglia express the corresponding chemokine receptor CXCR3, it has been suggested that microglia interact with neurons via these chemokines. Indeed, CXCR3 deficiency reduced microglial activation and neuronal loss in the entorhinal cortex lesion model [166], strongly supporting this hypothesis. Myelin-laden macrophages reside in CNS parenchyma adjacent to damaged neurons. Since myelin-laden macrophages in MS have anti-inflammatory properties [120], it is tempting to speculate that

myelin-ingestion by macrophages might promote communication with distressed neurons in MS, resulting in neuroprotection [219].

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. 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 fact that myelin-laden macrophages within the perivascular spaces express CCL2, CCL3, CCL4, and CCL5 [208,209], suggesting that they attract leukocytes into the perivascular spaces. This concept has previously been suggested for astrocytes [220,221], and is supported by the fact that myeloid cells within the perivascular cuffs of MS brain lesions express the chemokine receptors CCR2 and CCR5 [97,222].

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. Considering the anti-inflammatory phenotype of myelin-laden macrophages [120] and their ability to recruit myeloid cells, this implies that myelin-laden macrophages might exert their anti-inflammatory action through interaction with other cells. The results in the present study therefore provide further insight into the trafficking capacity of anti-inflammatory myelin-laden macrophages in MS.

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

Experimental procedures

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 $1x10^7$ cells/petri dish in DMEM (Cambrex) supplemented with 10% heat-inactivated FCS (Cambrex), 50 μM β-mercaptoethanol (Sigma), 100 units/ml penicillin and 100 μg/ml streptomycin (Cambrex). Bone marrow cells were differentiated into macrophages by stimulation with 10 ng/ml recombinant mouse M-CSF (Peprotech) on day one, and 5 ng/ml recombinant mouse M-CSF on day three. After seven days, differentiated macrophages were incubated with 25 μg/ml human myelin during 48 to 72 h. Macrophages which were not fed with myelin served as control macrophages. Cells were harvested by scraping them after incubation on ice in PBS/2mM EDTA for 30 min and used for further analysis.

Real time Quantitative PCR

mRNA expression was quantified using real-time quantitative reverse-transcription PCR as described [120]. 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.

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 analysed by CellQuest software (Becton Dickinson).

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 only 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 the cells within the pores using a 10x brightfield objective and the Cell Counter plugin from Image J biomedical image analysis software [223]. Chemokine-specific migration was calculated by normalizing for basal levels of migration. All experiments were performed in triplicate.

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.

Transwell migration assay

Migration of lymphocytes and myeloid cells towards myelin-laden macrophages was assessed using a transwell migration assay. $1x10^6$ 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.

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, $1x10^5$ 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. 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 duplicate. Migration towards medium without macrophages served as negative control.

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.

Acknowledgements

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Chapter | 5

Myelin-laden macrophages suppress Th1 differentiation and suppress experimental autoimmune encephalomyelitis

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Abstract

During MS, autoreactive T cells are (re)activated either in secondary lymphoid organs, in perivascular spaces within the CNS, or in the CNS parenchyma. Phagocytozing myelin-containing macrophages reside in all three compartments in close proximity to T cells. To date, it has not been addressed whether myelin-laden macrophages have the capacity to present antigens to T cells and whether this contributes to inflammation in disease. We demonstrate that human and mouse myelin-laden macrophages generated *in vitro* expressed MHC class I, MHC class II, and costimulatory molecules and are thus well equipped for antigen-presentation. Human myelin-laden macrophages exhibited normal endocytosis of particulate and soluble antigens, but showed quantitative differences in processed antigen at different time points. Increased myelin ingestion by human macrophages resulted in a dose-dependent increment in antigen-presenting capacity, enhancing proliferation of naïve as well as memory T cells. In addition to these *in vitro* data, we show that mouse myelin-laden macrophages induced naïve antigen-specific $CD4^+$ T cell proliferation *in vivo* and suppress Th1 differentiation. Functionally, MOG-loaded myelin-laden mouse macrophages reduced the severity of MOG-induced EAE. These data show that myelin uptake results in the induction of a population of regulatory macrophages, which protects against autoimmune-mediated disease by modulating T cell responses to CNS antigens. Such a regulatory function of these cells sheds a new light on the role of these macrophages in EAE and MS as these cells were previously considered as the primary aggressors in the development of disease.

Introduction

Myelin-laden myeloid cells reside in the parenchyma of the CNS, in perivascular spaces and within the brain-draining cervical lymph nodes during multiple sclerosis, where they are thought to present auto-antigens to autoreactive T cells [50,141-144,180]. In these different anatomical locations, distinct APC subsets can be identified in humans, monkeys and mice. Within the CNS parenchyma, the APC subsets include CD11b$^+$ microglia [143], DC-SIGN$^+$ dendritic cells [224], and CD163$^+$ macrophages [120,224]. In the perivascular spaces, the APC are characterized by the dendritic cell markers CD11c, CD1a, and DC-SIGN [144,225], and the macrophage markers CD68, CD11b and CD163 [120,224]. In addition, both DC-SIGN$^+$, and CD83$^+$ myelin-laden dendritic cells, as well as acid phosphatase-containing and CD68$^+$ myelin-laden macrophages are present within the cervical lymph nodes of MS patients [45,46], and of marmosets and rhesus monkeys with EAE [45].

The different contributions of these distinct APC subsets to (re)activation of encephalitogenic T cells upon myelin phagocytosis are currently unclear. APC activity has mostly been explored in terms of encephalitogenic activity. For instance, both CD11c$^+$ dendritic cells as well as CD11b$^+$ microglia contribute to EAE [16,50,143,144,226]. Recently, we have demonstrated that myelin-laden phagocytosing macrophages (foam cells) have anti-inflammatory function *in vivo* and *in vitro* [120]. These macrophages express various anti-inflammatory molecules *in vivo*, including TGF-β, IL-1 receptor antagonist, IL-10, and CCL18, which strongly suggests a local immunosuppressive mode of action. Furthermore, myelin-laden macrophages upregulate the anti-inflammatory molecules CCL18 and prostaglandin E_2 synthase *in vitro* and do not respond to pro-inflammatory stimuli such as LPS [120]. Within the paradigm of classically (M1) versus alternatively activated (M2) and regulatory macrophages, these myelin-laden macrophages are most reminiscent of the alternatively activated and regulatory macrophages [104,106,227]. We have therefore proposed that myelin-laden macrophages are involved in the resolution of inflammation and tissue repair [120]. Interestingly, myelin-laden macrophages express MHC class II and costimulatory molecules *in situ* [45,120,224,228] and are in close proximity to T cells [45,225,228], suggesting interaction between myelin-laden macrophages and T cells. Instead of stimulating the encephalitogenic process as suggested previously [112-114,143,202], this interaction may also contribute to regulation of T cell activity and diminished encephalitogenic potential.

Until now, it is unclear whether cognate interactions occur between the anti-inflammatory myelin-laden macrophages and T cells. We have therefore addressed the question whether myelin-laden macrophages, that were generated *in vitro*, are capable of inducing T cell responses and if so, whether this process would affect differentiation of functional T cell subsets. Considering the anti-inflammatory phenotype of myelin-laden macrophages in MS patients, we hypothesized that myelin-laden macrophages modulate T cell function, resulting in a protective response.

Results

Myelin-laden macrophages express MHC class I, MHC class II and costimulatory molecules

To confirm that differentiation of human blood-derived monocytes and mouse bone marrow generated macrophages, the expression of the macrophage markers CD68 and CD11b was determined by flow cytometry. Almost all monocyte-derived human macrophages (99.2±0.2%) expressed CD68 (Figure 1A), and the majority of mouse bone marrow-derived macrophages were $CD11b^+$ (82.1±7.6%) (Figure 1B), indicating that these cells were indeed macrophages.

To determine whether human myelin-laden macrophages are equipped to present antigens to T cells, the expression levels of MHC class I and II, and costimulatory molecules on *in vitro*-generated myelin-laden macrophages were assessed. In a series of experiments with different healthy donors, we observed that myelin ingestion modestly but significantly increased the expression level of MHC class I and CD80, whereas we consistently found similar expression levels of MHC class II and the costimulatory molecule CD40 (Figure 1A) by myelin-laden macrophages as compared to control macrophages (Figure 1A, Table 1). The expression levels of the costimulatory molecules CD86 (Figure 1A) and PD-L1 varied between donors and were not significantly different (Table 1).

In parallel with human myelin-laden macrophages, mouse myelin-laden macrophages also showed a significant increase in expression levels of MHC class I and CD80 (Figure 1B). In addition, MHC class II was upregulated, whereas the expression levels of CD40, CD86 (Figure 1B), PD-L1 and ICOS-L (data not shown) by myelin-laden macrophages were similar as control macrophages (Table 1). These data indicate that abundant myelin phagocytosis did not lead to shedding or downregulation of surface molecules that are pivotal for antigen-presentation.

Myelin ingestion does not affect subsequent endocytosis of particulate and soluble antigens

Since abundant amounts of myelin stored within the myelin-laden macrophage could hamper de novo uptake and processing of antigens, we determined whether myelin ingestion affects endocytosis of both particulate and soluble antigens by human macrophages. Myelin-laden macrophages endocytozed FITC-labeled polystyrene beads (1.0 μm diameter), used as particulate antigen, equally well as control macrophages (Figure 2A). Also FITC-labeled dextran (40 kDa) and OVA protein, used as soluble antigens, were endocytozed in similar quantities by myelin-laden macrophages and control macrophages (Figure 2B, C), indicating that myelin ingestion did not affect the endocytosis of additional antigens.

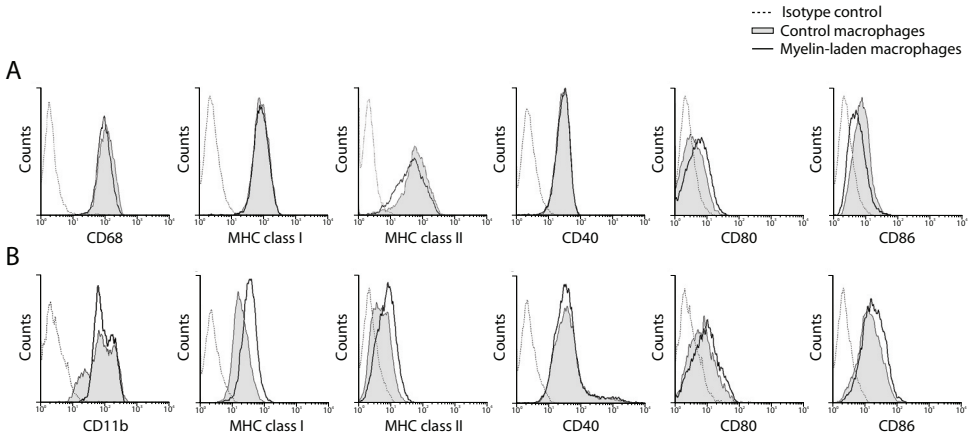


Figure 1. Myelin ingestion by macrophages increases expression of MHC class I and II and CD80. The expression of CD68, CD11b, MHC class I, MHC class II, and costimulatory molecules by monocyte-derived human macrophages (A) and bone marrow-derived mouse macrophages (B) was determined by flow cytometry. Dotted lines represent isotype controls, bold lines represent myelin-laden macrophages and shaded areas represent control macrophages, which were not incubated with myelin. Results are representative for one to four separate experiments with cells from different donors.

Table 1. Relative expression levels of MHC-class I and II, and costimulatory molecules by myelin-laden macrophages as compared to control macrophages

	Relative expression as compared to control macrophages	
Marker	Human	Mouse
MHC class I	1.07±0.05*	1.71±0.14*
MHC class II	0.85±0.15	1.38±0.07*
CD40	1.02±0.01	0.97
CD80	1.73±0.11*	1.72±0.38*
CD86	1.41±0.45	1.18±0.14
PD-L1	1.15±0.25	1.59±0.42
ICOS-L	n.d.	1.36±0.18

The relative mean fluorescence intensity (MFI) was calculated by dividing the MFI of myelin-laden macrophages by the MFI of control macrophages. Data are presented as the mean relative MFI ± SEM. The results are representative for one to five separate experiments with different donors. *p<0.05 (two-tailed Mann-Whitney U-test).

In vitro generation of myelin-laden macrophages resulted in a heterogeneous population consisting of macrophages that ingested different quantities of myelin. To assess whether large quantities of ingested myelin perturb phagocytosis, the macrophages were incubated with DiI-labeled myelin, allowing monitoring the quantity of myelin ingested by the macrophages. We defined cells that were strongly positive, weakly positive, or negative for DiI (Figure 2D). Myelin-laden macrophages were subsequently incubated with fluorescent beads. Figure 2E-F shows that the quantity of phagocytozed beads correlated with the quantity of ingested myelin. Myelin-laden macrophages that were strongly positive for DiI and thus had ingested large quantities of myelin, had also phagocytozed the highest number of beads (Figure 2E). Ingestion of large quantities of myelin therefore did not perturb the phagocytic capacity of the cells.

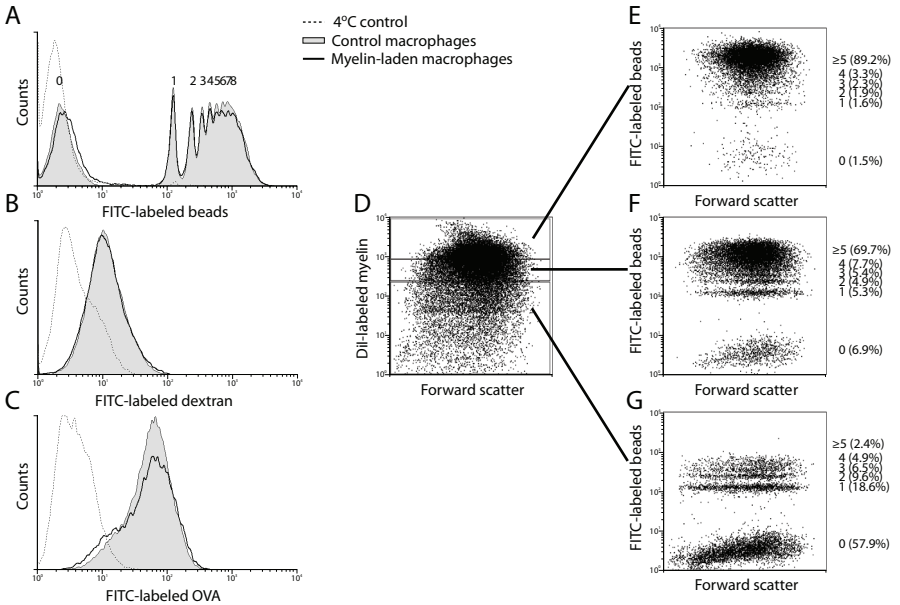


Figure 2. Myelin-laden macrophages demonstrate normal endocytosis. Human monocyte-derived myelin-laden and control macrophages were incubated with (A) FITC-labeled polystyrene beads (1.0 μm diameter), (B) FITC-labeled dextran (40 kDa) or (C) FITC-labeled OVA for 30 min to determine the endocytosis capacity. Endocytosis was determined by flow cytometry. The numbers in (A) represent the number of endocytosed beads reflected by the separate peaks. (D) Macrophages were fed with DiI-labeled myelin to determine the quantity of ingested myelin. Subsequently, we defined macrophages that ingested a (E) high quantity of myelin, (F) little myelin or (G) no myelin to determine the endocytosis capacity of FITC-labeled polystyrene beads in these three populations. The numbers in E-G represent the numbers of endocytosed beads and the percentage of cells which ingested this number of beads. Dotted lines represent negative controls, bold lines represent myelin-laden macrophages and shaded areas represent control macrophages. Results are representative for three separate experiments with cells from different donors.

Myelin-laden macrophages process antigen less efficiently than control macrophages

In addition to the endocytosis capacity, the capacity of myelin-laden macrophages to process antigens was assessed. To this end, macrophages were incubated with DQ-conjugated OVA. DQ is a self-quenching conjugate, which becomes fluorescent upon hydrolysis by proteases. A fluorescent signal therefore indicates proteolytic processing of OVA. The cells were allowed to process OVA for 30, 60, or 90 min to determine the quantity of processed antigen at different time points. Figure 3A shows that myelin-laden macrophages did not process as much OVA as control macrophages after 30 min of processing. After 60 and 90 min however, the quantity of OVA processed was similar between myelin-laden and control macrophages (Figure 3B, C). These data suggest that myelin-laden macrophages have slower processing kinetics than control macrophages.

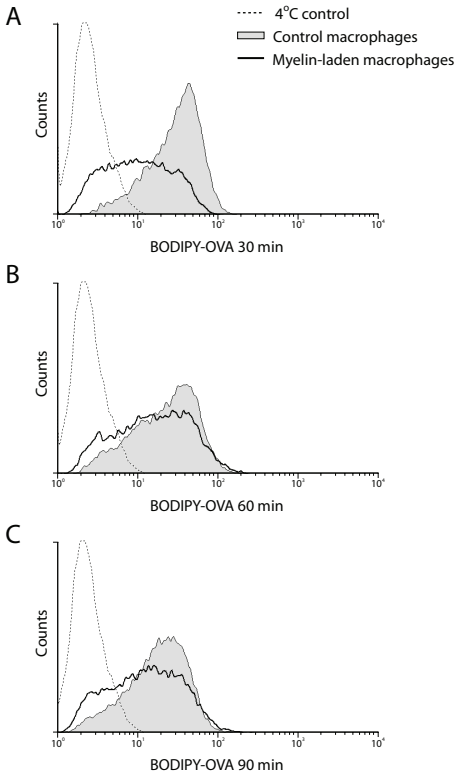


Figure 3. Processing kinetics are slower in myelin-laden macrophages. Human macrophages were incubated with DQ-conjugated OVA protein for 15 min after which the macrophages were allowed to process OVA protein for (A) 30, (B) 60, and (C) 90 min. Processing of DQ-conjugated OVA by myelin-laden and control macrophages was determined by flow cytometry. Dotted lines represent negative controls, bold lines represent myelin-laden macrophages and shaded areas represent control macrophages. Results are representative for three separate experiments with cells from different donors.

Human myelin-laden macrophages activate both naïve and memory T cells

As these data establish that myelin-laden macrophages have antigen-presenting equipment, and endocytozing and processing capacity, the question was addressed whether myelin-laden macrophages are capable of inducing T cell responses. Human naïve T cell activation by human myelin-laden macrophages was determined in an allogeneic MLR. Human T cells were coincubated for five days with human myelin-laden or control macrophages from another donor. T cell proliferation was determined by adding [^{3}H]-thymidine for the last 18 h of coculture. This revealed that T cells incorporated [^{3}H]-thymidine, indicating that myelin-laden macrophages were fully able to promote T cell proliferation. The quantity of ingested myelin dictated the extent of T cell activation, since increased myelin uptake correlated with enhanced T cell proliferation (Figure 4A). This phenomenon was not restricted to MHC class I or II as myelin-laden macrophages stimulated proliferation of both CD4$^+$ and CD8$^+$ T cells more than control macrophages as determined by BrdU incorporation (Figure 4B).

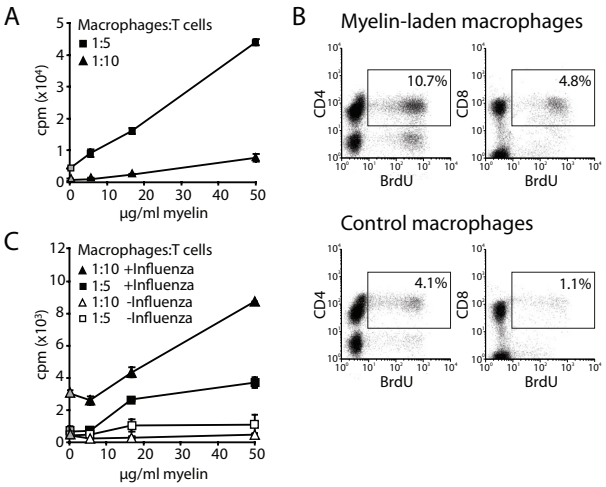


Figure 4. Myelin ingestion promotes APC function. (A) Myelin ingestion by human macrophages promoted naïve human T cell proliferation dose-dependently in an allogeneic mixed leukocyte reaction. Naïve human T cells were coincubated for five days with human myelin-laden macrophages from another donor in different macrophage:T cell ratios. [^{3}H]-thymidine was added during the last 18 h of coincubation (B) Both CD4$^+$ and CD8$^+$ naïve T cells were activated by myelin-laden macrophages in an allogeneic mixed leukocyte reaction. BrdU was added the last 48 h of coincubation. Incorporation of BrdU was determined using flow cytometry. (C) Increased myelin ingestion by human macrophages enhanced antigen-specific human T cell proliferation dose-dependently in an influenza recall response. Myelin-laden or control macrophages were loaded overnight with influenza vaccine. Subsequently, human T cells were incubated with the macrophages from the same donor in different macrophage : T cell ratios. [^{3}H]-thymidine was added during the last 18 h of coincubation. Results are presented as the mean ± standard deviation of triplicates and representative for at least five separate experiments with cells from different donors. Grey-colored rectangles and triangles represent control macrophages.

Allogeneic MLR responses are multifactorial, driven by many epitopes and hence do not properly reflect physiological T cell activation. Therefore, antigen-specific T cell activation by myelin-laden macrophages was investigated using an influenza recall response. During an influenza infection, immunological memory is established by $CD4^+$ and $CD8^+$ T cells [229]. Since the majority of the human population has been infected with influenza, we made use of this phenomenon in an influenza recall response. Macrophages were loaded with proteins from an influenza vaccine and subsequently coincubated with T cells from the same donor to assess proliferation against these influenza proteins. Figure 4C shows that myelin-laden macrophages also induced influenza-specific T cell proliferation. Similar to the allogeneic MLR, the quantity of ingested myelin correlated with the enhanced extent of T cell proliferation. Thus, myelin ingestion by human macrophages dose-dependently promoted their T cell activation capacity both in the setting of an allogeneic MLR and in a recall response against influenza virus.

Mouse myelin-laden macrophages suppress Th1-like T cell differentiation in vivo

Having demonstrated that myelin-laden macrophages stimulate human T cell function *in vitro*, we sought to assess their antigen-presenting function *in vivo*. In addition, we addressed the question whether T cells activated by myelin-laden macrophages undergo a specific functional differentiation. To this end, CFSE-labeled, OVA-TCR transgenic $CD4^+$ cells were transferred into naïve BALB/c mice. After 24 h, OVA protein-pulsed myelin-laden and control mouse bone marrow-derived macrophages were injected into the hind footpad. Three days later, the draining popliteal lymph nodes were isolated and dividing T cells were analyzed with respect to number of cell divisions and cytokine profile. Figure 5A demonstrates that myelin-laden macrophages induced OVA-specific T cell proliferation *in vivo*. The number of dividing T cells (67.5±17.6%) and the number of cell divisions was comparable to that induced by OVA-pulsed control macrophages (74.9±5.7%), indicating that the kinetics of division was similar.

In contrast to cell division, myelin-laden macrophages induced altered T cell differentiation. After 24 h of restimulation with OVA protein, lymph node cells from mice injected with myelin-laden macrophages produced less IL-10 and IFN-γ as compared to lymph node cells from mice injected with control macrophages ($p<0.05$). This decrease in cytokine production was less pronounced after 48 h and 72 h of restimulation. In contrast to IL-10 and IFN-γ, similar levels of IL-17A were released at all three time points. IL-4 could not be detected in the supernatants of the restimulated cells. Myelin-laden macrophages thus suppressed Th1 differentiation *in vivo*, but did not affect Th17 differentiation.

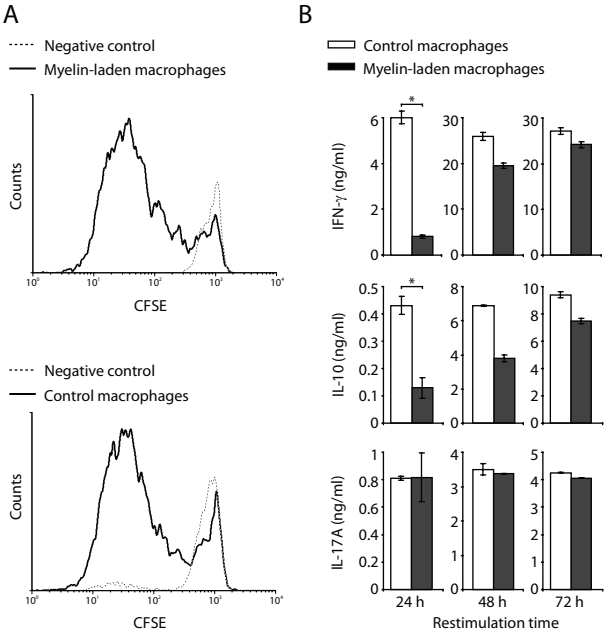
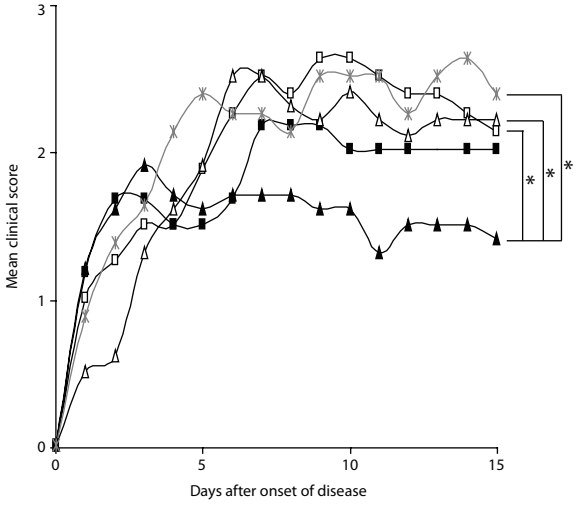


Figure 5. Myelin-laden macrophages skew T cell cytokine production *in vivo*. (A) BALB/c mice were injected intraveneously with 5x10^6 CFSE-labeled DO11.10 T cells one day before the mice received 2.5x10^6 OVA-pulsed myelin-laden or control mouse bone marrow-derived macrophages in the hind footpad. Three days later, CFSE profiles of CD4$^+$ DO11.10 T cells in the popliteal lymph nodes were determined using flow cytometry. Dotted lines represent T cell proliferation induced by macrophages which were not loaded with OVA, serving as negative control. Myelin-laden and control macrophages induced similar T cell proliferation *in vivo* (67.5±17.6% and 74.9±5.7%, respectively). Results are representative for two independent experiments. (B) Popliteal lymph node cells were restimulated *in vitro* during 24 h, 48 h and 72 h with OVA protein. Subsequently, supernatants were harvested and cytokine production was determined using ELISA. Results are presented as mean ± standard deviation of duplicates within the ELISA and are representative for two separate experiments. *p<0.05 as compared to control macrophages (two-tailed Mann-Whitney *U*-test).

Myelin-laden macrophages reduce EAE severity

Next, it was assessed whether the *in vivo* naïve T cell skewing by mouse myelin-laden macrophages away from the Th1 subset could accordingly limit EAE. Since myelin-laden macrophages affected differentiation of naïve T cells, we used an early treatment to allow interaction with naïve T cells reactive against MOG_{35-55}. Mouse myelin-laden or control bone marrow-derived macrophages were pulsed with MOG_{35-55}, and injected intraveneously three days before, during and four days after immunization. Mice treated with unpulsed macrophages, and mice treated with PBS served as negative controls. As shown in Figure 6, all these control mice showed a similar disease severity as expected. Mice treated with MOG_{35-55}-pulsed control macrophages showed a somewhat modest suppression of disease as compared to the negative controls, but this difference was not significant. In contrast, MOG_{35-55}-pulsed myelin-laden macrophages reduced EAE severity

significantly as compared to unpulsed macrophages and PBS (Figure 6; $p<0.05$). This was also reflected by the lowest area under the curve (Figure 6). In addition, MOG_{35-55}-pulsed myelin-laden macrophages delayed the onset of disease significantly (Figure 6; $p<0.05$). These data suggest suppression of Th1 differentiation by myelin-laden macrophages during the priming phase limits EAE *in vivo*.



Treatment	AUC	Day of onset
PBS	29.1 ± 2.7	10.5 ± 0.8*
Unpulsed control macrophages	32.7 ± 1.5	9.5 ± 0.69*
Unpulsed myelin-laden macrophages	28.1 ± 1.4	13.2 ± 2.0
MOG_{35-55}-pulsed control macrophages	31.3 ± 2.6	9.8 ± 0.5*
MOG_{35-55}-pulsed myelin-laden macrophages	24.1 ± 3.6	16.1 ± 0.7

Figure 6. Myelin-laden macrophages reduce EAE severity and delay the onset of disease. Intravenous injection of $1x10^6$ MOG_{35-55}-pulsed myelin-laden mouse bone marrow-derived macrophages three days before immunization, on the day of immunization itself and four days after immunization reduced EAE severity significantly as compared to mice which received unpulsed macrophages or PBS. Results from a representative experiment are shown as mean EAE scores. Data in the table are compiled of two separate experiments using five and ten animals per group and showing similar results. The data represent the mean area under the curve (AUC) and the mean onset of disease ± SEM. *$p<0.05$ as compared to MOG-pulsed mouse myelin-laden macrophages (variance analysis for repeated measurements SAS and two-tailed Mann-Whitney *U*-test).

Discussion

Inflammation in MS and EAE is considered to be driven by an excess of inflammatory autoreactive T cells. Since these T cells are instructed by APC, there is increasing attention for therapeutic strategies aimed at generating regulatory APC that will skew T cell towards a protective function. We here demonstrate that such protective regulatory APC are likely to be generated in MS as a result of CNS damage and subsequent phagocytosis of myelin debris.

Myelin-laden macrophages reside within CNS lesions, perivascular spaces and the brain-draining lymph nodes of MS patients, three critical locations of autoreactive T cell activation. Myelin-laden macrophages *in vivo* as well as *in vitro* have anti-inflammatory properties [120-122]. We therefore assessed whether interaction between myelin-laden macrophages and T cells results in differentiation of a specific T cell subset, and whether this suppresses or enhances the development of EAE. We demonstrate that myelin-laden macrophages generated *in vitro* effectively function as APC and that they promote T cell proliferation both *in vitro* and *in vivo*. In addition, they suppress Th1 differentiation *in vivo*. Importantly, administration of mouse myelin-laden macrophages delayed the onset of EAE and reduced clinical EAE in terms of disease burden. Such a regulatory function of myelin-laden macrophages sheds a new light on the role of these cells in EAE and MS as these cells were previously considered as the primary aggressors in the development of disease [112-114,143,202].

Myelin ingestion by human macrophages increased the expression levels of MHC class I and CD80. In parallel, mouse myelin-laden macrophages displayed an increase in MHC class I and CD80, and also in MHC class II (Figure 1), suggesting that this may have contributed to the observed increase in T cell proliferation. In addition, other mechanisms may be involved, such as the processing capacity [230]. Macrophages contain high levels of lysosomal proteases used to rapidly and completely degrade phagocytozed antigens, resulting in less efficient antigen-presentation as for instance dendritic cells [230]. To assess the processing capacity of myelin-laden macrophages, we incubated the cells with DQ-conjugated OVA, which becomes fluorescent after proteolytic hydrolysis. Myelin-laden macrophages had processed less OVA protein after 30 min than control macrophages, whereas this quantitative difference disappeared after 60 or 90 min. This suggests that myelin-laden macrophages demonstrate less aggressive degradation of OVA protein, which might result in more efficient antigen-presentation, at least in the influenza recall response. It can be envisaged that the abundant phagocytosis of myelin alters proteolytic activity within the lysosomes, causing the slower processing kinetics [231-233].

The fact that myelin-laden macrophages interact with T cells is a crucial finding as these cells reside abundantly at locations where autoreactive T cells in MS are (re)activated [50,141-144,180]. In the current study, we observed that mouse T cells activated by mouse myelin-laden macrophages produced reduced levels of IFN-γ after 24 h of *in vitro* restimulation, suggesting suppression of Th1 differentiation. This is in line with our previous findings showing that myelin-laden macrophages are involved in the resolution of inflammation. They have an anti-inflammatory phenotype and resemble alternatively activated and regulatory macrophages [120], which are associated with tissue repair, Th2 responses [104,106] and activation of regulatory T cells [105,234].

In addition to suppression of Th1 differentiation, we demonstrate that myelin-laden macrophages suppress EAE severity and delay the onset of disease. This delay may be caused by the delayed IFN-γ production induced by myelin-laden macrophages. Various APC-based studies have been performed to reduce EAE. Similar to our results, administration of TNF-α-treated dendritic cells, TGF-β-treated APC, and M-CSF-treated APC suppressed EAE in an antigen specific way [235-237]. In contrast, APC may exert their immunsuppressive function also without antigen-specificity, such as glatiramer acetate (copaxone)-treated monocytes [238]. This suggests that these monocytes act through bystander suppression, e.g. by production of suppressive cytokines [239]. Such bystander suppression may also be operational in our study. However, macrophages not pulsed with MOG_{35-55}, did not reduce EAE severity, suggesting antigen-specificity. This notion is also supported by the fact that macrophages which were not loaded with OVA protein did not induce *in vivo* T cell proliferation of mouse OVA-TCR-transgenic $CD4^+$ T cells (Figure 5A).

The APC used in our study and in the studies described above all direct T cells to produce less IFN-γ [235,237,238]. However, in contrast to myelin-laden macrophages, the tolerogenic APC used in the other studies skew T cells to produce increased IL-10 levels [235,237] and decreased IL-17A levels [237]. Despite decreased IL-10 and unaffected IL-17A production by myelin-laden macrophage-activated T cells, myelin-laden macrophages could still suppress EAE.

Our data extend previous findings showing that naturally occurring myelin-laden macrophages in MS brain have an anti-inflammatory phenotype and produce anti-inflammatory cytokines [120]. From the current study, we conclude that myelin ingestion results in the generation of a regulatory macrophage population that induces T cell proliferation but inhibits detrimental Th1 differentiation. In concordance, myelin-laden macrophages suppress EAE in an antigen-specific way. These data provide new insights into the self-limiting mechanisms during MS.

Experimental procedures

Mice

Female C57BL/6 mice and BALB/c mice (8–12 wk old) were purchased from Harlan. DO11.10 mice, backcrossed onto the BALB/c background were bred within the animal care facility of the Erasmus MC. All animal experiments were performed with approval of the Erasmus University Committee (Rotterdam, The Netherlands) for animal ethics. Mice were housed under specified pathogen-free conditions and received water and food *ad libitum*.

In vitro generation of human myelin-laden macrophages

Human monocyte-derived macrophages were generated from healthy donors as described previously [120]. Briefly, monocytes were isolated from human blood using Ficoll and Percoll density gradients. Next, the monocytes were differentiated into macrophages under non-adherent conditions using 5% human AB serum. After 5-7 days, differentiated macrophages were plated into 6-well or 96 well culture plates (Nunc) and non-adherent cells were removed after 24 h. This yielded a population of >95% macrophages as determined by macrophage-specific esterase staining [120]. The macrophages were incubated during 72 h with 50 μg/ml human myelin, derived from post-mortem white matter [171], unless otherwise indicated. Myelin-laden macrophages were compared with control macrophages from the same donor, which were not incubated with myelin.

In vitro generation of mouse bone marrow-derived myelin-laden macrophages

Femora and tibiae from adult C57BL/6 mice were cleaned of muscles and tendons and ground in a mortar using Dulbecco's Modified Eagles Medium (DMEM; Lonza) to obtain bone marrow. Single cell suspensions were obtained by passing the bone marrow through a 70 μm gauze. $1x10^7$ to $4x10^7$ bone marrow cells were seeded into a petri dish containing 10 ml DMEM with 5% heat-inactivated FCS (Lonza), 50 μM β-mercaptoethanol (Gibco), 100 units/ml penicillin, 100 μg/ml streptomycin (Lonza). Bone marrow cells were differentiated into macrophages as described [240]. Bone marrow cells were stimulated with 10 ng/ml recombinant mouse M-CSF (Peprotech) on day one and 5 ng/ml recombinant mouse M-CSF on day three. After seven days, non-adherent cells were removed and the differentiated macrophages were incubated with 13 μg/ml human myelin for 72 h in the presence of 5 ng/ml recombinant mouse M-CSF.

Immunophenotyping of macrophages

Intracellular expression of CD68, and surface expression of MHC class I, MHC class II and costimulatory molecules by human macrophages was detected using FITC-labeled anti-CD68 (DAKO), anti-MHC class I, anti-CD40, anti-CD80, and anti-CD86, PE-labeled anti-MHC class II (all from BD Biosciences), and biotinylated anti-PD-L1 (eBioscience) with FITC-labeled streptavidin (BD Biosciences) as conjugate. Mouse macrophages were stained with FITC-conjugated anti-CD40

(FGK45, Bioceros), anti-CD11b and anti-CD86 (both from BD PharMingen), PE-conjugated anti-MHC class II (M5/114), anti-CD80 (both from BD PharMingen), anti-PD-L1, anti-ICOS-L (both from eBioscience), and rat-anti MHC class I (M1/42) with a FITC-conjugated anti-rat secondary antibody (DAKO). For intracellular staining, cells were fixed with 4% paraformaldehyde and permeabilized with PBS/0.5% BSA/0.5% saponin. Subsequently, the cells were incubated with the primary antibody diluted in PBS/0.5% BSA/0.5% saponin for 30 min on ice. For surface staining, cells were incubated with primary antibodies and conjugates diluted in PBS/0.5% BSA/0.01% sodium azide for 30 min on ice. Isotype-matched primary antibodies of irrelevant specificity or first antibody omission served as negative controls. 20.000-30.000 events were measured using a FACSCalibur flow cytometer and analyzed by CellQuest software (BD Biosciences). Dead cells were excluded from analysis by staining with 7-aminoactinomycin (7-AAD, BD Biosciences).

Endocytosis

Myelin was labeled with the lipophilic dye 1.1''-diotadecyl-3,3,3',3'-tetramethylindocarbocyanide perchlorate (DiI; Molecular Probes) as described [241]. Macrophages were fed with 50 μg/ml DiI-labeled myelin to distinguish between macrophages that ingested different amounts of myelin.

To determine the endocytosis capacity for particulate and soluble antigens, macrophages were incubated with $40x10^6$ FITC-labeled polystyrene beads (Molecular Probes; 1.0 μm diameter), 1 mg/ml FITC-labeled dextran (Sigma; 40 kDa) or 5 mg/ml FITC-labeled OVA (Molecular Probes) for 30 min. Subsequently, cells were washed with ice-cold PBS and endocytosis was determined using flow cytometry as described above in the presence of 0.004% trypan blue (Flow Laboratories, Irvine, Scotland) to quench extracellular surface-bound antigen. Cells incubated at 4°C served as negative controls.

Processing

Intracellular proteolytic processing of OVA was determined with DQ- conjugated OVA (Molecular Probes) as described [242]. Briefly, cells were incubated with 100 μg/ml DQ-conjugated OVA for 15 min. Subsequently, the cells were thoroughly washed with ice-cold PBS and further processing was allowed for 30, 60, and 90 min. Fluorescence was determined using flow cytometry as described above. Cells incubated at 4°C served as negative controls.

Allogeneic mixed leukocyte reaction

Human macrophages were seeded into flat-bottomed 96-well plates (Nunc) at $12x10^4$, $6x10^4$ and $3x10^4$ cells/well and incubated with 50 μg/ml human myelin for 72 h, unless indicated otherwise. Subsequently, cells were washed and coincubated with $3x10^5$ T cells/well from a different donor. T cells were isolated from human blood using the human Pan T cell isolation kit II (Miltenyi Biotec) and Automacs (Miltenyi Biotec). After 3 days, BrdU was added to the cultures and allowed to incorporate for 48 h. Subsequently, T cells were stained as described above with APC-labeled anti-CD4, peridin

chlorophyll protein (PerCP)-labeled anti-CD3 and PE-labeled anti-CD8 (all from BD Biosciences). BrdU was stained using a BrdU proliferation kit (BD Biosciences) according to manufacturer's instructions. T cell proliferation was determined by overnight incorporation of [^{3}H]-thymidine (1 μCi/well; Amersham Biosciences) [165] to quantify proliferation or by flow cytometry to determine $CD4^+$ and $CD8^+$ T cell division.

T cell recall response against influenza vaccine

Human macrophages were incubated with myelin as described above. Subsequently, the cells were washed and loaded overnight with a predetermined optimal concentration of dialyzed inactivated influenza vaccine from season 1999/2000 (Influvac, Solvay Pharmaceuticals B.V.). Next, the cells were washed again and coincubated with $3x10^5$ T cells/well from the same donor. The T cells were isolated as described above. Macrophages not loaded with influenza vaccine served as negative control. After 5 days, T cell proliferation was determined by overnight incorporation of [^{3}H]-thymidine as described above.

In vivo determination of T cell proliferation

Bone marrow-derived macrophages of BALB/c origin were incubated for 72 h with 13 μg/ml human myelin to generate myelin-laden macrophages. Next, control and myelin-laden macrophages were loaded overnight with 250 μg/ml OVA protein (Worthington). Spleen and lymph nodes were isolated from DO11.10 mice and single cell suspensions were obtained. After red blood cell lysis using Gey's lysis buffer, OVA-TCR-transgenic T cells were isolated using the mouse Pan T cell isolation kit (Miltenyi Biotec) and Automacs. BALB/c mice received $5x10^6$ CFSE-labeled [243] $CD4^+KJ1\text{-}26^+$ (anti-OVA-transgenic TCR) T cells intraveneously. One day after transfer, $2.5x10^6$ OVA-loaded mouse macrophages were injected into the hind footpad. $2.5x10^6$ unpulsed mouse macrophages were injected into the other hind footpad of the same animal as a negative control. Popliteal lymph nodes were isolated 3 days later and single cell suspensions were analyzed for cell division using flow cytometry. In addition, $5x10^5$ popliteal lymph node cells were seeded into 24-well plates (Nunc) and restimulated with 250 μg/ml OVA protein. After 24 h, 48 h, and 72 h, supernatants were collected for further analysis.

EAE induction and treatment with myelin-laden macrophages

To induce EAE, female C57BL/6 mice (8-12 weeks) were immunized with 200 μg MOG_{35-55} (ABC Biotechnology) in CFA and treated with *Bordetella pertussis* toxin as described [165]. The day of immunization is denoted as day 0. Bone marrow-derived myelin-laden and control macrophages of C57BL/6 origin were loaded overnight with 100 μg/ml MOG_{35-55} peptide. At day –3, 0, and 4, $1x10^6$ macrophages were injected intraveneously to determine whether myelin-laden macrophages could modulate disease. Mice injected with mouse macrophages which were not loaded with MOG peptide and mice injected with PBS served as negative controls. Mice were weighed and scored

for clinical signs of EAE daily according to the following scoring system: 0, no disease; 0.5, partial tail paralysis; 1, complete tail paralysis; 1.5, limb weakness without tail paralysis; 2, limb weakness and tail paralysis; 2.5, partial limb and tail paralysis; 3, complete hind or front limb paralysis; 3.5, paraplegia; 4, quadriplegia; 5, death due to EAE. Paralyzed mice with EAE scores above 2.5 were afforded easier access to food and water.

ELISA

Cytokine production by mouse T cells was determined in culture supernatants. Cytokines in sera and supernatants were determined using commercial capture ELISA's according to manufacturers' instructions. OptEIA ELISA kits for IL-4, IL-10 and IFN-γ were obtained from BD Biosciences. IL-17A production was determined using a capture ELISA from R&D.

Statistics

Differences between groups were determined by a two-tailed Mann-Whitney *U*-test using the statistical software program SPSS, version 11.0. Differences in EAE course between groups were analyzed with a variance analysis for repeated measurements using statistical analysis software (SAS). P values < 0.05 were considered significant.

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Part IV

Summary and Discussion

Part IV

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Chapter | 6

General discussion

Preface

The recruitment of autoreactive lymphocytes into the CNS is characteristic for MS and contributes to CNS pathology and disease symptoms [2-4]. In view of understanding disease mechanisms and possible therapeutic interventions aiming to limit activation of autoreactive lymphocytes, it is crucial to know where these lymphocytes are activated. This may occur in distinct anatomical locations, namely in the secondary lymphoid organs (i.e. the CNS-draining lymph nodes and the spleen), in the perivascular spaces within the CNS, and in the brain parenchyma of MS lesions. We hypothesized that autoreactive T cells are activated in the CNS-draining lymph nodes, subsequent to drainage of CNS antigens to these lymph nodes. An intriguing twist is the tolerance-inducing capacity of cervical lymph nodes after antigen injection into the brain and upon administration through the nasal mucosa. Hence, autoreactive T cell activation in the CNS-draining lymph nodes during MS and EAE may be detrimental or beneficial to the disease. To test our hypothesis and to answer the question whether the immune responses during MS and EAE in the CNS-draining lymph nodes are tolerogenic or immunogenic, we combined analysis of MS and EAE tissues in conjunction with functional studies in distinct animal models for brain inflammation and injury. Furthermore, we paid special attention to the migrating capacity and immunological function of myelin-laden macrophages, which are observed in all three anatomical compartments of lymphocyte activation. The major conclusions of our studies are summarized in Box 1. Below, implications of our results are discussed in the light of other studies and future research.

Are CNS antigens present in the draining lymph nodes of MS patients and EAE animals?

A prerequisite for lymphocyte activation within the CNS-draining lymph nodes is the presence of CNS antigens in APC within these lymph nodes. Despite the fact that classical lymphatic vessels are lacking in the central nervous system [68,152], antigens drain to the CNS-draining lymph nodes and the spleen after injection into the CNS [63,68,74,126,152,179,244]. In keeping with this, we previously demonstrated that myelin antigens are present in the spleen and CNS-draining lymph nodes of MS patients and EAE-affected animals [45,46].

In **chapter 2**, we extended these findings towards neuronal antigens, based on the hypothesis that neuronal damage is characteristic for MS and EAE. Interestingly, the frequencies of neuronal antigen-containing cells in the cervical lymph nodes correlated with the extent of neuronal damage. Rhesus monkeys and Biozzi ABH mice, which demonstrate pronounced neuronal damage during the course of EAE [35,36], had significantly more neuronal antigen-containing cells in the cervical lymph nodes than animals without EAE. This was not the case for common marmoset monkeys, in which neuronal damage is limited [35].

The quantity of neuronal antigen-containing cells in the cervical lymph nodes of MS patients was also not significantly higher as compared to control subjects, despite a clear trend. This may be due to the fact that MS is a heterogeneous disease in which CNS inflammation differs between individuals and fluctuates in time, and to the fact that the lymph nodes could not be isolated at

set time points. Furthermore, the contribution of drainage via the olfactory route in humans is uncertain. Humans have much larger brains than rodents in relation to the size of the olfactory bulbs. The nasal lymphatics may therefore not be relevant to CSF absorption. Another issue is that the arachnoid villi are significantly more developed in humans than for example in rodents. Most of the CSF in humans therefore drains into the blood via arachnoid villi, providing a direct route of access to the spleen [67,244,245].

Box 1. Main conclusions of the studies performed in this thesis.

- Pro-inflammatory APC within the cervical lymph nodes of MS patients contain neuronal antigens.
- Myelin antigens are present in anti-inflammatory APC within the cervical lymph nodes of MS patients.
- The frequencies of CNS antigens in the CNS-draining lymph nodes correlate with the extent and type of CNS damage.
- Myelin-reactive naïve T cells are activated within the CNS-draining lymph nodes.
- Lymphadenectomy of the CNS-draining lymph nodes results in less severe relapse burden and histopathology in the spinal cord.
- Myelin-laden macrophages respond to the lymph node-homing chemokine CCL21 and express CCR7 *in vitro* and *in vivo* in the cervical lymph nodes.
- Myelin-laden macrophages recruit more myeloid cells *in vitro* than control macrophages.
- Myelin ingestion by macrophages promotes their antigen presenting capacity to naïve and memory T cells.
- Myelin-laden macrophages inhibit Th1 differentiation.
- Myelin-laden macrophages suppress clinical symptoms in EAE during the T cell-priming phase.

Do CNS antigens drain to the CNS-draining lymph nodes as a direct result of CNS damage?

The correlation between the extent of CNS damage and the frequencies of neuronal antigens in the cervical lymph nodes of EAE-affected animals strongly suggests that the degree of CNS drainage is reflected by the magnitude of CNS damage. To investigate this, we used four distinct CNS-insult models in which the type, location and extent of CNS damage differs. The results in **chapter 2** show that the frequencies of CNS antigen-containing cells in the cervical lymph nodes indeed correlated with the extent and type of CNS damage. In the first model, occlusion of the middle cerebral artery in mice induces massive CNS degeneration [147]. This was reflected by numerous NF-L and PLP-containing cells in the cervical lymph nodes in the MCAO model. Evident drainage to CLN occurred already 24 h after MCAO and was reduced after 72 h, indicating rapid and transient

drainage of CNS antigens. These results confirm previous studies, showing that tracers injected into the CSF and CNS parenchyma reach the cervical lymph nodes within minutes to hours [60,64,72,246,247].

Lesioning the perforant pathway in the entorhinal cortex results in axonal degeneration within the dentate gyrus of the hippocampus. Demyelination is absent or limited in the ECL model since the perforant pathway is not myelinated [173]. Analysis of the cervical lymph nodes of ECL mice revealed few CNS antigen-containing cells, reflecting the limited CNS damage. In addition, we analyzed the cervical lymph nodes of mice that underwent facial nerve axotomy. FNA induces retraction of the motoneurons in the brainstem [148]. As in the ECL model, demyelination is limited since the facial nucleus is not myelinated [174]. In concordance with this limited CNS damage, the cervical lymph nodes of FNA-mice contained hardly any CNS antigen-containing cells.

To determine whether, in addition to the extent of CNS damage, also the type of CNS damage correlates with the presence of CNS antigens in the cervical lymph nodes, the cuprizone model was used. Cuprizone-feeding induced pronounced demyelination in the brain and spinal cord [150], but only little neuronal degeneration [151]. This was reflected by a large quantity of myelin antigens in the cervical lymph nodes, but hardly any neuronal antigens. These results indicate that the frequencies and type of CNS antigens in the cervical lymph nodes is a direct and rapid result of CNS damage.

Our findings suggest that the presence of CNS antigens in the draining lymph nodes is a diagnostic marker for recent CNS damage in MS patients. Ultrasound-guided fine needle aspiration in MS patients has revealed that myelin-containing APC can be obtained from the cervical lymph nodes [46]. However, this method is rather invasive and may interfere with the balance between tolerogenic and immunogenic processes against mucosal and CNS-derived antigens. Instead, imaging techniques may be used to detect CNS antigens in the CNS-draining lymph nodes, such as Raman spectroscopy or magnetic resonance imaging.

Are the distinct CNS-draining lymph nodes similarly involved in CNS drainage?

As described in the general introduction of this thesis, CNS antigens drain to distinct CNS-draining lymphoid organs. These include the superficial cervical lymph nodes, the deep cervical lymph nodes, the lumbar lymph nodes, and the spleen. The results described in **chapter 2** show that CNS antigens preferentially drain to the deep cervical lymph nodes, and hardly to the superficial cervical lymph nodes in EAE-affected and cuprizone-treated mice. In contrast, this distinction between the deep and superficial cervical lymph nodes was not observed after MCAO, which may be caused by a different drainage route, or by the fact that the massive CNS damage has destroyed (part) of the drainage route. Differential drainage to distinct draining lymph nodes has also been described in other studies. Brain-injected Evans blue-labeled albumin or HRP preferentially drained to the ipsilateral deep cervical lymph nodes of rats [60]. Similarly, T cells injected into the lateral ventricle or the entorhinal cortex of mice drained specifically to one superficial cervical lymph node, irrespective of the injection site [248]. These data indicate that specific drainage routes are used

depending on the origin within the CNS.

The cuprizone model also revealed substantial drainage of CNS antigens to the lumbar lymph nodes (**chapter 2**). Antigens in the lumbar lymph nodes are likely derived from the spinal cord and the spinal fluid [74-77,249], in which cuprizone-induced demyelination also takes place. Since the spinal cord is also affected in MS patients, the spinal cord-draining lymph nodes may be important in MS as well. Future studies on the CNS-draining lymph nodes of MS patients will provide more information on the level of drainage to the distinct CNS-draining lymph nodes in humans.

Do CNS antigens reach the CNS-draining lymph nodes as solutes or within cells?

A major unresolved issue is whether APC can leave the CNS and gain access to draining lymph nodes. Do activated macrophages filled with debris survive, and migrate out of the CNS. Do dendritic cells phagocytose CNS antigens to present to T cells in the draining lymph nodes as has been described for other tissues [53-57]? Similarly, can microglia under strongly inflammatory and degenerative conditions acquire sufficient motility and migration capabilities to migrate out of the CNS?

There are three possible mechanisms for antigen transfer to the draining lymph nodes (Figure 1) [57,210,250]. First, antigens may drain as solutes to the lymph nodes, where they are phagocytozed by lymph node-resident APC. Second, antigens may be phagocytozed within the CNS by microglia, macrophages or dendritic cells, which then migrate to the lymph nodes where they take residence. Third, antigens are transferred within phagocytes, but upon arrival in the lymph nodes these cells transfer their cargo to lymph node-resident APC [250].

The results in **chapter 2** describe that a substantial part of myelin-laden cells in the cervical lymph nodes of MS patients and EAE-affected rhesus monkeys expressed the lymph node homing receptor CCR7, suggestive of recent migration. Furthermore, *in vitro* myelin ingestion by human (**chapter 2**) and mouse macrophages (**chapter 4**) increased CCR7 surface protein expression, and mouse myelin-laden macrophages demonstrated enhanced migration towards CCL21, one of the CCR7 ligands. Notably, myelin-laden cells in the cervical lymph nodes had an anti-inflammatory phenotype, as myelin-laden macrophages in MS lesions [120], although the myelin-laden cells in the cervical lymph nodes do not have the foamy appearance as in MS lesions. Together with the finding that myelin-laden cells are present in the CSF of MS patients [203,204], these results strongly suggest that myelin antigens are at least partly transferred within phagocytes. Myelin-laden cells may emigrate from the CNS in a CCR7-dependent mechanism, while retaining their immunosuppressive phenotype, but losing their foamy appearance. Nevertheless, CCR7 deficiency did not affect the quantity of myelin antigens in the cervical lymph nodes in EAE-affected mice (**chapter 2**), implying that other or additional chemokine receptors are involved or that myelin antigens are transferred as soluble antigens.

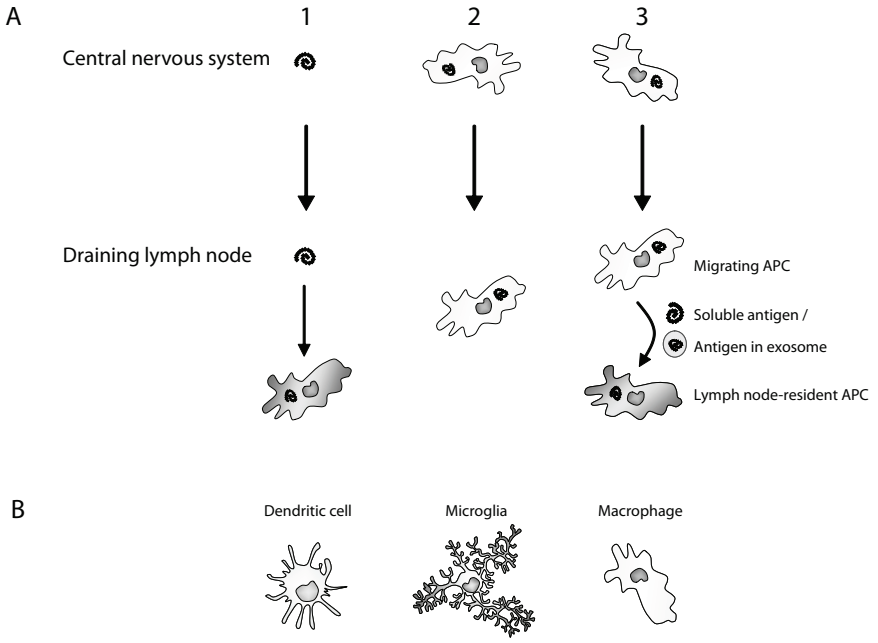


Figure 1. Putative transfer mechanisms of CNS antigens to the draining lymph nodes. (A) CNS antigens can reach the draining lymph nodes via three mechanisms. (1) Antigens can drain as solutes to the lymph nodes, where they are phagocytozed by lymph node-resident APC. This pathway is not contested. In contrast, cell-mediated transport from the CNS is more controversial: (2) Antigens may be phagocytozed within the CNS by APC, which subsequently transport the antigens to the lymph nodes where they take residence. (3) Antigens are transferred within phagocytes, but upon arrival in the lymph nodes these cells transfer their cargo to lymph node-resident APC, perhaps as soluble material and perhaps in exosomes. (B) The cells depicted in the figure are macrophages, but may also be dendritic cells or microglia. The findings of our study suggest that neuronal antigens likely drain via mechanism 1, and that myelin antigens can be transferred via all three mechanisms. Based on [57,210,250].

Evidence that CNS cells have the capacity to reach the CNS-draining lymph nodes comes from patients suffering from glioblastoma metastasis in the cervical lymph nodes [251,252]. Furthermore, also cells that are injected into the CNS parenchyma of experimental animals reach the CNS-draining lymph nodes [84,141,179,248,253]. These cells were injected in large volumes of 4-30 μl in mice and 100 μl in rabbits. Considering the calculated flow of interstitial fluid of 0.1-0.3 μl/min/gram of brain [61], it is likely that these volumes have disturbed the natural flow and created overpressure, resulting in forced drainage. However, that *in vitro*-generated microglia- and bone marrow-derived dendritic cells injected into the striatum of rats in a volume of 0.3 μl also had the capacity to leave the CNS parenchyma via the bloodstream to reach the peripheral lymph nodes and the spleen. In contrast, microglia did not leave the CNS, suggesting that they do not have the capacity to emigrate the CNS [254].

These results are in sharp contrast with other recent findings. Particulate fluospheres injected into the corpus striatum of mice were transferred to the perivascular spaces where they were ingested by perivascular macrophages. These macrophages did not emigrate the CNS, but instead stayed within the perivascular spaces [63]. Tracer-containing perivascular macrophages also stayed within the perivascular spaces over a 2-year period [64]. Furthermore, fluosphere-containing macrophages did not even emigrate the CNS after an inflammatory trigger [63], suggesting that the perivascular drainage pathway along the basement membranes does not have the capacity for the drainage of cells [63]. In addition, dendritic cells injected into the corpus callosum of rats remained in the CNS and were not observed in the CNS-draining lymph nodes [181].

The question remains whether macrophages, which are the most prominent phagocytic cells in MS and EAE lesions, have the capacity to emigrate from the CNS under inflammatory conditions, such as in MS and EAE. Interestingly, dendritic cells within the CSF show increased migration to the cervical lymph nodes of rats under the inflammatory conditions of EAE as compared to non-inflammatory conditions [255]. Injecting fluorescently-labeled macrophages into the striatum of rodents, similar as performed by Hochmeister and colleagues [254], would answer the question whether macrophages can leave the CNS. Furthermore, it remains unknown whether microglia can leave the CNS under inflammatory conditions. More insight into this can be obtained using GFP transgenic mice, in which GFP expression is driven by the CD11b promoter. CD11b is the alpha chain of the Mac-1 integrin, which is expressed in myeloid cells, including macrophages and microglia [256]. Irradiation and subsequent adoptive transfer of bone marrow will leave only the microglia GFP^+, thus allowing studying the migration of microglia to the periphery in animal models with inflammation in the CNS, such as EAE.

In contrast to myelin-laden cells, neuronal antigen-containing cells in the cervical lymph nodes of MS patients and EAE-affected rhesus monkeys did not express CCR7 and EAE-affected CCR7 deficient mice contained similar frequencies of neuronal antigens in the cervical lymph nodes (**chapter 2**). This may be due to different kinetics in processing or degradation of CNS compounds, or to different kinetics in migration of distinct APC subsets. The elevated presence of neuronal proteins in the CSF of MS patients and EAE mice as compared to healthy controls [156,157], strongly suggests that neuronal antigens drain as soluble compounds from the CNS to the periphery. Collectively, the available data suggest that CNS antigen-containing APC can emigrate from the CNS to the CNS-draining lymph nodes in addition to drainage of soluble antigens.

Are CNS antigen-specific immune responses initiated in the CNS-draining lymph nodes?

Antigens that are injected into the CNS readily drain to the CNS-draining cervical lymph nodes. A major disadvantage of this method is that the volume of injection often increases the pressure in the CNS, resulting in a non-physiological and forced drainage [39,152]. In this thesis, this problem was circumvented by making use of inflammatory CNS damage during MS and EAE, so without mechanical damage. The results described in **chapter 2**, showing that both myelin and neuronal

antigens are present in the cervical lymph nodes are thus physiologically relevant and implicate that CNS-antigen specific immune responses are initiated in the CNS-draining lymph nodes.

Chapter 3 indeed shows myelin-specific proliferation in the CNS-draining lymph nodes and the spleen of EAE-affected animals. Previous studies already demonstrated proliferation against the immunizing peptide [45,47,48]. We confirmed these findings in two distinct EAE models using different mouse strains. Proliferative responses against the immunizing peptide were also observed in the axillary and inguinal lymph nodes. These lymph nodes drain the adjuvant-antigen inoculation site, and a response against the immunizing peptide was therefore expected. Importantly, there was also proliferation against additional myelin peptides in the CNS-draining lymph nodes, which was not observed in the axillary and the inguinal lymph nodes. This implies intermolecular epitope spreading and indicates that naïve CNS antigen-specific T cells are activated in the CNS-draining lymph nodes.

Epitope spreading is the process by which reactivity to epitopes distinct from, and non-crossreactive with, the disease-inducing epitope is induced during chronic inflammatory processes [257]. Such diversification of autoreactive encephalitogenic T cell responses has been documented in MS patients [258] and in EAE-affected animals [258-260]. Epitope spreading in EAE has been associated with distinct periods of clinical worsening and implicated as the major functional cause of disease progression [258,260-262]. However, others find contrary results and claim that epitope spreading is not required for EAE relapses [263,264]. Hence, it remains unclear whether epitope spreading in the CNS-draining lymph nodes is critical to EAE progression.

Despite drainage of the neuronal antigen NF-L to the CNS-draining lymph nodes during EAE, **chapter 2** shows that no response against NF-L was detected in the CNS-draining lymph nodes of EAE-affected mice. This may have several reasons. First, the lack of proliferation against NF-L may be the result of tolerance against this antigen. Second, we could have analyzed the CNS-draining lymph nodes at a wrong time point, since the kinetics of NF-L drainage and/or presentation may be different from myelin antigens. And finally, intermolecular epitope spreading from myelin antigens to neuronal antigens might not take place in a MOG-induced EAE model. This latter hypothesis is supported by the fact that splenocytes from NF-L immunized mice did not proliferate against recombinant mouse MOG [140].

Our results are in contrast with the previous findings that naïve T cells are not activated within the cervical lymph nodes, but in the CNS itself [50]. This study employed transfer of naïve $PLP_{139-151}$-TCR-transgenic T cells into mice undergoing CNS inflammation caused by Theiler's virus or relapsing-remitting EAE. It is known that the CNS is capable of maintaining a strong re-stimulatory capacity due to the presence of APC once CNS inflammation is established [143,144,226,265]. Nevertheless, this study demonstrated that under experimental conditions, naïve T cells can be activated within the CNS.

Do the CNS-draining lymph nodes contribute to disease?

The results in **chapter 3** and research of others provide clear evidence that drainage of CNS antigens to the CNS-draining lymph nodes activates myelin-specific T cells [45,47,48]. Yet, the contribution of immune responses within the CNS-draining lymph nodes to disease remains unclear for two reasons. First, naïve T cells can also be activated within the CNS [50,143,226,265]. In fact, peripheral lymphoid tissue is dispensable for T cell-dependent neuroinflammatory disease during EAE [144]. Dendritic cells accumulate in CNS parenchyma of EAE-affected animals and MS patients [225,266], and perivascular dendritic cells are responsible for the retention and activation of naïve transgenic T cells against $PLP_{139-151}$ in the CNS in PLP-induced EAE [50]. Furthermore, lymphoid follicle-like structures within the meninges have been identified in EAE-affected animals and MS patients, which are associated with B cell responses [51,52]. These findings thus leave open the question to what extent the different anatomical compartments contribute to disease.

Second, the functional immunological outcome of CNS drainage to the draining lymph nodes is most likely dependent on a balance between inflammatory and tolerogenic immune responses, implying that drainage of CNS antigens to the CNS-draining lymph nodes will result in either detrimental immune reactions or tolerance against these antigens. Neurodegenerative disorders, traumatic CNS tissue damage and stroke cause inflammation in the CNS, which is associated with activation of microglia, cytokine production, and infiltration of leukocytes [267,268]. These findings imply that brain damage and release of antigens to the periphery initiate an immune response that seems to target the antigen source in the CNS. Indeed, peripheral leukocyte depletion or inhibition of leukocyte migration reduces tissue damage in stroke, indicating that the immune system contributes to the disease process [267]. However, CNS inflammation in these disorders is transient, implying immune tolerance or immune suppression [269], which is in line with the fact that CNS trauma does not correlate with the onset of MS [270]. In fact, immunodeficiency, leading to systemic bacterial infections, is a major problem in stroke patients [271].

Immune tolerance towards CNS antigens has been associated with drainage to the cervical lymph nodes. Tolerance, as measured by the inability to induce a delayed hypersensitivity response, was observed after intracerebral ovalbumin injection, and could be transferred to naïve recipients by intravenous administration of cervical lymph node cells [272]. Furthermore, mice with ECL suffered from less severe EAE as compared to mice without ECL. This was associated with increased T cell apoptosis within the cervical lymph nodes, possibly induced by the presence of neuronal antigens in these lymph nodes after ECL [182]. Thus, although CNS inflammation is characteristic for MS and EAE, the type of immune responses in the CNS-draining lymph nodes may be inflammatory or tolerogenic, possibly reflecting the local inflammatory microenvironment in the CNS.

The immunological significance of CNS drainage to the CNS-draining lymph nodes is emphasized by studies showing that removal of the cervical lymph nodes in rats interferes with B and T cells responses in the brain [49,83,183,273-275]. In **chapter 3**, the contribution of the CNS-draining lymph nodes to clinical EAE was tested by surgically removing these lymph nodes prior to EAE

induction. Three distinct EAE models were used, which demonstrate acute, chronic and chronic-relapsing EAE. This allowed functional analysis of the role of the CNS-draining lymph nodes in different phases of disease. Our studies revealed that the excision of the CNS-draining lymph nodes reduced and delayed, although not significantly, the relapse burden in chronic-relapsing EAE in Biozzi ABH mice. In addition, fewer lymphadenectomized mice demonstrated EAE pathology in the spinal cord as compared to sham-operated mice. These results are in concordance with previous findings showing that cervical lymphadenectomy in rats reduced the number of brain lesions in a cryolesion-enhanced EAE model [49], and conclusively demonstrate that the tolerance-inducing capacity of the CNS-draining lymph nodes is not operational in these three distinct EAE models.

Cervical and lumbar lymphadenectomy did not completely abolish EAE, which may have several explanations. First, antigens could have drained through a different route to more distal lymph nodes and the spleen. Second, naïve T cells may be activated within the CNS itself [50], which is emphasized by the finding that encephalitogenic T cells are activated in the absence of secondary lymphoid organs in an adoptive transfer EAE model [144]. Third, encephalitogenic T cells are continuously activated in the axillary and inguinal lymph nodes draining the adjuvant-antigen depots in the immunization sites. In fact, it has been shown that the immunization site is crucial for the maintenance of encephalitogenic T cells and clinical symptoms, by a drastic experiment in which the hind feet of guinea pigs, immunized with MBP, were amputated to remove the antigenic depots [187]. The problem of the continuous activation of naïve T cells in the adjuvant-antigen depots could be circumvented by using transgenic mice that spontaneously develop CNS inflammation and paralysis. Examples are MOG-TCR-transgenic mice crossed with MOG-specific immunoglobulin heavy chain knock-in mice. These animals spontaneously develop a severe form of EAE and demonstrate widespread demyelination within the CNS, mediated by T and B cell responses against myelin [31,32]. A spontaneously developing EAE model allows assessing the kinetics of immune responses against CNS antigens within the CNS-draining lymph nodes and the CNS.

Very recently, such a study was performed by the group of Lafaille [276], who used MBP-TCR-transgenic mice, crossed with either RAG-1 knock-out mice or T cell receptor α/β double knock-out mice. These mice develop spontaneous EAE of which the clinical symptoms start around the age of 40 days [277]. Interestingly, the quantity of MBP-specific T cells was highest in the deep cervical lymph nodes pre-clinically and dropped during clinical disease. Simultaneously, the frequencies of MBP-specific T cells was low in the CNS during the pre-clinical phase, but a sudden rise in number of MBP-specific T cells within the CNS was observed when the mice reached the sub-clinical phase, indicating that MBP-specific T cells are initially activated in the cervical lymph nodes [276]. Initial T cell activation within the deep cervical lymph nodes was also observed in a spontaneous EAE model using PLP-TCR-transgenic mice [278]. Excision of the deep cervical lymph nodes prior to clinical EAE at the age of 21 to 25 days significantly reduced disease severity [276]. Furthermore, excision of the deep cervical lymph nodes during established EAE significantly reduced MBP-specific T cell infiltration [276]. These results are in parallel with the results from our studies in **chapter 3** in terms of reduced EAE

severity, and confirm that the CNS-draining lymph nodes are involved in the initiation of immune responses against myelin antigens that cause detrimental CNS inflammation.

The development of EAE in the MBP-specific T cell receptor transgenic mice was also not completely abrogated by the excision of the deep cervical lymph nodes [276]. This may be due to the fact that initial T cell activation already took place before excision of the lymph nodes. Furthermore, the superficial cervical lymph nodes and the lumbar lymph nodes were not removed, while a pronounced response against MBP was observed in the lumbar lymph nodes in actively induced EAE [276]. Future experiments should thus aim to dissect the roles of the distinct CNS-draining lymph nodes and to the role of the CNS-draining lymph nodes at different time points during disease development. Timing of excision of the draining lymph nodes is crucial as illustrated by a study in which the pancreatic-draining lymph nodes are removed in non-obese diabetic (NOD) mice. Excision of the pancreatic-draining lymph nodes in three week old NOD mice completely abrogated disease, while sham-operated mice developed spontaneous diabetes around the age of 12 weeks. In contrast, excision of the lymph nodes at the age of 10 weeks did not have an effect on the severity of diabetes [175]. These results indicate that the initiation of immune responses in the pancreas-draining lymph nodes, which drain a relatively small organ with straightforward lymphatic vessels, has a strict time window. Collectively, the data support the notion that the CNS-draining lymph nodes are, at least partly, involved in detrimental immune reactions against CNS antigens during EAE.

Are the CNS-draining lymph nodes target organs for treatment strategies?

When considering MS treatment, the implicit is often that drugs should target the CNS to act locally in the inflamed tissue. Work in this thesis supports the notion that the CNS-draining lymph nodes provide an additional, or alternative target organ for treatment strategies. The CNS-draining lymph nodes are reached through the intranasal route [279]. This route is beneficial for three reasons. First, it provides a non-invasive and convenient therapeutic strategy. Second, the cervical lymph nodes provide a special opportunity in view of their specialized microenvironment driving tolerogenic responses upon intranasal delivery [78,79]. Third, compounds delivered intranasally do not only reach the cervical lymph nodes, but also the CNS through the olfactory sensory neurons, the intercellular clefts in the olfactory epithelium and the subarachnoid space. Via this route, significant concentrations are yielded in the CNS, the CSF, and the olfactory bulb [188,280,281]. Intranasal drug delivery may therefore be dually effective, targeting both the CNS as well as the CNS-draining lymph nodes, while bypassing the blood-CNS barrier.

Different animal studies tried to induce immunological tolerance against brain-derived peptides by intranasal administration. However, the results were inconsistent and effectiveness of this antigen-specific strategy in animal models therefore remains unclear [189-191]. Instead, efficacy may be improved by intranasal administration of existing or novel therapeutics, e.g. cytokines, blocking antibodies against surface molecules, or drugs promoting number and function of regulatory T cells [188,281,282]. Alternatively, systemic administration of therapeutics may target not only the cervical

lymph nodes, but also the spinal cord-draining lymph nodes and the spleen.

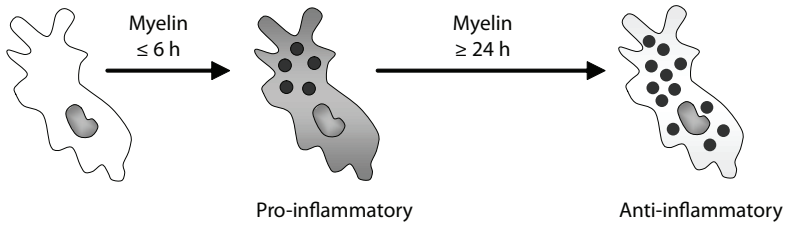
Although intranasal administration of drugs seems promising, some difficulties need to be overcome. The nasal epithelium has a low pH and is enzymatically and proteolytically active, increasing the risk of destroying molecules before they reach their target organs. In addition, large variability caused by nasal pathology, such as common cold or mucosal irritation, may hamper effective treatment [283]. Future animal studies using therapeutics intranasally will give more insight on efficacy of this delivery route in animals and possibly provide new treatment strategies for MS or other neurological diseases.

Table 1. Macrophages and microglia respond to myelin in a two-phase response.

A

		Myelin	
	LPS/IFN-γ	≤ 6 h	≥ 24h
Human Mϕ	-	nd	CCL18 ↑ [120] PGES ↑ [120]
	+		IL-12p35/p40 ↓ [120] TNF-α ↓ [120] IL-10 ↓↑ [120]
Mouse Mϕ	-		TGF-β ↑ [122] CCL11 ↑ [122]
	+	nd	nd
Mouse MG	-		TNF-α ↑ [122] CXCL1 ↑ [122] CCL2 ↑ [122] CCL3 ↑ [122]
	+	TNF-α ↑ [121] IL-1β ↑ [121] IL-10 ↑ [121] CXCL10 ↑ [121]	TNF-α ↓ [121] IL-1β ↓ [121] IL-10 =/↓ [121] CXCL10 ↓ [121] PGE_2 ↑ [121] ROS ↑ [121]

B



Human and mouse macrophages or microglia were incubated with myelin for a short period (≤ 6 h) and a longer period (≥ 24 h) with or without the pro-inflammatory stimuli LPS or IFN-γ before or after incubation with myelin. Based upon the pro-inflammatory response after 6 h of myelin incubation, which then reverts into an anti-inflammatory response after at least 24 h of myelin ingestion (A), it is proposed that myelin ingestion induces a two-phase response (B). Mϕ, macrophages; MG, microglia; nd, not determined; ↓ downregulated; ↑ upregulated; = no difference. This table combines results from [120-122].

Does myelin ingestion by macrophages result in anti-inflammatory action?

As described in the general introduction of this thesis, macrophage activation leads to a range of differentially activated macrophages, depending on the presence of microbial and endogenous ligands, and the cytokine environment. These functionally distinct macrophages are essential in triggering, instructing and terminating immune responses as a consequence of tissue-derived signals. Three hallmark functional phenotypes within a continuum of phenotypes have been identified for macrophages, which can be identified based on surface markers and release of cytokines and chemokines *in vitro*. Classically activated M1 macrophages are involved in the clearance of pathogens by the release of pro-inflammatory cytokines and reactive oxygen species, thereby often causing tissue damage. Alternatively activated M2 macrophages stimulate wound healing and tissue repair. Regulatory macrophages suppress immune responses through the production of IL-10 and by promoting development of regulatory T cells and skewing towards a Th2 phenotype [104-109].

Macrophages and activated microglia are key effectors for tissue injury during MS by the secretion of inflammatory cytokines and reactive oxygen species and interaction with T cells. Blockade of macrophage activation or inhibition of their migration into the CNS ameliorates clinical EAE and tissue injury [18], and depletion of macrophages blocks T cell infiltration into the CNS and suppresses EAE [112-114]. In contrast, macrophages stimulate myelin regeneration by clearance of myelin debris and the production of trophic factors [115-119].

Our group has shown that myelin-laden cells in MS brain lesions and *in vitro* have an immunosuppressive phenotype, implying that they are involved in the resolution of inflammation and tissue repair [120]. *In situ*, these macrophages express the anti-inflammatory molecules IL-1 receptor antagonist, TGF-β, IL-10, prostaglandin E_2 synthase, and CCL18. *In vitro*, macrophages demonstrate increased expression of prostaglandin E_2 synthase and CCL18 after at least 24 h of myelin ingestion. Furthermore, *in vitro*-generated myelin-laden macrophages do not respond to inflammatory stimuli such as LPS [120]. In parallel, the results in **chapter 2** show that the majority of myelin-laden macrophages in the cervical lymph nodes of MS patients and EAE-affected rhesus monkeys also had an anti-inflammatory phenotype as determined by the expression of TGF-β and IL-1 receptor antagonist and the lack of pro-inflammatory cytokines. In contrast to the myelin-laden macrophages in MS lesions, myelin-laden cells in the cervical lymph nodes did not have a foamy appearance acquired by the ingestion of lipids.

The anti-inflammatory function upon myelin ingestion has also been described for cultured mouse microglia. Strikingly, the expression of TNF-α, IL-1β, IL-10 and CXCL10 by IFN-γ-pre-activated microglia was increased shortly after incubation with myelin (≤ 6 h), but then rapidly decreased after 24 h, indicating a two-phase response to myelin ingestion shifting from a pro-inflammatory to an anti-inflammatory response (Table 1). In addition, microglia demonstrated an increase in anti-inflammatory reactive oxygen species and prostaglandin E_2 [121]. Furthermore, myelin phagocytosis has recently been described in a co-culture model of macrophages with mouse

central (optic) or peripheral (sciatic) nerve preparations. Peritoneal macrophages were introduced in this *ex vivo* system, where they phagocytozed myelin time-dependently from two to ten days of coculture. This coculture system initially released high amounts of IL-6, IL-10, CCL22, and CXCL1 after two days, followed by a decreased release of these molecules, also suggesting a two-phase response to myelin ingestion. In contrast, the release of TGF-β, CCL2, and CCL11 was sustained for ten days of coculture [122] and implies an environment in favor of Th2 cells.

Considering the results from these studies, the phenotype of myelin-laden macrophages and microglia seems to mostly resemble the alternatively activated and regulatory macrophages [104,106-109]. Myelin ingestion apparently provides signals directing macrophages to suppress inflammation and perhaps also to stimulate tissue repair.

Does myelin signal through engaging to nuclear receptors?

Inflammatory CNS lesions are the pathological hallmark of MS. As these lesions do not expand indefinitely and inflammation dampens over time, this suggests that endogenous mechanisms are operational to keep the inflammation under control and to start healing processes. Anti-inflammatory myelin-laden macrophages may well play an important role in this. As mentioned before, they reside in MS lesions and in the cervical lymph nodes, where they are optimally situated to interact with other leukocytes. Myelin-laden macrophages in MS have phagocytozed vast amounts of myelin, of which lipids constitute 70 percent of the dry weight (Table 2), suggesting that myelin lipids provides anti-inflammatory signals.

Nuclear receptors, such as liver X receptors (LXR) and peroxisome proliferator-activated receptors (PPAR) respond to endogenous lipid metabolites (Figure 2). They are expressed in high levels by macrophages and have emerged as key regulators of inflammation and lipid homeostasis in macrophages [284]. There is ample data on the role of LXR in the chronic inflammatory disease atherosclerosis. LXR drive cholesterol efflux from macrophages, and are therefore important players in foam cell formation. Indeed LXR-knock-out mice spontaneously demonstrate high numbers of foam cells in lung, spleen and arterial wall [285]. These knock-out mice also demonstrate aberrancies in their innate immune functions [286,287], caused by altered macrophage function. Importantly, LXR are negative regulators of inflammatory gene expression by macrophages, microglia and astrocytes [288-291] and inhibit toll-like receptor-mediated immune response via trans-repression of NF-κB pathways [292,293]. *In vivo*, synthetic LXR agonists inhibit inflammation [291] and atherosclerosis in mouse models [289].

Since cholesterol metabolites are endogenous ligands for LXR and cholesterol is the main component of myelin (Table 2), it seems likely that myelin is, or contains, a ligand for LXR. LXR are therefore likely to play a key role in the intracellular mechanisms responsible for the regulatory function of myelin-laden macrophages. Importantly, LXR agonist treatment before immunization protects against EAE [294], stressing the importance of LXR in inflammatory demyelinating disease. Preliminary data from our lab show that mRNA expression of LXR and the direct target gene

ATP-binding cassette transporter 1 (ABCA1) is significantly increased in MS lesions as compared to normal appearing white matter tissue from MS patients. Moreover, *in vitro* myelin ingestion by human macrophages significantly induces LXR and ABCA1 mRNA expression. Furthermore, LXR agonists increased the expression of CCR7 by macrophages in a mouse model for atherosclerosis [295], supporting our data that myelin ingestion increased CCR7 expression (**chapter 2 and 4**).

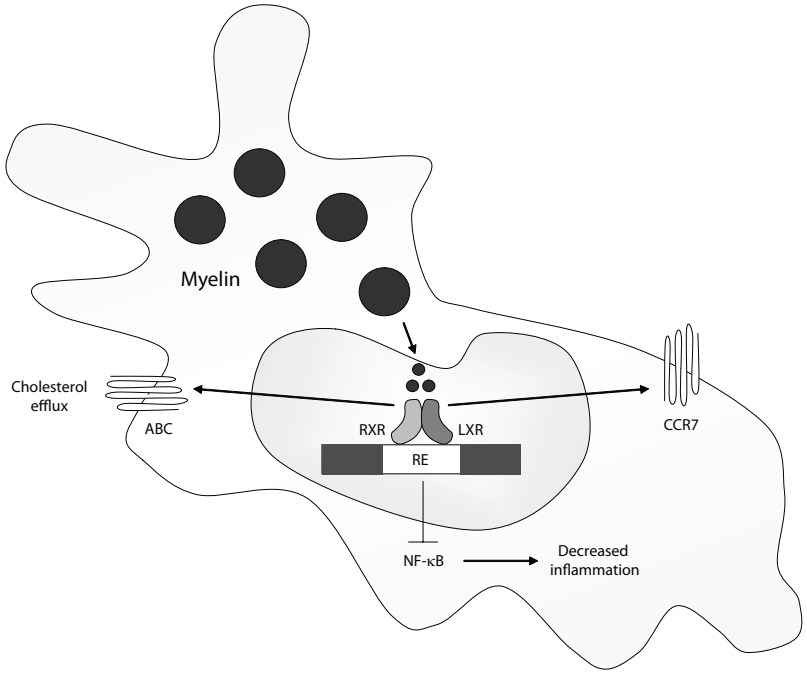


Figure 2. The nuclear receptor LXR is involved in suppression of inflammation. Liver X receptors (LXR) are cholesterol-sensing transcription factors. LXR form heterodimers with the retinoid X receptors (RXR) and bind to LXR-responsive elements (RE). In response to the binding of cholesterol metabolites, possibly derived from myelin, expression of ATP-binding cassette transporters (ABC) is induced. This stimulates the efflux of cholesterol. On the other hand, ligand activation of LXR inhibits NF-κB–dependent induction of inflammatory gene expression and promotes expression of CCR7. Based on [291,295].

Do myelin-laden macrophages modulate the function of other leukocytes?

Myelin-laden macrophages are present in MS lesions, perivascular spaces and the CNS-draining lymph nodes. Since they are in close proximity to a range of other immune and CNS resident cells, it is likely that they interact with these cells and possibly influence their function. In **chapter 4**, we have determined whether myelin-laden macrophages increase the production of chemokines to attract other immune cells. Myelin-laden macrophages attracted significantly more monocytes than control macrophages. This was accompanied by an increased production of CCL2, CCL3, and CCL4,

which however did not reach statistical significance. Increase in CCL2 and CCL3 production was also observed by mouse microglia after myelin ingestion [122]. The enhanced recruitment of myeloid cells by myelin-laden macrophages *in vitro*, suggests that this might also occur *in vivo*. These recruited myeloid cells might be involved in tissue damage. However, preliminary *in vitro* results from our lab reveal that myelin-laden macrophages seem to transfer unresponsiveness to pro-inflammatory stimuli to macrophages that did not ingest myelin. This is likely mediated via membrane(-bound) molecules, as cell-cell contact seem to be required for this transfer.

Further research is warranted to investigate whether this also holds true for monocytes and to determine the factor(s) responsible for this transfer. A possible candidate is TREM-2 (triggering receptor expressed on myeloid cells-2), a membrane receptor on myeloid cells, which is induced by alternative activation of macrophages [296]. Importantly, TREM-2 activation triggers increased phagocytosis or debris removal and creates an anti-inflammatory cytokine milieu, which reduces disease severity and tissue injury in EAE [297,298].

In contrast to the recruitment of monocytes, myelin-laden macrophages did not demonstrate increased capacity to recruit lymphocytes *in vitro* **(chapter 4)**. However, the results in **chapter 5** show that myelin-laden macrophages are potent activators of $CD4^+$ and $CD8^+$ T cells and that they can modulate T cell differentiation. Specifically, the production of IFN-γ by T cells was suppressed, indicating reduction of Th1 differentiation. In line with this, macrophages which ingested myelin upon co-culture with optic or sciatic nerves presented a distinct macrophage phenotype, which mainly skews to a Th2-supporting environment [122]. The expression of both IL-6 and TGF-β by myelin-laden macrophages [120,122] could assist Th17 development [19], but our results show that Th17 production by myelin-laden macrophages-activated T cells is similar to T cells activated by control macrophages. This is possibly caused by lack of IL-23 production by myelin-laden macrophages, which has not been determined yet.

Strikingly, EAE severity was reduced upon administration of myelin-laden MOG-loaded macrophages during EAE induction with a MOG peptide **(chapter 5)**. This was likely mediated through interaction with naïve MOG-reactive T cells, since myelin-laden macrophages which were not loaded with MOG did not have this beneficial effect on disease. Myelin-laden MOG-loaded macrophages possibly inhibited Th1 skewing *in vivo*, as we observed in *in vitro* experiments. In contrast to naïve T cells, memory T cells could not be skewed *in vitro*, which may explain the fact that myelin-laden MOG-loaded macrophages did not affect EAE severity once disease was already established (preliminary data, not shown).

In addition to $CD4^+$ and $CD8^+$ T cells, myelin-laden macrophages may act on NKT cells via the presentation of glycolipids. In particular, CD1a, b and d-restricted glycolipid presentation to (NK)T cells has gained attention with respect to MS and EAE [299]. Importantly, activation of CD1d restricted NKT cells by an artificial ligand protects mice against EAE [300-302]. Moreover, the number of invariant

NKT cells is significantly reduced in the blood and CNS lesions of relapsing-remitting MS patients [303-305] and expanded less in response to the non-endogenous ligand α-galactosyl ceramide [305]. Endogenous lipids responsible for CD1d-restricted NKT cell activation are still unknown, although there are some clues on possible candidates, such as the glycolipid sulfatide, a major component of myelin [306-308].

In addition to NKT cell activation, B cell responses against myelin lipids occur. Lipid-array analysis of myelin-specific lipids revealed lipid-specific antibodies against sulfatide, sphingomyelin and oxidized lipids in CSF from individuals with multiple sclerosis. Sulfatide-specific antibodies were also detected in SJL/J mice with acute EAE [309]. Immunization of mice with sulfatide plus myelin peptide resulted in a more severe disease course of EAE, and administration of sulfatide-specific antibody exacerbated EAE [309]. Furthermore, immunization of guinea pigs with galactocerebroside plus MBP resulted in aggravated demyelination [310]. Together, these data suggest that the timing and route of lipid administration are important factors in the outcome of lipid-based immunomodulatory agents.

Myelin-laden macrophages may present lipid antigens to NKT cells, thereby contributing to resolution of inflammation, while 'free' lipids may be captured by B cells, evoking a robust inflammatory response. We have preliminary data showing high expression of CD1d on myelin-laden macrophages in MS lesions and *in vitro*. Furthermore, human myelin-laden macrophages loaded with α-galactosyl ceramide, activated the mouse NKT-celline DN32. However, further investigation is needed to further explore this topic and to determine whether endogenous myelin lipids or metabolites bind to CD1d and activate NKT cells or their function.

Table 2. Lipids in human myelin.

Protein			30
Lipid			70
	Cholesterol		19.4
	Galactolipids		19.3
		Cerebroside	15.9
		Sulfatide	2.7
	Phospholipids		30.2
		Ethanolamine posphatides	10.9
		Lecithin	7.8
		Sphingomyelin	5.5
		Phosphatidylserine	3.4
		Phosphatidylinositol	0.4

Numbers in percent dry weight. Adapted from [324].

Are myelin-laden macrophages neuroprotective?

In addition to interaction with leukocytes, myelin-laden macrophages likely also interact with CNS-resident cells, such as neurons. Distressed neurons express and upregulate CXCL10 and CCL21 *in vivo* [166,214,215] and *in vitro* [215-218], although this has not yet been found in MS lesions. Since microglia express the corresponding chemokine receptor CXCR3, it has been suggested that microglia interact with neurons via these chemokines. Indeed, CXCR3 deficiency reduced microglial activation and neuronal loss in the entorhinal cortex lesion model [166], strongly supporting this hypothesis. Myelin-laden macrophages reside in CNS parenchyma adjacent to damaged neurons and upregulate CXCR3 expression *in vitro* **(chapter 4)**. It is therefore tempting to speculate that myelin-ingestion by macrophages might promote communication with distressed neurons in MS, resulting in neuroprotection [219]. Co-cultures with myelin-laden macrophages and neurons would provide considerable information on the role of myelin-laden macrophages in neuroprotection.

In addition, myelin-laden macrophages may be neuroprotective through the production of neurotrophic factors. For instance, macrophages and microglia promote axonal sprouting through the production of glial cell-derived neurotrophic factor (GDNF) and brain-derived neurotrophic factor (BDNF) [311]. Furthermore, these two compounds protect dopaminergic neurons against neurotoxins in a rat model for Parkinson's disease and improve animal behaviour [312]. It would therefore be interesting to determine the production of GDNF and BDNF by myelin-laden macrophages and to incubate neuronal cultures with supernatants from myelin-laden macrophages to investigate whether neuroprotection takes place via trophic factors.

Furthermore, recent attention is given to neurogenesis in multiple sclerosis lesions. A pronounced increase in mature interneuron density was observed in subcortical white matter lesions, which is most likely the result of neurogenesis [313]. Importantly, immune cells seem to be required for neurogenesis in the hippocampus [314], and the subventricular zone [315], probably through the excretion of soluble mediators, which stimulate the proliferation and differentiation of neural stem cells. To determine whether myelin-laden macrophages have enhanced capacity to promote neurogenesis or axonal sprouting, cocultures with neural stem cells, or culturing neural stem cells in the presence of myelin-laden macrophage-supernatants would provide significant information.

Could lipid mediators be used as a treatment strategy for MS?

There is increasing evidence that lipid mediators play a key role in the resolution of inflammation [316-320]. The phagocytosis of myelin by macrophages may therefore result in the production of anti-inflammatory lipid mediators, in parallel or addition to the production of anti-inflammatory cytokines [120-122]. For example, myelin ingestion by macrophages enhances expression of prostaglandin E_2 synthase [120] and APC promote Th2 responses in the presence of prostaglandin E_2 [321,322].

The main source for lipid mediators are membrane glycerophospholipids, which contain a vast proportion of arachidonic and docosahexaenoic acid. These precursor lipids are converted

enzymatically by lipases and lipoxygenases, or through oxidation, into a plethora of intermediate products, which have distinct functions in inflammatory processes. These intermediate products, such as prostaglandins and leukotrienes, in turn actively switch on the transcription of enzymes required for the generation of lipid mediators such as resolvins, protectins, and lipoxins, which are anti-inflammatory and critical to resolving inflammation [319,320,323]. The active switch into the generation of anti-inflammatory lipid products underscores the ability of leukocytes to program the self-limiting response to inflammation.

Hence, the inflammatory cells itself actively resolve inflammation after an infection, likely via the secretion of resolvins, protectins, and lipoxins [319,320,323]. It is conceivable that autoimmune disease entails difficulties in appropriate production of endogenous compounds, thereby disturbing the self-limiting capacity of inflammation. Instead of inhibiting the inflammatory processes by for instance blocking antibodies, the use of agonists that stimulate the key points within the control of endogenous mechanisms for resolving inflammation has the potential to open new roads for therapy. Lipid-mediators, such as resolvins, protectins, lipoxins, and LXR ligands likely are such agonists and may therefore provide an interesting target for treatment strategies in autoimmunity [323].

Box 2. Outstanding questions.

- Is drainage of CNS antigens in normal circumstances involved in maintaining tolerance against these antigens, as proposed for other tissues?
- Can different APC subsets leave the CNS and gain access to the draining lymph nodes? Do macrophages filled with debris survive and migrate out of the CNS? Do dendritic cells transfer CNS antigens to present to T cells in the draining lymph nodes? Can microglia under strongly inflammatory and degenerative conditions acquire sufficient motility to emigrate the CNS?
- The deep cervical lymph nodes drive mucosal tolerance and in tolerance against brain-injected antigens. This is, however, not established for the superficial cervical lymph nodes, the lumbar lymph nodes, or the spleen. Are the distinct CNS-draining secondary lymphoid organs therefore similarly involved in the induction of immunity against CNS antigens?
- Are the CNS-draining lymph nodes a target organ for treatment strategies in neuroinflammatory diseases, such as MS? For instance, should drugs be administered intranasally?
- Are myelin-laden macrophages capable of transmitting their anti-inflammatory properties to other CNS-resident or infiltrating immune cells?
- Are myelin-laden macrophages neuroprotective? Do they promote tissue repair, remyelination, and astrogliosis?
- Do myelin-laden macrophages acquire their anti-inflammatory characteristics through lipid-mediators, such as lipoxins, resolvins or protectins? Can inflammation during MS be inhibited by the administration of such lipid-mediators?

Closure

The experiments performed in this thesis have provided a deeper insight in the role of the CNS-draining lymph nodes during CNS inflammation and in the anti-inflammatory action of myelin-laden macrophages present in and outside the CNS at crucial locations for immune activation. We have demonstrated that CNS antigens drain to the CNS-draining lymph nodes during CNS insult. At least part of these antigens are present in APC, implying that CNS antigen-specific immune responses can be generated in the CNS-draining lymph nodes rather than that drainage just occurs as a side effect of CNS damage. In concordance, myelin antigen-reactive proliferation was found in the CNS-draining lymph nodes of EAE-affected animals, suggesting that immune responses initiated within these lymph nodes contribute to CNS inflammation. Indeed, removing the CNS-draining lymph nodes prior to EAE induction reduced the relapse burden of EAE. The intriguing observations that the majority of myelin-laden cells have an anti-inflammatory phenotype, as myelin-laden cells in MS brain lesions, and that part of the myelin-laden APC within the cervical lymph nodes express the lymph node-homing receptor CCR7 raises the question whether phagocytes are capable of actively transporting CNS antigens from the CNS to the draining lymph nodes.

Furthermore, the anti-inflammatory phenotype of myelin-laden macrophages suggests that they are involved in limiting detrimental immune responses or even in the resolution of inflammation during MS. Our findings that myelin-laden macrophages inhibit Th1 differentiation and suppress EAE severity support this hypothesis. However, myelin-laden macrophages also produce chemoattractants that attract monocytes *in vitro*. Does this also occur *in vivo*? Do they transfer their anti-inflammatory phenotype to these newly attracted monocytes? Do myelin-laden macrophages communicate with other cells within the CNS? These and other outstanding questions are listed in Box 2. To better understand pathogenic mechanisms and to find new roads for therapeutic interventions for MS and other neuroinflammatory diseases future studies will need to answer these questions.

Chapter | 7

Summary | Samenvatting

Summary

Multiple sclerosis (MS) is characterized by demyelination and irreversible neuronal damage within the central nervous system (CNS). Although the cause of this damage is not yet elucidated, autoreactive lymphocytes against myelin and neuronal antigens are likely instrumental. In view of understanding MS pathogenesis and possible therapeutic interventions aiming to limit activation of autoreactive lymphocytes, it is crucial to know in what anatomical locations these lymphocytes are activated. This may occur in the CNS-draining secondary lymphoid organs, in the perivascular spaces within the CNS, and in the parenchyma of MS lesions. We hypothesized that autoreactive lymphocytes are activated within the CNS-draining lymph nodes following drainage of CNS antigens, thereby contributing to inflammation during MS and its animal model EAE. To test this, we combined analysis of human MS lymph nodes with functional studies, using distinct animal models for brain inflammation and injury.

We previously described the presence of myelin antigen-containing cells in the cervical lymph nodes of MS patients and EAE-affected animals. In **chapter 2**, we extended these findings to neuronal antigens and showed that the frequencies of CNS antigens within the cervical lymph nodes correlated with the extent and type of CNS damage in the CNS of mice with distinct types of CNS damage. Furthermore, we found differential drainage to the distinct CNS-draining lymph nodes in these mouse models, indicating that CNS antigens follow specific drainage routes.

Next, we determined that neuronal antigens were present in antigen presenting cells. Nevertheless, cells isolated from the CNS-draining lymph nodes of EAE-affected mice did not proliferate against neuronal antigens *in vitro*. Surprisingly, all neuronal antigen-containing cells in the cervical lymph nodes of MS patients and EAE-affected rhesus monkeys were pro-inflammatory, whereas the majority of myelin-laden cells expressed anti-inflammatory molecules. These data suggest that the antigen or the inflammatory environment direct the phenotype of the APC. This may reflect a different origin of the cells or different drainage mechanisms. Indeed, we show that neuronal antigen-containing cells in human cervical lymph nodes did not express the lymph node homing receptor CCR7, whereas myelin antigen-containing cells *in situ* and *in vitro* did. Nevertheless, the cervical lymph nodes of EAE-affected CCR7-deficient mice contained equal quantities of CNS antigens as wild type mice.

In **chapter 3**, we surgically removed the superficial cervical lymph nodes, deep cervical lymph nodes and the lumbar lymph nodes prior to EAE induction to functionally assess the role of the CNS-draining lymph nodes. We used three mouse EAE models, representing acute, chronic, and chronic-relapsing EAE to study the effect of lymphadenectomy in distinct phases of EAE. Excision of the CNS-draining lymph nodes in chronic-relapsing EAE reduced and delayed the relapse burden and EAE pathology within the spinal cord, indicating that immune reactions against CNS antigens in the CNS-draining lymph nodes contribute to disease.

To test whether immune reactions against CNS-antigens are indeed initiated within the CNS-draining lymph nodes, we restimulated lymph node cells isolated from EAE-affected mice *in vitro*. The superficial cervical lymph nodes from EAE-affected mice demonstrated proliferation against the immunizing peptide, and the deep cervical lymph nodes, lumbar lymph nodes, and spleen demonstrated additional proliferation against additional myelin peptides, indicating intermolecular epitope spreading. Proliferation was highest in the lumbar lymph nodes, reflecting the fact that EAE lesions are predominantly present in the spinal cord.

Myelin-laden macrophages are present in the CNS-draining lymph nodes, MS lesions and in the CSF of MS patients and EAE-affected animals. This suggests that myelin-laden macrophages actively migrate within and between the different anatomical compartments. The aim of **chapter 4** was therefore to elucidate whether myelin ingestion promotes the migrating capacity of macrophages. Mouse bone marrow-derived macrophages increase CCR7 and CXCR3 surface protein expression after myelin ingestion *in vitro*. Concordantly, myelin-laden macrophages demonstrated enhanced migration towards CCL21 and CXCL10, suggesting that myelin-laden macrophages are apt at migrating between CNS parenchyma, perivascular spaces, CSF, and the CNS-draining lymph nodes.

Furthermore, myelin-laden macrophages are optimally positioned to recruit and interact with other leukocytes. The second aim of **chapter 4** was therefore to analyze whether myelin-laden macrophages secrete inflammatory chemokines involved in the recruitment of leukocytes into the CNS. Myelin ingestion resulted in a slightly higher mRNA expression of CCL2, CCL3 and CCL4. In parallel, myelin-laden macrophages attracted significantly more myeloid cells, but not lymphocytes, as compared to control macrophages. These *in vitro* data suggest that myelin-laden macrophages might contribute to myeloid cell recruitment into the CNS *in vivo*, thereby contributing to the regulation of CNS inflammation.

In **chapter 5**, we investigated whether myelin-laden macrophages interact with T cells. Since myelin-laden macrophages have an anti-inflammatory phenotype, we hypothesized that interaction with T cells results in a protective response. We show that both human and mouse myelin-laden macrophages generated *in vitro* are equipped for antigen presentation. Strikingly, increased myelin ingestion by human macrophages resulted in a dose-dependent increment in antigen-presenting capacity, enhancing proliferation of both naïve T cells as well as memory T cells. In addition to these *in vitro* data, mouse myelin-laden macrophages induced naïve ovalbumin-specific $CD4^+$ T cell proliferation *in vivo* and suppressed Th1 differentiation. Functionally, myelin-laden mouse macrophages reduced EAE severity when administered during immunization.

In conclusion, our data imply that encephalitogenic immune reactions within the CNS-draining lymph nodes contribute to detrimental inflammation during EAE. These results suggest that nasal administration of medication is an attractive therapeutic strategy in MS, targeting both the CNS-draining lymph nodes as well as the brain. Furthermore, our data show that myelin uptake results in the induction of a population of regulatory macrophages, which modulate T cell skewing and protect against autoimmune-mediated disease.

Samenvatting

Multipele sclerose (MS) kenmerkt zich door demyelinisatie en neuronale schade aan het centrale zenuwstelsel (CNS). Hoewel de oorzaak onbekend is, spelen autoreactieve lymfocyten waarschijnlijk een belangrijke rol. Om de pathogenese van MS te begrijpen en potentiële therapieën te ontwikkelen die gericht zijn tegen activatie van autoreactieve lymfocyten, is het cruciaal te weten waar in het lichaam deze lymfocyten worden geactiveerd. Dit kan plaatsvinden in de CNS-drainerende lymfeklieren, in de perivasculaire ruimten in het CNS, of in het parenchym van MS laesies. Onze hypothese was dat autoreactieve lymfocyten worden geactiveerd in de CNS-drainerende lymfeklieren na drainage van CNS antigenen, en dat dit bijdraagt aan ontsteking in MS en in het diermodel voor MS (EAE). Om deze hypothese te testen hebben we humaan MS weefsel geanalyseerd en dit gecombineerd met functionele studies, waarin we gebruik maakten van verschillende diermodellen voor hersenontsteking en hersenschade.

Eerder hebben we beschreven dat myeline antigenen aanwezig zijn in de cervicale lymfeklieren van MS patiënten en dieren met EAE. In **hoofdstuk 2** is beschreven, dat ook neuronale antigenen aanwezig zijn in de cervicale lymfeklieren. Daarnaast lieten we zien dat het aantal en type CNS antigenen in de lymfeklieren correleert aan de omvang van de breinschade en dat CNS antigenen in verschillende hoeveelheden draineren naar de verscheidene CNS-drainerende lymfeklieren.

Vervolgens hebben we bepaald dat neuronale antigenen aanwezig zijn in antigeen presenterende cellen. Desondanks vonden we geen proliferatie tegen neuronale antigenen na restimulatie van lymfekliercellen *in vitro*. Alle cellen met neuronale antigenen hadden een pro-inflammatoir fenotype, terwijl de meeste cellen die myeline antigenen bevatten anti-inflammatoire moleculen tot expressie brachten. Dit suggereert dat de antigenen uit een verschillende inflammatoire omgeving komen, of dat de antigenen door middel van verschillende drainage mechanismen naar de CNS-drainerende lymfeklieren zijn gedraineerd. Cellen met neuronale antigenen brengen inderdaad niet de chemokine receptor CCR7 tot expressie, terwijl myeline-bevattende cellen *in situ* en *in vitro* dat wel doen. Desalniettemin bevatten de cervicale lymfeklieren van CCR7-deficiënte muizen evenveel CNS antigenen als de cervicale lymfeklieren van wild type muizen.

Om de rol van de CNS-drainerende lymfeklieren functioneel te bepalen, hebben we de diepe cervicale, oppervlakkige cervicale, en lumbale lymfeklieren operatief verwijderd voordat EAE geïnduceerd werd. Hiervoor hebben we gebruik gemaakt van drie verschillende EAE muismodellen die acute, chronische, en relapsing-remitting ziekte representeren. In **hoofdstuk 3** laten we zien dat verwijdering van de CNS-drainerende lymfeklieren de ziekteverschijnselen gedurende de relapsing fase van de ziekte vermindert en vertraagt. Dit duidt op initiatie van CNS antigeen-specifieke afweerreacties in de CNS-drainerende lymfeklieren. Om dit te onderzoeken hebben we lymfeklieren

geïsoleerd uit muizen met EAE en cellen uit deze lymfeklieren *in vitro* gerestimuleerd met myeline antigenen. Cellen uit de oppervlakkige cervicale lymfeklieren prolifereerden alleen tegen het peptide waarmee de muizen geïmmuniseerd waren. De diepe cervicale lymfeklieren, de lumbale lymfeklieren en de milt prolifereerden bovendien ook tegen andere myeline antigenen, wat duidt op epitoop spreiding.

Myeline-geladen macrofagen zijn aanwezig in de CNS-drainerende lymfeklieren, in MS laesies en in het hersenvocht van MS patiënten en dieren met EAE. Dit suggereert dat deze macrofagen migreren tussen deze anatomische compartimenten. Het doel van **hoofdstuk 4** was daarom om te onderzoeken of myeline opname de migratiecapaciteit van macrofagen promoot. Muis macrofagen brengen de chemokine receptoren CCR7 en CXCR3 verhoogd tot expressie na myeline opname *in vitro*. In overeenstemming hiermee migreren myeline-geladen macrofagen *in vitro* meer naar CCL21 en CXCL10. Deze resultaten suggereren dat myeline-geladen macrofagen inderdaad kunnen migreren tussen het parenchym, het hersenvocht en de drainerende lymfeklieren.

Myeline-geladen macrofagen in de verschillende anatomische compartimenten zijn optimaal gepositioneerd om andere afweercellen te rekruteren en om daarmee interactie aan te gaan. Het tweede doel van **hoofdstuk 4** was daarom om te onderzoeken of myeline opname de productie van chemokines induceert die betrokken zijn bij de rekrutering van afweercellen in het CNS. Myeline opname zorgde voor een bescheiden verhoging van CCL2, CCL3, en CCL4 mRNA expressie. Bovendien trokken myeline-geladen macrofagen meer myeloïde cellen aan, maar geen lymfocyten, vergeleken met controle macrofagen. Deze *in vitro* data suggereren dat myeline-geladen cellen bijdragen aan de rekrutering van afweercellen in het CNS tijdens MS.

In **hoofdstuk 5** hebben we onderzocht of myeline-geladen macrofagen interactie aangaan met T cellen. Aangezien myeline-geladen macrofagen *in vivo* en *in vitro* een anti-inflammatoir fenotype hebben, hypothetiseerden we dat interactie met T cellen zou resulteren in een beschermende respons. Onze resultaten laten zien dat humane en muis macrofagen na myeline opname de moleculen tot expressie brengen die nodig zijn voor antigeen presentatie. Bovendien zorgde myeline opname door macrofagen voor een dosis afhankelijke toename van T cel proliferatie van zowel naïeve als geheugen T cellen *in vitro*. Ook *in vivo* induceerden muis myeline-geladen macrofagen naïeve T cel proliferatie en onderdrukten ze T helper 1 differentiatie. Bovendien verminderden myeline-geladen macrofagen de ziekteverschijnselen van EAE.

Concluderend kunnen we zeggen dat afweerreacties tegen CNS antigenen worden geïnitieerd in de CNS-drainerende lymfeklieren en dat dit bijdraagt aan ontsteking in het CNS tijdens EAE. Deze resultaten suggereren dat medicijnen intranasaal toegediend kunnen worden, zodat zowel de CNS-drainerende lymfeklieren, als het CNS bereikt worden. Daarnaast laten onze data zien dat myeline opname resulteert in een populatie regulatoire macrofagen, die T cel differentiatie beïnvloeden en beschermen tegen autoimmuun ziekte.

Part V

Appendices

Part V

Appendices

Appendix I

References

Appendix I

References

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Appendix II

Dankwoord

Appendix II

Dankwoord

Dankwoord

H eel veel mensen hebben bijgedragen aan dit proefschrift en mijn fantastische promotietijd:
A ls eerste Leonie, copromotor, een goede combinatie van expertise en ontspanning
R ob, betrokken en daadkrachtig
T evens Jon, promotor, uniek in je gedrevenheid en positivisme
E e802, supercollega's
L etterlijk en figuurlijk is Marie-José een bron van energie
I n haar doen en laten is Annet een rots in de branding
J uweel: Marjan blinkt uit in haar kunde en karakter
K eer op keer klaarstaan, dat is Jane

B ergen werk verzet dankzij 'mijn' studenten Ilona, Petra, Benjamin, Marijke en Sara
E n natuurlijk Ruth, samen proeven doen en brainstormen tijdens etentjes
D ierverzorgers van het EDC, jullie vakkundigheid staat buiten kijf
A ltijd behulpzaam en goed geregeld, karakteristiek voor Wendy
N iemand die zo snel en nauwkeurig tekst kan redigeren als Joke
K ey collaborators: Sandra, Bert, Rogier, Janneke, Edward, Ingo, Uta, Christiane, Christian, Andreas
T ar, voor de allermooiste foto's

A io's, in het bijzonder Els, Esther, Fleur en Karin. Weekenden en borrels, Rudi en Frank
L ab Medische Fysiologie, hele fijne nieuwe collega's
L eslie en Caroline, paranimfen waar ik trots op ben
E en heleboel lieve vrienden en familie, voor de nodige ontspanning
M ałgosia i Zbyszek, dziękuję za serce i cierpliwość
A nsporen tot verder studeren en alsmaar doorleren. Papa, je hebt me altijd gesteund
A an jou liefde heb ik veel gehad. Mama, bedankt voor je bemoedigende woorden en kaartjes
L iefste Dominik, jou vertrouwen gaf me alle moed die nodig was om dit af te ronden

Appendix III

Curriculum vitae

Appendix III

Curriculum vitae

Marloes van Zwam was born on June 17th 1979 in Rotterdam, the Netherlands. She completed secondary school in 1997 at the City College St. Franciscus in Rotterdam and proceeded to study Human Nutrition and Health at the University of Wageningen. During her study, she performed an internship at Numico B.V. in Wageningen under supervision of Dr. I.F. Palm and Dr. E.M. van der Beek, where she developed a rat model for female subfertility. Subsequently, she did an internship at the department of Toxicology of the University of Wageningen in collaboration with the department of Health Risk Analysis and Toxicology of the University of Maastricht under supervision of Dr. G.M. Alink, Dr. Y.E.M. Dommels, Dr. T.M.C.M. de Kok en Dr.ir. H.J.J. Moonen, where she investigated the role of fatty acids on DNA adduct formation.

Because of her interest in immunology, she did an extra internship at the University of British Columbia and the Vancouver General Hospital in Vancouver, Canada under supervision of Prof.dr. K. Dorovini-Zis en Prof.dr. I.M.C.M. Rietjens. Here, Marloes started her research on multiple sclerosis, specifically on the expression of the immunoproteasome and MHC class I molecules by human brain microvessel endothelial cells. In 2003, she obtained her master's degree and started her PhD described in this thesis. She performed this PhD at the department of Immunology of the Erasmus MC under supervision of Prof.dr. J.D. Laman en Dr. L.A. Boven, and as part of the MS centre ErasMS.

During her PhD, Marloes attended several conferences in the Netherlands and abroad to present her data. Furthermore, she did research at the Institute of Cell Biology and Neurobiology of the Charité Humboldt University in Berlin, Germany under supervision of Prof.dr. I. Bechmann, and at the Institute of Clinical Pharmacology of the Charité Humboldt University in Berlin, under supervision of Dr. C. Meisel.

Since December 2007, Marloes works as a postdoc at the department of Medical Physiology of The University Medical Centre Groningen under supervision of Prof.dr. H.W.G.M. Boddeke en Prof.dr. K.P.H. Biber on the functional role of the orphan chemokine receptor CCX-CKR.

Marloes van Zwam werd op 17 juni 1979 geboren te Rotterdam. In 1997 behaalde zij haar gymnasium diploma aan het City College St. Franciscus te Rotterdam en startte zij de studie Humane Voeding en Gezondheid aan Wageningen Universiteit te Wageningen. Tijdens haar studie verrichtte zij een afstudeervak bij Numico B.V. te Wageningen onder begeleiding van Dr. I.F. Palm en Dr. E.M. van der Beek, waar zij een rat model voor vrouwelijke subfertiliteit heeft ontwikkeld. Haar tweede afstudeervak voerde zij uit bij de leerstoelgroep Toxicologie van Wageningen Universiteit in samenwerking met de afdeling Gezondheids Risico Analyse en Toxicologie (GRAT) van de Universiteit van Maastricht onder begeleiding van Dr. G.M. Alink, Dr. Y.E.M. Dommels, Dr. T.M.C.M. de Kok en Dr.ir. H.J.J. Moonen, alwaar zij de rol van vetzuren op DNA adduct vorming onderzocht.

Vanwege haar interesse in de immunologie, heeft zij een extra stage gelopen bij de Universiteit van British Columbia en het Vancouver General Hospital in Vancouver te Canada onder begeleiding van Prof.dr. K. Dorovini-Zis en Prof.dr. I.M.C.M. Rietjens. Hier werd de interesse van Marloes naar multipele sclerose aangewakkerd. Zij bestudeerde de expressie van het immunoproteasoom en MHC klasse I moleculen door humane endotheel cellen van bloedvaten uit het brein. In 2003 behaalde zij haar doctoraaldiploma en begon zij aan haar promotieonderzoek, beschreven in deze dissertatie, op de afdeling Immunologie van het Erasmus MC onder begeleiding van Prof.dr. J.D. Laman en Dr. L.A. Boven, onderdeel uitmakend van het MS centrum ErasMS.

Gedurende haar promotieonderzoek heeft Marloes verscheidene congressen in binnen- en buitenland bezocht om haar data te presenteren. Daarnaast heeft zij buitenlandse werkbezoeken gebracht aan The Institute of Cell Biology and Neurobiology, Charité Humboldt Universiteit te Berlijn, Duitsland waar zij onderzoek verrichtte onder begeleiding van Prof.dr. I. Bechmann, en aan The Institute of Clinical Pharmacology, Charité Humboldt Universiteit te Berlijn, Duitsland waar zij onderzoek verrichtte onder begeleiding van Dr. C. Meisel.

Vanaf december 2007 werkt Marloes als postdoc op de afdeling Medische Fysiologie onder begeleiding van Prof.dr. H.W.G.M. Boddeke en Prof.dr. K.P.H. Biber, alwaar zij de functionele rol van de chemokine receptor CCX-CKR bestudeert.

Appendix IV

PhD portfolio

Appendix IV

PhD portfolio

Name PhD student	Marloes van Zwam
Erasmus MC Department	Immunology
PhD period	August 2003 – November 2007
Promotor	Prof.dr. J.D. Laman
Copromtor	Dr. L.A. Boven

PhD training

General academic skills	
Biomedical English Writing and Communication	2004
Ethics and Scientific Integrity	2006
Management for PhD Students and Postdocs (NIBI [1])	2007
Research skills	
Laboratory Animal Science (Article 9)	2001
Basic Radiation Protection 5B	2001
Biomedical Research Techniques (MolMed [2])	2003
Flow Cytometry: From Calibration to Application (Nova Knowledge)	2003
In Vivo Imaging (MolMed)	2004
Classical Methods for Data Analysis (NIHES [3])	2005
Practical Introduction into Laser Scanning Microscopy (AOIC [4])	2007
In-depth courses	
Molecular Immunology (MolMed)	2004
5th ESNI course (European School for Neuroimmunology)	2004
Immunity in the Central Nervous System (MolMed)	2005
Inflammatory Mechanisms in Neurodegenerative Disease (MolMed)	2005
Didactic skills	
Basisdidactiek voor het Hoger Onderwijs (OEC Rotterdam [5])	2005
(Inter)national conferences	
Dutch MS Research Meeting, Paris, France (Dec 9-10)	2003
Annual Meeting NVVI [6], Noordwijkerhout, the Netherlands (Dec 18-19)	2003
8th MolMed Day, Rotterdam, the Netherlands (Jan 21)	2004
Annual meeting NVVI, Lunteren, the Netherlands (April 1-2)	2004
7th International Congress of Neuroimmunology, Venice, Italy (Sept 28-Oct 2)	2004
JAMI [7], Maastricht, the Netherlands (Oct 20-23)	2004

Dutch MS Research Meeting, Amsterdam, the Netherlands (Dec 23-24)	2004
9th MolMed Day, Rotterdam, the Netherlands (Feb 9)	2005
Annual meeting NVVI, Lunteren, the Netherlands (April 7-8)	2005
Dutch MS Research Meeting, Amsterdam, the Netherlands (Nov 24-25)	2005
10th MolMed Day, Rotterdam, the Netherlands (Feb 6)	2006
Annual meeting NVVI, Lunteren, the Netherlands (March 23-24)	2006
8th International Congress of Neuroimmunology, Nagoya, Japan (Oct 15-19)	2006
Dutch MS Research Meeting, Rotterdam, the Netherlands (Nov 16-17)	2006
Annual meeting NVVI, Noordwijkerhout, the Netherlands (Dec 7-8)	2006
11th MolMed Day, Rotterdam, the Netherlands (Feb 15)	2007
Annual Meeting NVVI, Lunteren, the Netherlands (March 22-23)	2007
Keystone Symposia, Colorado, USA (April 24)	2007
Dutch MS Research Meeting, Hasselt, Belgium (Nov 15-16)	2007

Seminars

Dept. Immunology, Erasmus MC, Rotterdam, the Netherlands (Feb 22)	2005
Dept. Neurology, Charité Humboldt University, Berlin, Germany (June 21)	2006
Dept. Medical Physiology, UMCG, Groningen, the Netherlands (Feb 9)	2007
Dept. Immunology, Erasmus MC, Rotterdam, the Netherlands (Dec 16)	2008

Presentations

Alternative activation of macrophages containing self-antigen during central nervous system autoimmunity (8th MolMed Day 2004)	Poster
A regulating role for CNS antigen-containing antigen presenting cells in the cervical lymph nodes during MS (7th International Congress of Neuroimmunology 2004)	Poster
A potential regulating role for CNS antigen-containing antigen-presenting cells in the cervical lymph nodes during MS (Annual MS Research Meeting 2004)	Oral
CNS antigen-containing antigen presenting cells in the cervical lymph nodes may regulate multiple sclerosis (JAMI 2004)	Poster
A potential regulatory role for central nervous system antigen-containing antigen-presenting cells in MS (9th MolMed Day 2005)	Poster
Brain-derived neuronal antigens are present in cervical lymph nodes in MS and EAE (Annual MS Research Meeting 2005)	Oral
Brain-derived antigens in the cervical lymph nodes: implications for multiple sclerosis? (10th MolMed Day 2006)	Poster
Neuronal antigen-containing antigen-presenting cells in cervical lymph nodes of multiple sclerosis patients and EAE animals are functionally distinct from myelin antigen-containing cells (8th International Congress of Neuroimmunology 2006)	Poster

Antigen presentation by foamy macrophages results in enhanced activation of both naïve and memory T cells (Annual MS Research Meeting 2006)	Oral
Antigen presentation by foamy macrophages results in enhanced activation of both naïve and memory T cells (11th MolMed Day 2007)	Poster
Antigen presentation by foamy macrophages results in enhanced activation of both naïve and memory T cells (Keystone Symposia 2007)	Poster
CNS-draining lymph nodes drive relapse severity in EAE (Annual MS Research Meeting 2007)	Oral

Teaching activities

Supervising practicals

Colon en colon-carcinoma	2003
Cellular interactions during the immune response	2004-2007
Hemopoiese	2004-2007
Spleen and mucosa-associated lymphoid tissue (MALT)	2007

Supervising three trainees from middle and higher laboratory education	2004-2007
Supervising two master students writing their thesis	2005-2007

[1] Netherlands Institute for Bioscience

[2] Postgraduate school Molecular Medicine

[3] Netherlands Institute for Health Sciences

[4] Applied Optical Imaging Centre

[5] Onderwijsexpertise Centrum (Educational Expertise Centre)

[6] Nederlandse Vereniging voor Immunologie (Dutch Society for Immunology)

[7] Joint Annual Meeting of the German and Dutch Societies for Immunology

Appendix V

Publications

Appendix V

Publications

Van Zwam M, Huizinga R, Wierenga-Wolf AF, Melief MJ, Van Meurs M, Voerman JS, Biber KPH, Boddeke HWGM, Hintzen RQ, 't Hart BA, Amor S, Laman JD and Boven LA. Brain antigens in functionally distinct antigen-presenting cell populations in cervical lymph nodes in MS and EAE. *Journal of Molecular Medicine (in press)*

Van Zwam M, Huizinga R, Heijmans N, van Meurs M, Wierenga-Wolf AF, Melief MJ, Hintzen RQ, 't Hart BA, Amor S, Boven LA and Laman JD. Surgical excision of CNS-draining lymph nodes reduces relapse severity in chronic-relapsing experimental autoimmune encephalomyelitis. *Journal of Pathology (in press)*

Van Zwam M, Samsom JN, Nieuwenhuis EE, Melief MJ, Wierenga-Wolf AF, Van Ham P, Van Meurs M, Voerman JS, Laman JD and Boven LA. Myelin-laden macrophages suppress Th1 differentiation and suppress experimental autoimmune encephalomyelitis. *Submitted for publication*

Van Zwam M, Wierenga-Wolf AF, Melief MJ, Schrijver B, Laman JD and Boven LA. Myelin ingestion by macrophages promotes their motility and capacity to recruit myeloid cells. *Submitted for publication*

De Geus ED, **Van Zwam M**, Van Meurs M, Assink A, Voerman JS, Hintzen RQ, Laman JD and Boven LA. Adenosine receptor expression is altered in foamy macrophages in multiple sclerosis lesions and in vitro, resulting in an enhanced anti-inflammatory function. *Manuscript in preparation*

Van Hamburg JP, De Bruijn M, Ribeiro de Almeida C, **Van Zwam M**, Van Meurs M, De Haas E, Boon L, Samsom JN and Hendriks RW. Enforced expression of GATA3 allows differentiation of IL-17-producing cells, but constrains Th17-mediated pathology. *European Journal of Immunology,* 2008; 38(9):2573-2586

Mutlu L, Brandt C, Kwidzinski E, Sawitzki B, Gimsa U, Mahlo J, Aktas O, Nitsch R, **Van Zwam M**, Laman JD and Bechmann I. Tolerogenic effect of fiber tract injury: Peripheral apoptosis of T cells and reduction of EAE severity following entorhinal cortex lesion. *Experimental Brain Research,* 2007; 178(4):42-53

Boven LA, Van Meurs M, **Van Zwam M**, Wierenga-Wolf AF, Hintzen RQ, Boot RG, Aerts JM, Amor S, Nieuwenhuis EE and Laman JD. Myelin-laden macrophages are anti-inflammatory, consistent with foam cells in multiple sclerosis. *Brain,* 2006; 129(Pt 2):517-526

Moonen HJJ, Dommels YEM, **Van Zwam M**, Van Herwijnen MHM, Kleinjans JCS, Alink GM and De Kok TMCM. Effects of polyunsaturated fatty acids on prostaglandin synthesis and cyclooxygenase-mediated DNA adduct formation by heterocyclic aromatic amines in human adenocarcinoma colon cells. *Molecular Carcinogenesis,* 2004; 40(3):180-188