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Macrophages as mediators of protective autoimmunity in multiple sclerosis

Proefschrift voorgelegd tot het behalen van de graad van Doctor in de Biomedische Wetenschappen, te verdedigen door:

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universiteit hasselt

It is a mark of an educated mind to be able to entertain a thought without accepting it.

Aristotle (384 BC - 322 BC)

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Table of Contents

Table of contents I
List of Figures V
List of Tables VII
List of Abbreviations VIII

CHAPTER 1. INTRODUCTION AND AIMS 1

1.1 Basic concepts of Multiple sclerosis 1
1.1.1 Clinical aspects and diagnosis 2
1.1.2 Animal model of MS 4
1.1.3 Genetic and environmental aspects of MS 5
1.1.4 Immunopathogenesis 7
1.1.5 Lesion pathology 12
1.1.6 Therapeutic strategies 14
1.2 Macrophages in MS 17
1.2.1 Monocyte and macrophage function and heterogeneity 17
1.2.2 Macrophages in disease 18
1.2.3 Macrophages and microglia in MS 20
1.2.4 Role of macrophages and microglia in demyelination 23
1.2.5 Role of macrophages and microglia in axonal degeneration 25
1.2.6 Role of macrophages and microglia in CNS repair 27
1.2.7 Foamy macrophages in MS 30
1.3 Aims of the study 33

CHAPTER 2. MYELIN-PHAGOCYTOSING MACROPHAGES MODULATE AUTOREACTIVE T CELL PROLIFERATION 37

2.1 Introduction 39
2.2 Materials and methods 41
2.2.1 Animals 41
2.2.2 Isolation of peritoneal macrophages 41
2.2.3 Myelin isolation 41

2.2.4 Immunization and *in vivo* myelin treatment 42
2.2.5 Generation of antigen-specific lymphocytes 42
2.2.6 CFSE-labeling of lymphocytes 43
2.2.7 Coculture of macrophages with lymphocytes 43
2.2.8 [^{3}H]Thymidine incorporation 44 44
2.2.9 Nitrite formation 44
2.2.10 Histology and immunohistochemistry 44
2.2.11 Statistics 45

2.3 Results 46

2.2.1 Myelin-laden macrophages inhibit lymphocyte proliferation 46
2.2.2 Inhibition of lymphocyte proliferation is independent of the phagocytosis process or myelin-antigen presentation 46
2.2.3 Macrophages inhibit lymphocyte proliferation by the production of nitric oxide 50
2.2.4 Myelin modulates lymphocyte proliferation *in vivo* 51

2.4 Discussion 55

CHAPTER 3. MYELIN-DERIVED LIPIDS MODULATE MACROPHAGE ACTIVITY BY LIVER X RECEPTOR ACTIVATION **61**

3.1 Introduction 63
3.2 Materials and Methods 65

3.2.1 Animals 65
3.2.2 Myelin isolation 65
3.2.3 Cell culture 65
3.2.4 RNA isolation 66
3.2.5 Microarray analysis 66
3.2.6 Quantitative PCR 67
3.2.7 Cholesterol efflux assay 68
3.2.8 Nitrite formation and IL-6 production 69
3.2.9 Statistics 69

3.3 Results 70

3.3.1 Differentially regulated genes, biological processes and pathways in myelin-phagocytosing macrophages 70
3.3.2 QPCR validation of differentially expressed genes 74
3.3.3 Myelin-phagoctyosing macrophages have an increased capacity to dispose intracellular cholesterol 75
3.3.4 Myelin alters the macrophage phenotype by activating the LXRβ isoform 76
3.4. Discussion 79

CHAPTER 4. PHOSPHATIDYLSERINE PRESENT IN MYELIN ALTERS THE INFLAMMATORY PROPERTIES OF MYELIN-PHAGOCYTOSING MACROPHAGES BY ACTIVATING PPARS **83**

4.1 Introduction 85
4.2 Materials and methods 87
4.2.1 Animals 87
4.2.2 Myelin isolation 87
4.2.3 Preparation of liposomes 87
4.2.4 Cell culture 88
4.2.5 Nitrite formation and cytokine concentration 88
4.2.6 Induction of EAE and systemic liposome treatment 88
4.2.7 [^{3}H]Thymidine incorporation 89
4.2.8 Quantitative PCR 89
4.2.9 Immunohistochemistry 91
4.2.10 Statistics 91
4.3 Results 93
4.3.1 PS in myelin modulates the macrophages phenotype by activating PPARs 93
4.3.2 Systemically administered liposomes home primarily to splenic macrophages and ameliorate EAE 95
4.3.3 PSLs modulate T cell proliferation and the expression of pro- and anti-inflammatory mediators in the spleen 99
4.3.4 Myelin-phagocytosing macrophages display increased activation of PPARs in active MS lesions 100

4.4 Discussion 104

CHAPTER 5. INDUCTION OF COLEC12 EXPRESSION ON MYELIN-PHAGOCYTOSING MACROPHAGES AND IN ACTIVE MS LESIONS **109**

5.1 Introduction 111
5.2 Materials and methods 112
5.2.1 Myelin isolation and labelling 112
5.2.2 Cell culture 112
5.2.3 Myelin phagocytosis assay 112
5.2.4 Immunohistochemistry 113
5.2.5 Statistics 113
5.3 Results 114
5.3.1 COLEC12 is upregulated in a LXR- and PPAR-independent manner in myelin-phagocytosing macrophages 114
5.3.2 COLEC12 does not facilitate myelin internalization by macrophages and microglia 115
5.3.3 COLEC12 expression in increased in the center and rim of active MS lesion 117
5.4 Discussion 119

CHAPTER 6. SUMMARY AND GENERAL DISCUSSION **123**

CHAPTER 7. NEDERLANDSE SAMENVATTING **139**

Reference list 149
Curriculum Vitae 184
Bibliography 185
Dankwoord

List of Figures

Figure 1.1	The "inside-out" and "outside-in" models for MS	8
Figure 1.2	The immunepathogenesis of multiple sclerosis	11
Figure 1.3	Macrophage heterogeneity	19
Figure 1.3	(Myelin-phagocytosing) macrophages and microglia in MS pathogenesis	31
Figure 2.1	Myelin-phagocytosing macrophages inhibit TCR-triggered lymphocyte proliferation in an antigen-independent manner	47
Figure 2.2	Nitric oxide secreted by myelin phagocytosing macrophages inhibits lymphocyte proliferation *in vitro*	48
Figure 2.3	Myelin homing to CD169$^+$ macrophages in popliteal lymph nodes	51
Figure 2.4	Myelin inhibits lymphocyte proliferation in OVA-immunized animals	52
Figure 2.5	MBP-immunized animals demonstrate an aggravated disease course following myelin treatment	53
Figure 2.6	OVA does not influence lymphocyte reactivity to MBP in MBP-immunized animals	54
Figure 3.1	Quantitative PCR validation	74
Figure 3.2	Myelin- phagocytosing macrophages have an increased capacity to transfer intracellular cholesterol towards HDL	75
Figure 3.3	Myelin and T09 affect the expression of LXR response genes in a similar manner	76
Figure 3.4	Myelin alters the macrophage phenotype by activating the LXRβ isoform	78
Figure 4.1	Characteristics of myelin and liposomes	93
Figure 4.2	Myelin modulates the macrophages phenotype by PPARβ/δ activation	94
Figure 4.3	Homing properties of liposomes following systemic administration	95
Figure 4.4	Systemically administered liposomes home primarily to	

	splenic marginal zone and red pulp macrophages	96
Figure 4.5	Intravenously administered PSLs reduce CNS infiltration of immune cells and ameliorate EAE	97
Figure 4.6	PSLs have a therapeutic effect on EAE	98
Figure 4.7	PSLs suppress splenic cognate antigen specific proliferation	99
Figure 4.8	PSLs affect splenic expression of TNFα, iNOS and ARG-1	101
Figure 4.9	PPARs are activated in myelin-phagocytosing macrophages during active MS	102
Figure 5.1	COLEC12 is upregulated in a LXR- and PPAR-independent manner in myelin-phagocytosing-macrophages	114
Figure 5.2	COLEC12 is not involved in phagocytosis of myelin	115
Figure 5.3	COLEC12 expression in increased in the center and rim of active MS lesion	116
Figure 5.4	COLEC12 is highly expressed on foamy macrophages in perivascular cuffs	117
Figure 6.1	Summary of findings from this thesis	135

List of Tables

Table 3.1 Quantitative PCR primers 68

Table 3.2 Top 20 up- and downregulated genes in myelin-phagocytosing macrophages 71

Table 3.3 Overrepresented canonical pathways and biological functions (IPA) 72

Table 3.4 Overrepresented KEGG pathways and biological functions (DAVID) 73

Table 4.1 Quantitative PCR primers 90

List of Abbreviations

1-MT	1-Methyl-L-tryptophan
18S	18S subunit ribosomal RNA
7AAD	7 aminoactinomycin D
ABCA1	ATP-binding cassette transporter A1
ABCG1	ATP-binding cassette transporter G1
ActB	Beta actin
ADRP	Adipose differentiation related protein
ANOVA	Analysis of variance
APC	Antigen presenting cell
ApoE	Apolipoprotein E
ARG1	Arginase 1
ARR	Annual relapse rate
AUC	Area under the curve
BBB	Blood-brain barrier
BSA	Bovine serum albumin
cAMP	Cyclic adenosine monophosphate
CFSE	carboxyfluorescein diacetatesuccinimidyl ester
CLEC16A	C-type lectin-domain family 16 member A
CNS	Central nervous system
CNTF	Ciliary neurotrophic factor
COLEC12	Collectin sub-family member 12
ConA	Concanavalin
CRD	Carbohydrate recognition domain
CSF	Cerebrospinal fluid
CTP1a	Carnitine palmitoyltransferase 1a
CycA	Cyclophilin A
DAPI	4,6′-diamidino-2-phenylindole
DAVID	Database for annotation, visualization and integrated discovery
DC	Dendritic cell
DC-SIGN	Dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin
DiI	1,1″-diotadecyl-3,3,3′,3′,-tetramethylindocarbocyanide perchlorate
DMSO	Dimethylsulfoxide
DMT	Disease-modifying therapy
Dpi	Days post-immunization
EAE	Experimental autoimmune encephalomyelitis
EBNA	Epstein-Barr nuclear antigen
EBV	Epstein-Barr virus
EDTA	Ethylenediamine tetraacetic acid
ELISA	Enzyme-linked immunosorbant assay
FCS	Fetal calf serum
FDA	Food and drug administration
FITC	Fluorescein isothiocyanate
Foxp3	Forkhead box P3

GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GATA-3	GATA binding protein 3
GFAP	Glial fibrillary acidic protein
GM	Gray matter
HDL	High-density lipoprotein
HLA	Human leukocyte antigen
HMBS	Hydroxymethyl-bilane synthase
ICAM-1	Intercellular adhesion molecule 1
IDO	Indoleamine 2,3-dioxygenase
IFN	Interferon
Ig	Immunoglobulin
IGF-1	Insulin-like growth factor 1
IL	Interleukin
IPA	Ingenuity pathway analysis
iNOS	Inducible nitric oxide synthase
IRF8	Interferon regulatory factor 8
JC virus	John Cunningham virus
KEGG	Kyoto encyclopedia of genes and genomes
LDL	Low density lipoproteins
LFA-1	Lymphocyte function-associated antigen 1
LFB	Luxol fast blue
LN	Lymph node
L-NMMA	N^G-Monomethyl-L-arginine
LPS	Lipopolysaccharide
LXR	Liver X receptor
Ma	Macrophage
MAC-1	Macrophage antigen 1
MAG	Myelin associated glycoprotein
MBP	Myelin basic protein
MCP-1	Monocyte chemoattractant protein 1
MERTK	C-mer proto-oncogene tyrosine kinase
MHC	Major histocompatibility complex
MIAME	Minimum information about a microarray experiment
MMP	Metalloproteinase
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
MOBP	Myelin associated oligodendrocyte basic protein
MOG	Myelin oligodendrocyte glycoprotein
MRI	Magnetic resonance imaging
MS	Multiple sclerosis
mTOR	Mammalian target of rapamycin
Mye-Ma	Myelin-phagocytosing macrophage
NAWM	Normal-appearing white matter
NO	Nitric oxide
NOHA	N^G-Hydroxy-L-arginine
OPC	Oligodendrocyte precursor cell
OVA	Ovalbumin
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction

PC	Phosphatidylcholine
PCL	Phosphatidylcholine-containing liposome
PDK4	Pyruvate dehydrogenase kinase isozyme 4
PECAM-1	Platelet endothelial cell adhesion molecule 1
PEC	Peritoneal exudate cell
Pgk1	Phosphoglycerate kinase 1
PLP	Proteolipid protein
pMA	Peritoneal macrophage
PPAR	Peroxisome proliferator-activated receptor
PP-MS	Primary-progressive multiple sclerosis
PR-MS	Primary-relapsing multiple sclerosis
PS	Phosphatidylserine
PSL	Phosphatidylserine-containing liposome
QPCR	Quantitative polymerase chain reaction
RhoA	Ras homolog gene family member A
RGC	Retinal ganglion cells
RMA	Robust multichip average
RNA	Ribonucleic acid
RNS	Reactive nitrogen species
RORγt	RAR-related orphan receptor gamma
ROS	Reactive oxygen species
Rpl13A	Ribosomal protein L13A
RR-MS	Relapse-remitting multiple sclerosis
RXR	Retinoid X receptor
SCD1/2	Stearyl-CoA desaturase 1/2
SCS	Subcapsular sinus
SEL	Slowly expanding lesions
SEM	Standard error of the mean
SP-MS	Secondary progressive multiple sclerosis
SR	Scavenger receptor
T-bet	T-box transcription factor
Tbp	TATA box binding protein
TCR	T cell receptor
TGF	Tumor growth factor
Th cell	T helper cell
TNF	Tumor necrosis factor
TNFRSF1A	Tumor necrosis factor receptor family member 1a
Treg	Regulatory T cell
T09	T0901317
VCAM-1	Vascular-cell adhesion molecule 1
VLA-4	Very late antigen 4
WM	White matter
YWHAZ	Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation

1

Introduction and aims

1.1 Basic concepts of multiple sclerosis

1.1.1 Clinical aspects and diagnosis

The man who brought Multiple Sclerosis (MS) with all its complexities to the attention of the medical society is Jean Martin Charcot, who according to two of his students first familiarized himself with the disease by asking a woman showing motor problems to be his housemaid [1]. As a result, he was able to follow the disease course on a day-to-day basis up to her death, after which he arranged to examine her brain and spinal cord. In 1868 he described MS as an accumulation of inflammatory cells in a perivascular distribution in the brain and spinal cord of patients with episodes of neurologic dysfunction [2]. Since then, advances in life sciences have resulted in a detailed analysis of the etiology and pathogenesis of MS.

Clinical aspects of MS

Nowadays, MS is appreciated to be a chronic inflammatory disease of the central nervous system (CNS), characterized by multiple sites of inflammation, axonal degeneration and astrogliosis. The insulating myelin sheath surrounding the axons is destructed, resulting in impaired signaling between neurons. This reduced neuronal communication gives rise to a variety of symptoms such as muscle weakness, sensory disturbances, bowel and bladder dysfunction, spasticity and tremor, difficulty maintaining balance, cognitive impairments and fatigue [3, 4].

The prevalence of MS is approximately 2 million people worldwide with an incidence of about 1:1000 in Europe and Northern America [5, 6]. It is the most common cause of non-traumatic neurologic disability among young adults and women are more frequently affected than men [7-9]. The prognosis is variable, however, half of the patients require help with walking within 15 years after disease onset [4, 10]. According to clinical manifestations, MS patients can be subdivided into either relapsing-remitting (RR) MS, secondary-progressive (SP) MS, primary-progressive (PP) MS or progressive-relapsing (PR) MS [11-13]. The majority of patients (85%) develop RR-MS, which is marked by relapses of clinical symptoms that can last for days, weeks or months, followed by periods of remission. During remission the patient fully or partially recovers from the

deficits acquired during the flare-ups. The RR-MS subtype has a female predominance of approximately 2:1 [4]. The majority of the RR-MS patients will eventually develop SP-MS. This subtype is characterized by a steady progressive neurological deterioration with or without relapses and minor remissions and plateaus. In 10-20% of patients, the relapsing phase is lacking and the disease is progressive from the onset (PP-MS). Finally, less than 5% of patients suffer from PL-MS, which is progressive from the start, with intermittent flare-ups of worsening symptoms along the way. Whereas inflammation and the development of new lesions are regarded to be the substrate for RR-MS, neuronal degeneration and atrophy are hallmarks of progressive MS [12].

MS diagnosis

There is no single test for diagnosing MS. The main criteria for diagnosis are the occurrence of at least two different episodes in the disease course (time dissemination) and evidence of inflammation in at least two different sites in the CNS (space dissemination). Magnetic resonance imaging (MRI), evoked potentials and biochemical analysis of the cerebrospinal fluid (CSF) are used for evaluating these criteria [11, 14-16]. MRI has proven to be pivotal in identifying the number, size, inflammatory activity and the evolution over time of brain lesions [17]. However, the typical appearance of multiple lesions on MRIs is not specific for MS [7]. CSF analysis is performed to identify the presence oligoclonal bands, which consist of high concentrations of immunoglobulins (Igs), generated by activated B cells in the CNS or CSF [18, 19]. In approximately 90% of MS patients oligoclonal bands are detected [20]. Moreover, in patients with clinical isolated inflammatory demyelinating syndrome the presence of oligoclonal bands are predictive for acquiring clinically definite MS [21]. Similar to the appearance of CNS lesions, oligoclonal band are not specific for MS [22], and can merely be used as a screening tool for the diagnosis of MS. Finally, evoked potentials can be used as a screening tool for MS [23]. However, similar to MRI and CSF analysis, abnormal evoked potentials can occasionally occur in other conditions.

1.1.2 Animal models of MS

MS appears to be a unique human disorder, as no animals spontaneously develop a similar condition. Nonetheless, several different models are appreciated to mimic the clinical pathological and neurological features of MS [24, 25]. Experimental autoimmune encephalomyelitis (EAE) is the most extensively studied model. EAE was discovered accidently in humans during vaccination against rabies, using viruses grown on rabbit spinal cords. Individuals receiving the rabies virus vaccine developed encephalomyelitis due to hypersensitivity to the CNS debris contaminating the vaccine [26]. These observations resulted in the development of the first model of EAE for which monkeys were immunized with a CNS homogenate [27]. Since then, models in mice, rats, guinea pigs, rabbits, pigs, chickens and the common marmoset have been established and proven their value as a model to study MS [24, 25]. EAE can be induced by active immunization with whole CNS homogenate or myelin proteins such as myelin-basic protein (MBP), proteolipid protein (PLP) and myelin oligodendrocyte glycoprotein (MOG) or by adoptive transfer of myelin-reactive T cells [24, 25]. The clinical, pathological, and immunological response varies considerably, depending upon the mode of sensitization, the nature of the immunogen, and the genetic background of the animal model used. Also, infection with Theiler's murine encephalomyelitis virus has been reported to mimic MS pathogenesis [28]. Finally, mice expressing an MBP-specific T cell receptor (TCR) spontaneously develop EAE when challenged with microbial stimuli [29].

The value of EAE as a model of MS has been an active topic of debate, due to its inability to mimic some of the pathologic, immunologic, and chronic features of MS [26, 30, 31]. For instance, whereas IFNγ protected against chronic-progressive EAE [32], it worsened disease in clinical trials with MS patients [33]. Similarly, anti-tumor necrosis factor-α antibodies ameliorated EAE but were detrimental in MS patients [34, 35]. Nevertheless, the EAE model has also proved itself remarkably useful, as it has directly led to the development of several approved MS therapeutics [36]. Overall, EAE is a valuable tool for understanding particular aspects of MS pathogenesis and evaluating potential new therapeutics. However, investigators should be cautious with extrapolating findings in animals to humans.

1.1.3 Genetic and environmental aspects of MS

The precise etiology of MS is unclear, however, according to the current hypothesis, the disease develops in genetic susceptible individuals and requires additional exposure to environmental factors.

Genetic predisposition

The genetic contribution to the susceptibility of developing MS has been studied extensively [37]. Population, family and twin studies have demonstrated that first-, second- and third-degree relatives of MS patients are at higher risk to develop MS [38]. Moreover, elevated concordance rates were demonstrated in monozygotic twins (31%) compared to dizygotic (5%) and non-twin siblings (3%) [39]. Although the exact genes that are responsible for MS are still unknown, identified susceptibility genes are related to the immune response. The major histocompatibility complex (MHC) class II related human leukocyte antigens (HLA)-DR15/DQw6 (HLA-DRB1*1501, HLA-DQB1*0602 and HLA-DQA1*0102), located on chromosome 6p21, have been associated with an increased risk of getting MS [37, 40-42]. These alleles encode molecules involved in T cell antigen recognition. Notably, both carriage of HLA-DRB1*1501 and the presence of oligoclonal bands have been reported to hasten disease progression [43]. In addition to HLA class II genes, genome wide association studies also identified MHC class I, interleukin-2 receptor alpa (IL-2rα), IL-7r, IL-12a, IL-12b, CD6, CD58, CD86, C-X-C chemokine receptor type 5 (CXCR5), C-type lectin-domain family 16 member A (CLEC16A), c-mer proto-oncogene tyrosine kinase (MERTK) tumor necrosis factor receptor family member 1a (TNFRSF1A) and interferon regulatory factor 8 (IRF8) as potential risk genes for the development of MS [42, 44-47]. These data indicate polygenic influences on MS susceptibility and suggest that numerous genes, each with modest effects, contribute to MS risk.

Environmental factors

Disease discordance in monozygotic twins points towards an environmental importance in MS pathogenesis. In agreement, $CD4^+$ T cells from disease-discordant monozygotic twins do not show genetic, epigenetic or transcriptome differences that explain disease outcome [48]. Interestingly, epidemiological studies have demonstrated that MS occurrence follows a geographic pattern, as

prevalence raises with increasing distance from the equator [9, 49]. Moreover, migration from high- to low-risk areas before adolescence reduces the risk of acquiring MS [50, 51]. This protective effect is potentially due to vitamin D, which is produced after the exposure to ultraviolet radiation. Indeed, several studies have reported a positive relationship between vitamin D from the environment (sunlight or diet), circulating vitamin D status, and improved symptoms or prevention of MS [52-58]. Additionally, the seasonal fluctuations of circulating vitamin D at high latitudes may explain the month-of-birth effect in MS [59]. Experimental animal models of MS have validated the beneficial effects of vitamin D and 1,25(OH)(2)D(3) [57, 58]. Apart from vitamin D, hygiene has been linked to MS vulnerability in industrialized countries. This hypothesis holds that a relative lack of evolutionarily normal childhood infectious exposures, due to improved hygiene in developed countries, may predispose susceptible individuals to allergic and autoimmune diseases later in life [60, 61]. Finally, nutrition plays a role in the initiation and pathogenesis of MS and EAE [62, 63]. For instance, caloric restriction ameliorates EAE and neurologic deterioration is prevented in a subset of MS patients on a low fat diet [64, 65]. Additionally, preliminary results of our group show an aggravated disease severity of animals on a high fat diet. Other environmental factors like smoking, stress and drugs can also impact MS susceptibility and severity, and explain geographical differences.

Infectious agents

Several viruses have been considered to be involved in the pathogenesis of MS. Additionally, the risk of relapses and neurologic dysfunction is increased by infections [66, 67]. Recent attention has focused on Epstein-Barr virus (EBV), which is strongly associated with MS. For instance; virtually all MS patients are seropositive for EBV [68-70], EBV seroconversion occurs prior to development of MS [71], mononucleosis infected individuals have an increased risk of MS [72, 73], disease onset is correlated with an increased presence of Epstein-Barr nuclear antigen (EBNA)-specific (EBNA) antibodies [74, 75], EBV-infected B cells are enriched in MS brain tissues [76] and clonally expanded EBNA1-specific T cells show cross-reactivity with myelin antigens [77, 78]. Thus, there is a convincing association between MS and EBV infection. Nonetheless, a causal relationship has not been

established. Also viral infections with varicella–zoster virus, human herpesvirus-6, torque teno virus and human endogenous retroviruses, like MS-associated retroviral agent have been associated with MS pathogenesis. However, similar to EBV, no causal relationship between these viruses and MS has been recognized [79, 80]. Mechanisms proposed to explain how viruses might trigger autoreactive immune responses in MS include bystander activation, molecular mimicry and superantigen activation [79]. In summary, MS is a complex heterogenous disease in which genetic and environmental factors synergistically impact disease initiation and progression.

1.1.4 Immunopathogenesis

Although the precise etiology of MS is unknown, MS is traditionally considered to be an autoimmune inflammatory disorder mediated by an aberrant T cell attack against CNS elements, particularly myelin [81]. The major cause of the permanent neurologic disabilities is considered to be axon loss, caused by inflammatory mediators, loss of myelin-derived support, disruptions of axonal ion concentration, energy failure or Ca^{2+} accumulation [82, 83]. The most generally accepted "outside-in" hypothesis states that myelin-reactive T cells become activated in periphery, potentially by one or more of the previously mentioned immunogenetic or environmental factors (figure 1.1). Although both healthy and MS patients have comparable frequencies of circulating autoreactive myelin-specific T cells in their blood [84], some functional differences are reported when comparing T cell responses to myelin antigens. For example, differences were reported with respect to cytotoxic properties, costimulation requirement and avidity for the autoantigen [85-88]. Moreover, myelin-basic protein (MBP) reactive T cells show a higher level of activation in MS patients as compared to healthy individuals [89]. More recently this "outside-in" model has been challenged by a competing view that states that the initial trigger for multiple sclerosis is primary CNS cytodegeneration and that the subsequent release of antigenic constituents promote inflammation and autoimmunity [82, 90, 91]. The underlying mechanism for this "inside-out" hypothesis remains to be clarified (figure 1.1). Irrespective of the initial trigger, autologous T cells are indisputable pivotal in orchestrating the immunopathological processes involved in myelin sheath damage in both models [92-94].

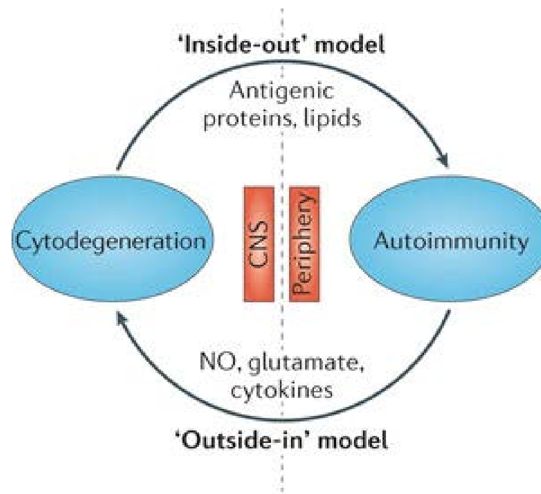

Figure 1.1 The "inside-out" and "outside-in" models for multiple sclerosis. MS has for a long time been considered to be an autoimmune disease in which peripheral activation of myelin-reactive T cells is the initial trigger ("outside-in" model). Following activation, auto-reactive T cells cross into the CNS and together with other immune cells they destroy CNS elements. Recently, local CNS cytodegeneration has been postulated to be the initial event in MS ("inside-out" model). The subsequent release of antigen constituents promotes inflammation and autoimmunity. Reprinted with permission from Nature Publishing Group [91].

CD4$^+$ T helper cells in MS

Upon activation autoreactive T cells upregulate the expression of endothelial adhesion molecules like, such as vascular-cell adhesion molecule 1 (VCAM-1), intercellular adhesion molecule 1 (ICAM-1) and E-selectin [4], allowing diapedesis across the BBB. Within the CNS, likely in the Virchow-Robin space [95], autoreactive T cells are reactivated by local antigen-presenting cells (APCs), such as perivascular macrophages and dendritic cells [8, 96, 97]. Candidate auto-antigens, including MBP, proteolipid protein (PLP), myelin associated glycoprotein (MAG), myelin associated oligodendrocyte basic protein (MOBP) and myelin oligodendrocyte glycoprotein (MOG), are presented to infiltrating T cells in the context of MHC class II molecules [87, 98-101]. In the brain parenchyma, reactivated T cells release inflammatory cytokines and chemokines resulting in increased activation of microglia and the recruitment of monocytes and lymphocytes to the area of inflammation [102]. Several of these inflammatory

cytokines, such as IFN, TNFα, IL-6, IL-12, and IL-23, have been described to worsen disease severity [103-107]. The inflammatory cascade directed against the myelin sheaths eventually leads to demyelination and neurodegeneration [94].
Based on the strong disease susceptibility association with MHC class II alleles, MS is traditionally regarded as a CD4$^+$ T helper cell 1 (Th1)-mediated disease (figure 1.2). This idea is further supported by the observation that IL-12 neutralizing antibodies prevent EAE and IL-12p40-deficient mice are resistant to EAE [108-111]. Moreover, the number of IFNγ-producing cells are increased in the blood of MS patients and treatment of MS patients with IFNγ exacerbates disease [33, 112]. Finally, the presence of pro-inflammatory Th1-related cytokines within active MS lesions point towards an important role for these T cells in MS pathogenesis [113]. Paradoxically, mice lacking either IFNγ and IL-12p35 were not protected, but highly susceptible to EAE induction [114, 115]. This paradox was explained by the double usage of the IL-12p40 subunit, which is associated with either a IL-23p19 or IL-12p35 chain for IL-23 and IL-12, respectively. Indeed, mice lacking the IL-23p19 subunit are resistant to EAE, like IL-12p40 deficient mice [106]. IL-23 drives the maintenance and expansion of IL-17-producing Th17 cells [116]. Since their discovery, numerous studies have linked Th17 cells and the cytokines they secrete to the pathogenesis of both EAE, MS and other autoimmune diseases [117-125]. Although these findings suggest that Th1 cells are redundant in MS, recent studies reported that they are highly pathogenic, required for the entry of Th17 cells into the CNS, present in spontaneous mouse EAE models and that the Th17:Th1 ratio determines the site of CNS inflammation [126-130]. Notably, the pathogenic role of Th17 cells has already been challenged, as IL-17A and IL-17F only marginally contributed to the development of CNS autoimmunity [131].

CD8$^+$ cytotoxic T cells in MS

CD8$^+$ T cells outnumber CD4$^+$ T cells by far in MS lesions, regardless of the stage or activity of disease [12, 132]. In MS plaques they are located near APCs at the lesion border and in the proximity of oligodendrocytes and demyelinated axons [12, 133, 134]. These findings strongly suggest that infiltrating CD8$^+$ T cells are activated in the CNS and directly damage oligodendrocytes and neurons. Whereas the latter is in agreement with the elevated expression of MHC class I

proteins on these cells in MS lesions [135-137], the former corresponds with the observed memory phenotype that CD8$^+$ T cells display within CNS tissue and CSF [138, 139]. Direct neuronal damage by CD8$^+$ T cells is further supported by the correlation between CD8$^+$ T cells within the lesion and the extent of acute axonal damage [140]. This potential detrimental role of activated CD8$^+$ T cells in MS has been validated and extended in animal models [141-145]. These studies support the idea that CD8$^+$ T cells contribute to MS lesion development.

B cells in MS

Several lines of evidence also implicate a pathogenic role of B cells and antibodies in MS. As mentioned, oligoclonal bands containing Igs are detected in approximately 90% of MS patients [18, 19]. Secondly, elevated levels of Igs in CSF correlate with episodes of MS worsening and aggressive forms of MS [146, 147]. In agreement, demyelinating antibodies are present in a sizeable proportion of MS patients and clonally expanded B cells accumulate in the CSF and CNS of MS patients [148-152]. The presence of ectopic B-cell follicles in the meninges of MS patients indicates that B-cell maturation can be sustained locally within the CNS and contribute to the establishment of a humoral immune response in MS [153, 154]. The most convincing evidence is perhaps that rituximab, a FDA approved monoclonal antibody that depletes B cells, reduces active inflammation and relapses in RR-MS patients [155]. These studies point towards a critical role of B cells and their products in MS pathogenesis.

Regulatory T cells in MS

Immunoregulatory cells are reported to be dysfunctional in MS. For instance, CD4$^+$ regulatory T cells (Tregs) show a loss of functional suppression in RR-MS patients [156-158]. This loss of suppression by CD4$^+$ Tregs in RR-MS patients is associated with a diminished expression of Foxp3, the master regulator of Tregs [159, 160]. In accordance with these findings, depletion or adoptive transfer of CD4$^+$ Tregs worsens or ameliorates EAE, respectively [161, 162]. Similarly, CD8$^+$ Tregs in MS patients show a reduced suppressive capacity as compared to healthy controls [163]. Moreover, peripheral CD8$^+$ Tregs negatively correlate with lesion development in MS patients [164]. Also, the anti-inflammatory effects of glatiramer

acetate, an FDA approved MS therapeutic, may in part be due to the activation of $CD8^+$ Tregs [165].

In summary, these studies demonstrate the critical involvement of $CD4^+$ T cells, $CD8^+$ T cells and B cells in the immunopathogenesis of MS. However, apart from the adaptive branch of the immune system, the innate immune system also plays a pivotal role in MS. In section 1.2, the impact of infiltrating monocytes and microglia on inflammation, demyelination and neurodegeneration in MS will be discussed in detail.

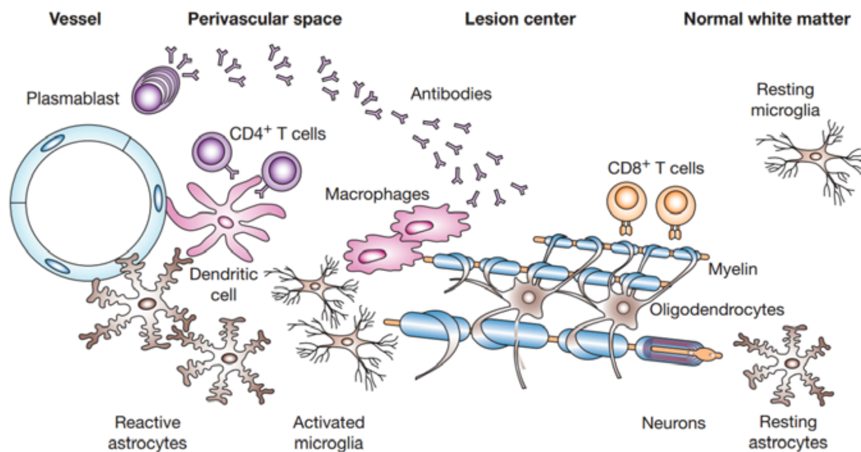

Figure 1.2 The immunopathogenesis of multiple sclerosis. Schematic illustration of the involvement of immune and CNS cells in MS pathogenesis. Following activation, autoreactive $CD4^+$ T cells cross the BBB and become reactivated by local APCs presenting CNS antigens. The subsequent release of inflammatory cytokines and other mediators attracts macrophages and B cells to the CNS. Infiltrated B cells release antibodies against CNS antigens, which can upon binding trigger the complement cascade or antibody-mediated internalization by macrophages and microglia. In addition, activated microglia and macrophages secrete inflammatory and toxic mediators capable of directly damaging oligodendrocytes and neurons. Upon diapedesis, $CD8^+$ T cells engage and destruct MHC class I expressing neurons and oligodendrocytes. Reprinted with permission from Nature Publishing Group [166].

1.1.5 Lesion pathology

The major diagnostic hallmark of MS is focal demyelination, which is present in all stages of the disease [12]. Demyelination will eventually result in acute axonal injury and loss [167, 168], which can be partially counteracted by remyelination [169]. Although the size, number and morphology of demyelinated areas varies among MS patients, they have a predilection for optic nerves, subpial spinal cord, brainstem, cerebellum and periventricular white matter regions [170]. MS patients can exhibit a mixture of lesion types, depending on the stage of MS, velocity of plaque development and extent of remyelination [171]. It should be noted that our knowledge concerning lesion pathology in MS is primarily based on post-mortem material from patients with long-lasting MS. Whether lesions from these patients precisely reflect lesion pathology in the initial stages of MS remains elusive.

White matter lesions

Classic active lesions are predominantly found in RR-MS patients [172, 173]. Histochemically, they are defined by the accumulation of activated myelin-containing macrophages and microglia throughout the lesion (active lesion) or at the rim of the lesion (chronic active lesion). Also, profound BBB leakage, axonal damage and infiltration of T cells and B cells is apparent in active plaques [167-169, 174]. The architecture of chronic active lesions is more complex, consisting of a zone of early tissue injury (prephagocytic area) which surrounds a zone of early myelin internalization (early active area), a zone of advanced myelin digestion (late active) and an inactive center [171, 174]. Remyelination occurs in the inactive center of chronic active lesions and is associated with the appearance of newly recuited oligodendrocytes [175, 176]. Nonetheless, remyelinated lesions are rare due to their instability during active inflammation [177, 178]. Lesional activity can also be predicted by the presence of specific myelin degradation products within macrophages [179-181]. The presence of major myelin proteins, like MBP and PLP, in myeloid cells are indicative for late active lesions, while the presence of smaller myelin proteins, like MOG and 2′,3′-cyclic nucleotide 3′-phosphohydrolase, denotes early active demyelination.

Slowly expanding lesions (SELs) are predominantly seen in progressive MS [172, 173, 178]. They likely reflect the expansion of pre-existing lesions, as gadolinium-enhancing lesions, an MRI measure suggestive of acute inflammatory changes

associated with breakdown of the BBB, are scarce in patients with progressive MS [182, 183]. The inactive lesion center of SELs is characterized by demyelination, dense astrocytic scarring and axonal loss [171, 174, 184]. Although regarded to be inactive, ongoing axonal degeneration has been reported in SELs [185]. Activated microglia and macrophages, containing early myelin degradation products, are scarce and primarily located at the lesion border [172]. A major hallmark of SELs is the massive loss of macrophages and microglia [174]. The consequence of this profound loss of phagocytes on lesion pathology is unclear.

The most frequent lesion type in all stages of MS is the inactive lesion. Like SELs they are devoid of myelin, lack remyelination, and show extensive axonal damage and astrocytic scarring. In contrast to classic active lesions and SELs, immune cell infiltration and inflammation is rare. Moreover, the density of microglia within the lesion is lower as compared to surrounding normal-appearing tissue [171].

Finally, demyelinated areas can be repaired by remyelination, resulting in so called shadow plaques. Remyelination can occur during early stages of lesion formation, even when demyelination is still active [186-188]. Interestingly, a recent study demonstrated that extensive remyelination was found not only in patients with RR-MS, but also in a subset of patients with progressive disease [189]. Effective remyelination depends on the recruitment of oligodendrocyte precursors cells (OPCs), which after maturation generate new myelin sheaths [190-192]. However, the progressive nature of MS may indicate that remyelination efficacy declines over time. Non-disease-related factors, like age [193-195], sex [196] and genetic background [197], and disease-related factors, such as a deficiency in precursor cells [198], a failure of precursor cell recruitment [199], or a failure of precursor cell maturation [200, 201], have been associated with the failure of remyelination in MS. Notably, remyelinated areas may also be more prone to inflammatory demyelination compared with the WM outside plaques [178].

Grey matter lesions

In addition to the more commonly described WM lesions, MS plaques are also located in the gray matter (GM), particularly in the cortex and deep GM nuclei [202-204]. The appearance of cortical lesions is most prominent in progressive MS, affecting up to 68% of the total forebrain cortical area in some extreme

examples [173, 204, 205]. Cortical GM lesions are heterogeneous in morphology, which is illustrated by the proposed classification into four subtypes [204]. Similar to WM lesions, cortical GM lesions in chronic MS patients are well-defined, and demonstrate oligodendrocyte and neuronal degeneration [202, 204, 206, 207]. Although the functional consequences of cortical demyelination are unclear, reports suggest that cortical damage is clinically significant in MS [173, 208]. Additional findings indicate that GM lesions occur independently of white matter lesions [173, 209, 210], that meningeal inflammatory infiltrates drive their formation [153, 211] and that GM lesions lack immune cell infiltration, complement deposition and BBB breakdown [202, 204, 207]. Notably, microglia display an activated morphology in cortical lesions and are located near neurites and neuronal cell bodies [202, 204, 207, 209, 212]. The observed lack of infiltrating immune cells led to the hypothesis that GM demyelination and neurodegeneration proceeds independent of inflammation. However, recent studies have demonstrated that cortical demyelination and neurodegeneration is already apparent in early MS [213, 214], possibly explaining the cognitive impairments and epilepsy in RR-MS patients [215, 216]. More importantly, in contrast to cortical lesions in progressive MS, early cortical GM lesions are characterized by BBB damage and the presence of myelin-containing macrophages and lymphocytes closely associated with neurons and neuritis [217, 218]. The highly inflammatory character of early GM lesions argues against a primary neurodegenerative process and suggests that neurodegeneration in MS runs on a background of inflammation. The apparent difference in demyelination in progressive and early MS can be explained by the extensive remyelination in early cortical MS lesions [219, 220]. Interestingly, in some patients cortical lesions appear before inflammatory lesions in the WM, suggesting that the pathological processes underlying MS originate in the cortex [213, 218].

1.1.6 Therapeutic strategies

In the past decades our understanding of MS pathogenesis has significantly increased. This has resulted in development of several FDA-approved disease-modifying therapies (DMTs) [221]. Although current drugs can prevent exacerbations and the formation of new lesions in RR-MS patients, none of them

can halt the neurodegenerative changes associated with the chronic phase of the disease.

First-line therapeutics

Immunomodulating therapeutics are most often used as first-line drugs. The commonly used immunomodulatory first-line DMTs are interferon-βs (IFN-βs) and glatiramer acetate. The anti-inflammatory effect of IFN-β is thought to be due to a decreased MHC class II expression, upregulated IL-10 production, reduced Th1 and Th17 differentiation, increased production of nerve growth factors and suppressed trafficking of inflammatory cells across the BBB [222]. Subcutaneous IFN-β1b (Betaseron™), intramuscular IFN- β1a (Avonex™) and subcutaneous IFN-β1b (Rebif™) reduce annualized relapse rates (ARRs), delay progression of disability and lower the number of new or enlarging MRI lesions [223-225]. However, for the described IFN-β drugs neutralizing antibodies can develop, which impact their therapeutic efficacy [226]. Glatiramer acetate (GA, Copaxone™) is a synthetic copolymer composed of a random mixture of four amino acids. GA stimulates the production Th2 cells, Tregs and regulatory CD8+ T cells, inhibits antigen-specific T cell activation, promotes regulatory B-cell properties and skews macrophages towards an anti-inflammatory phenotype [227-230]. Treatment with GA reduces ARRs and prevents the development of new lesions in patients with RR-MS [231, 232]. There are no adverse events in patients receiving GA therapy [233].

Second-line therapeutics

When available first-line DMTs fail, the chemotherapeutic drug mitoxanthrone (Novantrone™) can be used. It intercalates into DNA helixes, inducing strand breakage and inhibition of DNA repair enzymes. In MS, it eliminates white blood cells, thereby decreasing progression of disability and clinical exacerbations. The most serious adverse effects are potential cardiotoxicity and leukemia [234-237]. Natalizumab (Tysabri) is a humanized monoclonal antibody against leukocyte α4 integrins, which blocks the entrance of inflammatory leukocytes to the CNS. It reduces ARRs, the risk of disability progression and the formation of new lesions [238]. The most notable potential adverse effect of natalizumab is the development of progressive multifocal leukoencephalopathy, caused by the John Cunningham

virus [239]. Finally, fingolimod (FTY220) is an oral sphingosine-1 phosphate (S1P) receptor modulator and has recently been approved by the FDA [230]. Functionally, fingolimod prevents the egress of lymphocytes from the lymph nodes and has a direct cytoprotective effect in oligodendrocyte progenitors [240-242]. Treatment reduces ARRs, the risk of disability progression and the formation of new lesions [243]. However, adverse events have been observed [244].

Off-label and upcoming therapeutics

Diverse therapeutics have been used off label as DMTs in MS. Examples of such agents are mycophenolate mofetil [245], azathioprine [246, 247], methotrexate [248], rituximab [155, 249, 250], intravenous immunoglobulin [251-253] and corticosteroids [254, 255]. These drugs are used in individuals who need intensification of therapy, cannot endure the adverse effects of other MS-associated DMTs or people who cannot afford FDA-approved therapeutics [230]. However, large-scale trials are lacking for these DMTs, limiting the use of these drugs. Moreover, systemic adverse events, such as increased risk of malignancy and opportunistic infections, hamper their frequent use in the clinic as MS therapeutics. New upcoming drugs which have entered or completed phase II and III clinical trials are laquinimod [256], teriflunomide [257], dimethyl fumarate [258], alemtuzumab [259], daclizumab [260] and ocrelizumab [261]. Laquinimod (quinoline-3-carboxamide), teriflunomide (active metabolite of leflunomide) and dimethyl fumarate are novel oral immunomodulatory DMTs. Whereas laquinimod inhibits leukocyte migration into the CNS, modulates cytokine production and increases axonal integrity and levels of neurotrophic factors [262], teriflunomide inhibits DNA replication of leukocytes [257]. Dimethyl fumarate ameliorates disease severity by defending against oxidative-stress-induced neuronal death, protecting the BBB and supporting maintenance of myelin integrity in the CNS [258]. Alemtuzumab, daclizumab and ocrelizumab are humanized antibodies which target CD52 (monocytes and lymphocytes), CD25 (activated T cells) or CD20 (B cells), respectively [259-261].

1.2 Macrophages in multiple sclerosis

1.2.1 Monocyte and macrophage function and heterogeneity

Macrophages are phagocytic cells that play a crucial role in homeostasis and host defense [263]. In fact, primitive organisms completely rely on macrophages for their defense, as they are the only cells responsible for engulfment and elimination of threats [264]. However, during phylogeny host defense becomes progressively more complex, resulting in both innate immunity and lymphocyte-mediated adaptive immunity. Despite the development of adaptive immunity, macrophages still play an indispensable role in maintaining immune homeostasis, providing immediate defense against intruding pathogens, influencing the adaptive immune system and orchestrating many metabolic functions in health and disease.

Monocytes heterogeneity

Circulating monocytes are generally considered to give rise to tissue-resident macrophages following their extravasation during inflammation and in the steady state [265, 266]. Recent studies have reported that monocytes adopt two distinct fates following exit from the bone marrow. In mice, $CXCR3^{hi}LYC6^{lo}$ monocytes have a "patrolling" function, as they crawl on the vascular endothelium in search of damage or infections [267]. Importantly, due to lack of CCR2 expression, they cannot respond to the proinflammatory chemokine monocyte chemoattractant protein-1 (MCP-1) [266, 268]. In agreement with the latter, $LYC6^{lo}$ monocytes are recruited at a late phase to the ischemic myocardium, where they are proposed to exert a reparative function [269]. By contrast, the $CXCR3^{lo}LY6C^{hi}$ monocytes are linked to inflammation, express CCR2 and are rapidly mobilized following inflammation [266, 268-270]. The spleen has been identified to harbor a large number of $LY6C^{hi}$ monocytes that rapidly migrate to inflammatory sites [271]. Human monocytes are suggested to have a similar heterogeneity, mouse inflammatory $CCR2^{hi}CXCR3^{lo}LY6C^{hi}$ monocytes are believed to resemble human $CCR2^{hi}CD14^{hi}$ $CD16^{lo}$ cells and non-inflammatory $CCR2^{lo}$ $CXCR3^{hi}LY6C^{lo}$ monocytes the human $CCR2^{lo}CD14^{hi}$ $CD16^{hi}$ subset [272, 273].

Macrophage heterogeneity

As mentioned, monocytes give rise to variety of tissue resident macrophages during inflammation and in the steady state. However, although previously suggested to be replenished by hematopoietic cells [274-276], maintenance and local expansion of microglia, the CNS resident macrophages, has recently been described to rely solely on the self-renewal of CNS lineage-committed precursors in non-pathological conditions [277-280]. Disregarding their origin, virtually all tissues accommodate macrophages with specialized functions, depending on the requirements of the tissue [281-284]. This apparent tissue-related heterogeneity of macrophages is further amplified by the capacity of divergent stimuli, or combinations of stimuli, to exert a profound effect on macrophage physiology and function [265, 285-287]. The identified macrophage subsets were originally referred to as classically (M1) or alternatively activated (M2) (figure 1.3). The latter cells were subsequently subdivided into M2a, M2b and M2c macrophages to discriminate between phenotypes induced by different stimuli. Broadly, these divergent macrophage polarizations can be classified based on three different homeostatic activities; host defense, wound healing and immune regulation (figure 1.3) [288]. However, macrophages can likely obtain a spectrum of activated phenotypes rather than discrete stable subpopulations, analogous to the secondary colors in the color wheel in figure 1.3. This is underlined by the more recently described M2d (tumor-associated macrophages), M4 (CXCL4-induced macrophages) and Mox (oxidized phospholipid-induced macrophages) [289-293], which all slightly differ from the phenotypes already described. Notably, macrophages retain their plasticity after polarization, indicating they can adapt to a changing environment [294, 295].

1.2.2 Macrophages in disease

In contrast to their role in host defense and homeostasis, the macrophage phenotype has been reported to exert a profound effect on the progression of a diverse set of diseases. Tumor-derived factors have, for instance, been described to induce anti-inflammatory macrophages capable of supporting metastasis, tissue remodeling and cell proliferation, and inhibiting antitumor immune responses [296, 297]. Furthermore, diet-induced obesity leads to a shift in the activation state of macrophages, hereby promoting insulin resistance and

metabolic inflammation [298]. Likewise, atherosclerosis is appreciated to be both a lipid disorder and an inflammatory disease, with inflammatory foamy macrophages playing a central role in plaque progression [299, 300]. Finally, macrophage polarization is regarded to contribute to several inflammatory autoimmune disorders, including Crohn's disease [301, 302], rheumatoid arthritis [303, 304] and MS [305]. Strikingly, skewing of these macrophages towards their opposite phenotype results in amelioration of several of the abovementioned disorders [227, 306-313], indicating a pivotal role for macrophage function in the pathogenesis of these disorders.

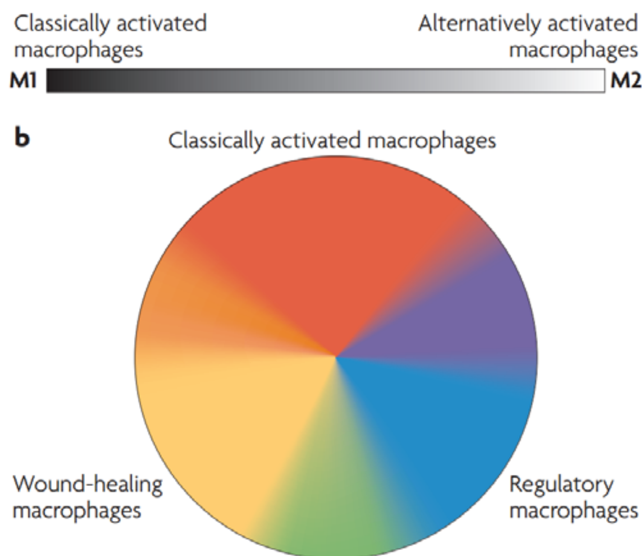

Figure 1.3 Macrophage heterogeneity. (a) Schematic illustration of the linear model of macrophage phenotypes. Classically activated (M1) and alternatively activated (M2) macrophages were regarded to be pro-inflammatory or anti-inflammatory, respectively. (b) In the newly proposed classification macrophages are arranged based on three different homeostatic activities; host defense, wound healing and immune regulation (analogous to the primary colors). Secondary colors may represent M4-, Mox- and tumor-associated macrophages, which share characteristics of multiple "primary" phenotypes. Reprinted with permission from Nature Publishing Group [288].

1.2.3 Macrophages and microglia in MS

During active MS, autoreactive lymphocytes are detected in both the perivascular cuffs and to a lesser extent in the parenchyma. Interestingly, activated macrophages and microglia are the dominant cell type in such lesions and outnumber lymphocytes by far [314-316], suggesting that they play an essential role in the MS pathogenesis. Since they appear morphologically indistinguishable when activated [317], it has been difficult to assess their individual contribution to CNS autoimmunity. However, experiments using bone marrow chimeras have demonstrated that microglial activation and proliferation precedes the onset of CNS autoimmunity and that microglial paralysis inhibits the development and maintenance of inflammatory CNS lesions [318, 319]. These findings argue for an essential role for microglia in disease onset and development. Consistent with the idea that monocytes and macrophages are pivotal in MS development, depletion or blockage of their entry into the CNS ameliorates EAE [280, 320-326]. Furthermore, enrichment of inflammatory LY6C^{hi} monocytes in the circulating pool is associated with an earlier onset and increased severity of EAE [327]. The major pathogenic effector functions of infiltrating macrophages and microglia likely depends on their location, phenotype and the local microenvironment. In the following sections I will focus on the detrimental role of macrophages and microglia on demyelination, neurodegeneration and neuroinflammation. In addition, recent studies reporting a beneficial impact of macrophages and microglia on these pathological mechanisms will be discussed.

Monocyte infiltration and migration

The CNS is an immune-priveleged organ [328], yet large numbers of monocytes and lymphocytes accumulate in the CNS during MS and EAE. Disruptance and alterations of the BBB, which normally restricts the movement of soluble mediators and leukocytes from the periphery to the CNS, are apparent in MS and contribute to leukocyte infiltration into the brain [329]. In MS, changes in the expression and activities of adhesion molecules on both the blood vessels and monocytes have been reported, which likely also play a role in monocyte trafficking towards the CNS. Adhesion molecules that have prominent roles include those associated with endothelial tight junctions, members of the selectin family (endothelial (E)-Selectin, leukocyte (L)-selectin, and platelet (P)-

selectin), cell adhesion molecules of the Ig superfamily (intercellular adhesion molecule 1 (ICAM-1), vascular cell adhesion molecule 1 (VCAM-1), and platelet endothelial cell adhesion molecule 1 (PECAM-1)) and the integrin family (very late antigen 4 (VLA-4), macrophage antigen 1 (MAC-1) and lymphocyte function-associated antigen 1 (LFA-1)) [329, 330]. BBB alterations have been reported to be induced by inflammatory cytokines, reactive oxygen species (ROS) and metalloproteinases are (MMPs), produced by inflammatory immune cells [329]. Together with adherence molecules, chemokines and their receptors contribute to the trafficking of leukocytes across the BBB and regulate their transfer to lesion sites [331-334]. Apart from monocyte chemoattractant protein-1 (MCP-1 or CCL2), which is upregulated in MS and EAE lesions and can attract inflammatory CCR2^{+} monocytes [326, 335-337], chemokines such as CCL1, CCL3, CCL4, CCL5, CCL7, CXCL1, CXCL9, CXCL10, CXCL11 and CXCL16, and their respective receptors, play a role in lesion-directed chemotaxis of monocytes in MS and EAE [331-334].

Microglial migration

As mentioned in section 2.1.2, microglia depend solely on the self-renewal of CNS lineage-committed precursors in non-pathological conditions [277-280]. Like macrophages, they can secrete a plethora of cytokines, phagocytose, present antigens and adopt multiple phenotypes [338-340]. In a resting state microglia display a ramified morphology and actively survey the environment for abnormal events. When activated, they adopt an amoeboid shape, similar to blood-derived macrophages, and produce numerous inflammatory and toxic mediators [341]. Upon any detection of signs for brain lesions or nervous system dysfunction, activated microglia can migrate to sites of injury, proliferate and phagocytose cells and cellular compartments [342]. Mouse and human microglia express the chemokine receptor CXCR3 [343, 344]. Interestingly, CXCR3-CXCL10 interaction has been implicated in the chemotaxis of microglia following injury [345]. Ample evidence suggests that this interaction also affects microglial migration in MS and EAE. For instance, CXCL10 levels are elevated in the CSF and lesions of patients with active MS [346]. In agreement, CNS levels of CXCL10 correlate to clinical relapses in EAE [347-349]. The CXCR3-CXCL10 interaction has also been reported to effect chemotaxis of T cells in the CNS [346, 350]. Similar to CXCL10,

CCL21, which is upregulated in neurons undergoing degeneration, can trigger migration of microglia through CXCR3 [351]. Although definite prove is still lacking, these findings point towards an role of the CXCL10- and CCL21-CXCR3 axes in lesion-directed microglial chemotaxis.

Antigen presentation

CNS reactivation of autoreactive lymphocytes by local antigen presenting cells displaying myelin antigens is thought to initiate and maintain the inflammatory cascade observed in the brain of MS patients [93, 94, 352]. Perivascular macrophages, microglia and infiltrated monocytes are ideally positioned to influence infiltrating and infiltrated myelin-reactive lymphocytes. More importantly, myelin-containing macrophages and microglia are situated in both the perivascular spaces and the brain parenchyma in MS patients [314-316]. Perivascular macrophages are located in the Virchow-Robin space, which is located in between the basal lamina of the blood vessel and the glia limitans. Perivascular macrophages have been demonstrated to (re)activate both naïve and primed myelin-reactive T cells [96, 97]. Their potent ability to present antigen is illustrated by a constitutively high expression of MHC class II [353-356]. Indeed, *ex vivo* they are demonstrated to be competent APCs [357]. Interestingly, MHC class II expression is elevated on these cells near MS lesions [358]. Similar to perivascular macrophages, microglia can act as antigen presenting cells [359]. In non-pathological conditions they constitutively express MHC class II molecules and co-stimulatory molecules [360], which are upregulated after damage and inflammation [361-363]. *In vitro*, microglia can present myelin antigens to T cells [364]. Finally, infiltrating monocytes are likely to contribute to the reactivation of autoreactive T cells in the CNS. Interestingly, in MS lesions high HLA-DR expression seems to be associated with myelin-containing macrophages [365]. The presence of brain antigen-containing phagocytes in secondary lymph nodes in MS and EAE further emphasizes a possible crucial role of these cells in modulating the autoreactive T cells during MS and EAE pathogenesis [366-368]. How brain antigens gain excess to CNS draining secondary lymph nodes, either chemotactically in the context of phagocytes or as soluble products, remains to be clarified [365, 369, 370]. Interestingly, myelin internalization enhances expression of CCR7 [370], which is involved in lymph-node homing of leukocytes [371],

suggesting that myelin can induce lymph node-directed chemotaxis of macrophages upon internalization.

1.2.4 Role of macrophages and microglia in demyelination

Myelin internalization

The major pathological hallmark of MS is focal demyelination. In classical acute lesions activated macrophages and microglia contain degenerated myelin products [314-316], indicating active involvement of these cells in demyelination. In the last three decades numerous studies have validated the capacity of macrophages and microglia to internalize myelin *in vitro* [365, 372-378]. Interestingly, the phagocytic capacity of microglia was regarded to be limited as compared to macrophages [373]. In contrast, in a more recent study human microglia were found to be more competent in phagocytosing myelin as compared to blood-derived macrophages [379]. Being regarded as the principal effector mechanism in MS, several studies have reported mediators capable of affecting myelin phagocytosis by macrophages and microglia [380-385]. Myelin has even been described to negatively regulate its own clearance by microglia and macrophages [386]. This negative feedback loop is, however, in contrast with the induced oxidative burst of macrophages following myelin internalization and the necessity of oxygen species in macrophage-mediated internalization of myelin [372, 387].

Complement, Fc and scavenger receptors (SRs) are the principal receptors for recognition and internalization of myelin components [372, 374, 388-392]. The contribution of Fc receptor-mediated myelin internalization is supported by the facts that reactive microglia in MS lesions have an increased expression of Fc-receptors [393], anti-myelin antibodies are present in MS patients [394], IgG depositions are consistently observed on macrophages in inflammatory demyelinating areas [395-397] and anti-myelin antibodies cause demyelination *in vitro* and in animal models of MS [398-401]. The massive activation of complement in demyelinating areas during antibody exacerbated EAE and active MS [395-397, 402], and the reduced demyelination in $SRA^{-/-}$ EAE mice and antibody-exacerbated EAE rats treated with a complement inhibitor [403, 404], supports an additional role for complement and SR-receptors on macrophages in demyelination. More

recently, the low-density lipoprotein receptor-related protein has been added to the growing list of receptors involved in myelin internalization [405].

Inflammatory and toxic mediators

In addition to internalizing myelin, activated macrophages and microglia can produce a plethora of toxic and inflammatory mediators capable of damaging myelin [406, 407]. Of particular interest are reactive oxygen and nitrogen species (ROS and RNS), as they are cytotoxic to oligodendrocytes and enhance myelin breakdown [408-415]. Interestingly, increased oxidative stress is apparent in EAE animals and MS patients and ROS production is elevated in activated mononuclear cells of MS patients [415-420]. The importance of ROS is further underlined by the fact that anti-oxidants are protective in EAE [421]. Consistent with the idea that macrophage-produced NO contributes to MS pathogenesis, induced levels of inducible nitric oxide synthase (iNOS) and its product nitric oxide (NO) in active MS lesions and EAE animals are associated with macrophages and microglia, and correlate with EAE severity [422-427]. The effect of iNOS inhibitors on EAE pathology has been controversial [428-430], likely due to the fact that, apart from promoting cytotoxicity, NO can exert an additional immunosuppressive effect [431-433]. In addition to directly affecting myelin integrity, RNS and ROS-mediated vasodilation and disturbance of the BBB may promote passage of inflammatory cells into the CNS, thereby augmenting demyelination [434-438].

In concert with RNS and ROS, the secretion of pro-inflammatory cytokines by activated macrophages and microglia has been indicated to influence demyelination and disease activity [406, 407]. TNF is a typical pro-inflammatory mediator produced by classically activated macrophages [265, 285, 286, 288]. MS patients have elevated concentrations of TNF in serum, CSF [439-441] and within active MS lesions [442, 443]. Macrophages and microglia are the predominant cells expressing TNF in EAE and MS lesions [444, 445]. Interestingly, CNS-specific inhibition or overexpression of TNF ameliorates EAE or results in spontaneous demyelination, respectively [446-449]. In agreement with the latter, TNF has been demonstrated to mediate myelin and oligodendrocyte damage *in vitro* [450, 451]. Remarkably, monoclonal antibodies directed against TNF were found to be detrimental in MS patients [35, 452], illustrating that the EAE model does not

necessarily mimic MS pathogenesis in all aspects. Similar to TNF, the pro-inflammatory cytokines IL-1 and IL-23 have been shown to be induced in activated macrophages and microglia in EAE and MS lesions [106, 453-455]. *In vivo*, IL-1 is cytotoxic to oligodendrocytes, induces demyelination, affects the integrity of the BBB and aggravates disease severity [456-459]. Likewise, mice deficient in IL-23p19subunit, lacking IL23 but not IL-12, are protected from EAE development [106]. However, during MS and EAE, macrophage-derived IL-23 likely affects demyelination indirectly by maintaining and promoting the generation of inflammatory Th17 cells [116, 460], instead of directly influencing myelin integrity.

Inflammatory products are regarded to activate macrophages and microglia in MS lesions [265, 285, 286, 288]. Moreover, myelin phagocytosis as such has also been reported to result in activation of macrophages and microglia. For instance, macrophages and microglia undergo an oxidative burst and show an elevated production of TNF, IL-1 and IL-6 afer myelin internalization [372-374]. More recently it was demonstrated that myelin increases the production of toxic and inflammatory mediators through activation of FAK/PI3K/Akt/NF-kappaB signaling pathways and that CR3 contributes to this myelin-induced PI3K/Akt/NF-kappaB activation [377]. These findings suggest the presence of a positive feedback loop, in which initial internalization of myelin by activated macrophages and microglia fuels additional demyelination by increasing the secretion of inflammatory and toxic mediators.

Glutamate is the most abundant excitatory neurotransmitter in the CNS. In addition to its role as an excitatory amino acid, it plays a role in long term neuronal potentiation [461]. However, excessive activation of glutamate receptors, which are expressed by oligodendrocytes, can induce oligodendroglial death and demyelination [462, 463]. In MS, glutamate is increased in the CSF, peripheral blood and acute MS lesions [464-466]. More importantly, activated macrophages and microglia release large amounts of glutamate in MS [467-469]. In support of a role of glutamate in demyelination, glutamate receptor antagonists limit oligodendrocyte loss and demyelination in EAE-affected animals [470, 471]. Finally, macrophages and microglia release metalloproteinases (MMPs) which damage neurons and oligodendrocytes [305].

1.2.5 Role of macrophages and microglia in axonal degeneration

While classically regarded as a demyelinating disease, it is now appreciated that MS pathology is much more complex. Besides demyelination, axonal injury and degeneration occur commonly in acute inflammatory lesions in both the WM and GM [169, 472, 473]. Importantly, axonal loss is closely associated with neurological deficits in MS and chronic EAE [82, 474-476], suggesting that axonal degeneration is at least partly responsible for disease progression. Ample evidence suggests that macrophages and microglia affect axonal integrity in MS and EAE. For instance, terminal axonal ovoids, suggestive of axonal transection, are often surrounded by activated macrophages and microglia containing neuronal antigens [167, 477]. Furthermore, axonal damage is correlated to the number of macrophages and microglia [140, 472]. Finally, macrophage- and microglia-conditioned media induce neuronal cell death *in vitro* [478-480]. These studies point towards a critical role of macrophages and microglia, and in particular the toxic and inflammatory mediators they secrete, in axonal degeneration.
Continuous removal of the myelin sheath surrounding the axons increases the vulnerability of axons [481]. If remyelination fails, this will eventually lead to a temporary conduction block and axonal degeneration, resulting in progressive disability as observed in MS patients [167, 168, 192, 481, 482]. Thus, macrophage-mediated demyelination can indirectly affect axonal integrity by increasing its vulnerability (see section 2.4). The presence of autoantibodies specific for axonal antigens further indicates that they can directly damage denuded axons by Fc receptor-mediated internalization [483-487]. Moreover, macrophages and microglia-secreted mediators have been described to negatively influence axonal integrity directly. For instance ROS and RNS have been reported to trigger axonal degeneration [410, 419, 488-491]. Moreover, *in vivo* neutralization or inhibition of ROS and RNS reduces axonal damage and inflammation in neuroinflammatory disorders [415, 492-495], and rescues axons that entered the degenerative process [488]. Secondly, pro-inflammatory cytokines secreted by macrophages and microglia, like TNF, IFN and IL1, have been reported to be neurotoxic [496-501]. Next, apart from its role in demyelination, glutamate causes excitotoxicity in neurons resulting in neuronal cell death *in vitro* and *in vivo* [467, 470, 502-504]. Finally, macrophage-produced MMPs have been suggested to affect axonal integrity in MS and EAE [505-509].

Interestingly, demyelination is not a prerequisite for axonal degeneration. For example, macrophage depletion also ameliorates acute EAE [321, 322], which is characterized by negligible demyelination [510]. Moreover, EAE experiments using animals with slowly progressing myelin degeneration demonstrated transient axonal injury in the absence of demyelination [511]. Here, iNOS expression in perivascular macrophages was associated with a transient functional disturbance of axons. In agreement, by using *in vivo* imaging, a recent study demonstrated that axonal degeneration is observed in axons with intact myelin sheaths [488]. In this study macrophage-derived ROS and RNS triggered mitochondrial pathology, which seemed to be an early event in axonal degeneration. These studies indicate that demyelination is not necessary for macrophages to contribute to axonal dysfunction. Thus, inflammatory and toxic mediators are able to circumvent the protective myelin barrier, for example, by diffusing through membranes or by acting at the nodes of Ranvier [484, 512].

It should be noted that axonal degeneration does not only take place in the context of early lesions but continues to occur during later stages of disease in which acute inflammatory episodes are generally absent [172, 513, 514], as evidenced by the gradual increase in clinical severity in progressive MS. The underlying mechanisms for axonal damage in the absence of inflammatory infiltrates are unclear. However, the absence of the insulating myelin sheath may lead to a lack of axonal support and an increased intra-axonal calcium concentration eventually resulting in axonal degeneration [515-517]. In contrast, the extent of diffuse axonal loss in normal-appearing WM in MS patients correlates with both the density of MHC class II expressing microglia in the normal-appearing WM and T cells in the meninges [513]. Both processes indicate that persisting diffuse inflammation in progressive MS occurs and likely contributes to the continuous axonal degeneration.

1.2.6 Role of macrophages and microglia in CNS repair

The relapsing-remitting nature of early MS indicates the presence of naturally-occurring regulatory mechanisms keeping the disease in check. Indeed, remyelination, as evidenced by the presence of shadow plaques, and axonal regeneration are apparent in MS and EAE [186-188, 518-521]. These findings indicate that the inflammatory microenvironment can also stimulate CNS repair, as

opposed to being purely detrimental. In contrast to their apparent detrimental role in MS, monocytes and macrophages have been described to play a key role in repair during MS and EAE. Although two seemingly mutually exclusive processes, studies have reported such a dual role of monocytes and macrophages in both injury and repair. For instance, in a mouse ischemic injury model, a orchestrated mobilization of functionally divergent monocyte subsets was observed [269]. Similarly, distinct macrophage phenotypes contribute to kidney injury and repair [522]. Finally, tumor-infiltrating myeloid-derived suppressor cells have been reported to have a pleiotrophic character, as they bear both M1 and M2 characteristics [523].

Macrophages and microglia promote remyelination

Myelin regeneration in the adult CNS is mainly mediated by oligodendrocyte progenitor cells (OPCs). Upon demyelination, OPCs are rapidly recuited to demyelinated areas where they subsequently give rise to mature myelinating oligodendrocytes [192, 524]. Macrophages and microglia are suggested to play a crucial role in remyelination, as remyelination is often correlated with large numbers of macrophages and microglia in an inflammatory microenvironment [525-530]. Increasing evidence indicates that macrophages and microglia merely fulfil a scavenging role in MS. For instance, CNS myelin debris inhibits OPCs maturation *in vitro* [531]. Since macrophages and microglia actively phagocytose myelin debris, local clearance of myelin debris in the centre or vicinity of lesions can be a necessary prerequisite for axonal remyelination after demyelination. This hypothesis is supported by the fact that monocyte depletion and a consequent inability to clear the microenvironment of myelin debris, causes an impairment of OPC differentiation *in vivo* [532, 533]. In addition, repair and resolution of EAE is promoted when myeloid cells are genetically modified to increase their phagocytic capacity [534]. Notably, as OPCs are present in most chronic demyelinated MS lesions, myelin debris attributes only to an arrest of the differentiation and not the recruitment of OPCs [200]. In a recent paper the decline in remyelination that occurs with advancing age has been associated with the reduced capacity of aging monocytes to clear myelin debris, potentially explaining the absence of recovery in MS patients over time [195]. Although several studies attempted to unravel the underlying mechanisms of myelin-

mediated inhibition of oligodendrocyte maturation, the myelin-associated proteins responsible are elusive. Nevertheless, transcriptional alterations in oligodendrocytes by myelin debris have been well documented [535, 536].
Macrophages and microglia might also influence survival, proliferation and differentiation of oligodendrocytes and reduce axonal degeneration by secreting a plethora of anti-inflammatory and neurotrophic factors, including tumor growth factor (TGF), insulin-like growth factor (IGF-1) nerve growth factor, neurotophin-3, brain-derived neurotrophic factor, ciliary neurotrophic factor and leukemia inhibitory factor [537-546]. Strikingly, the appreciated age-associated delay in remyelination efficiency has also been linked to changes in the inflammatory mediator response [547]. This hypothesis is supported by the fact that chronic lesions show negligible remyelination and display a dampened inflammatory environment. Moreover, non-remyelinating situations can be transformed to a remyelinating one by inducing acute inflammation [548]. In accordance, non-immunological toxin-mediated myelin-damage models show that remyelination is impaired in transgenic animals lacking inflammatory mediators, like TNF and IL1 [526, 549, 550]. The remyelination-enhancing effects of IL-1 and TNF may be direct or indirect by increasing IGF-1 [526, 549, 551-553]. The latter indicates that besides being crucial mediators of demyelination, macrophage-secreted inflammatory mediators are necessary for repair processes in the CNS.

Axonal regeneration is stimulated by macrophages and microglia

Axonal growth is required during development to establish a functional neuronal network. However, neurological recovery after CNS trauma also relies on the capacity of damaged axons to reconnect to their physiological targets [519, 554]. Apart from inhibiting OPC maturation, myelin contains several molecules capable of inhibiting axonal outgrowth, such as Nogo [555, 556], MAG [557] and OMgp [558]. The importance of myelin-associated inhibition of axonal outgrowth *in vivo* is illustrated by the fact that neutralization or knockout of Nogo A or LINGO-1, which is a functional component of the receptor-complex mediating the inhibitory effects of Nogo A, MAG and OMgp, promotes axonal integrity and sprouting in EAE and a spinal cord injury model [559-562]. Thus, rapid removal of myelin-associated inhibitors will create an environment susceptible for axonal

regeneration. In line with this hypothesis, increased axonal regeneration in subcortical white matter MS lesions and in a optical nerve injury model is accompanied by an augmented infiltration of macrophages [518, 563].

In addition to indirectly promoting axonal outgrowth by clearing myelin debris, activated macrophages secrete factors that stimulate damaged retinal ganglion cells (RGCs) to regenerate their axons [564, 565]. Subsequent studies demonstrated that macrophage-derived oncomodulin and EphB3 induce axonal regeneration in injured RGCs [563, 566]. Similarly, the pro-inflammatory cytokine IL-6 can protect neurons from insults, as well as stimulate neuronal differentation, growth, and survival [567-569]. Finally, neurotrophic mediators (described in section 2.5.1) can have a profound effect on axonal regeneration and sprouting.

1.2.7 Foamy macrophages in MS

Although little is known about the exact phenotype of macrophages in MS lesions, it is evident that macrophages infiltrating MS lesions confer, at least for a certain period of time, a classically activated phenotype [179, 422, 570]. Apart from the potential impact of the inflammatory environment in polarizing macrophages and microglia [265, 285, 286, 288], myelin internalization as such has been reported to induce a classically activated phenotype in macrophages and microglia [372-374, 377]. Interestingly, a recent study demonstrated the presence of anti-inflammatory, myelin-containing macrophages in the centre of MS lesions [365]. These M2-resembling foamy macrophages have an increased expression of MHC class II and express a variety of anti-inflammatory cytokines, like TGFβ, IL-4, IL-10 and IL-1 receptor antagonist, while lacking expression of pro-inflammatory cytokines like TNF, IL-1 and IL-12. Likewise, macrophages invading sciatic and nerve tissue *in vitro*, readily incorporate myelin and obtain an unique anti-inflammatory phenotype [376]. Also, the inflammatory potential of microglia is suppressed after myelin internalization [375]. Interestingly, whereas in the initial few hours myelin induced the secretion of inflammatory mediators, microglia adopted a less inflammatory phenotype after prolonged incubation with myelin. Moreover, the p47–PHOX-mediated production of ROS after myelin phagocytosis reduced the production of inflammatory mediators by microglia, suggesting that ROS functions as a negative feedback loop to limit neuroinflammation. Consistent with this immunomodulatory role of ROS, mice with experimental

autoimmune arthritis and EAE show enhanced autoimmunity and a reduced oxidative burst when they carry mutations in the neutrophil cytosolic factor 1 gene, which encodes a key adaptor of NADPH–oxidase [571]. Finally, myelin

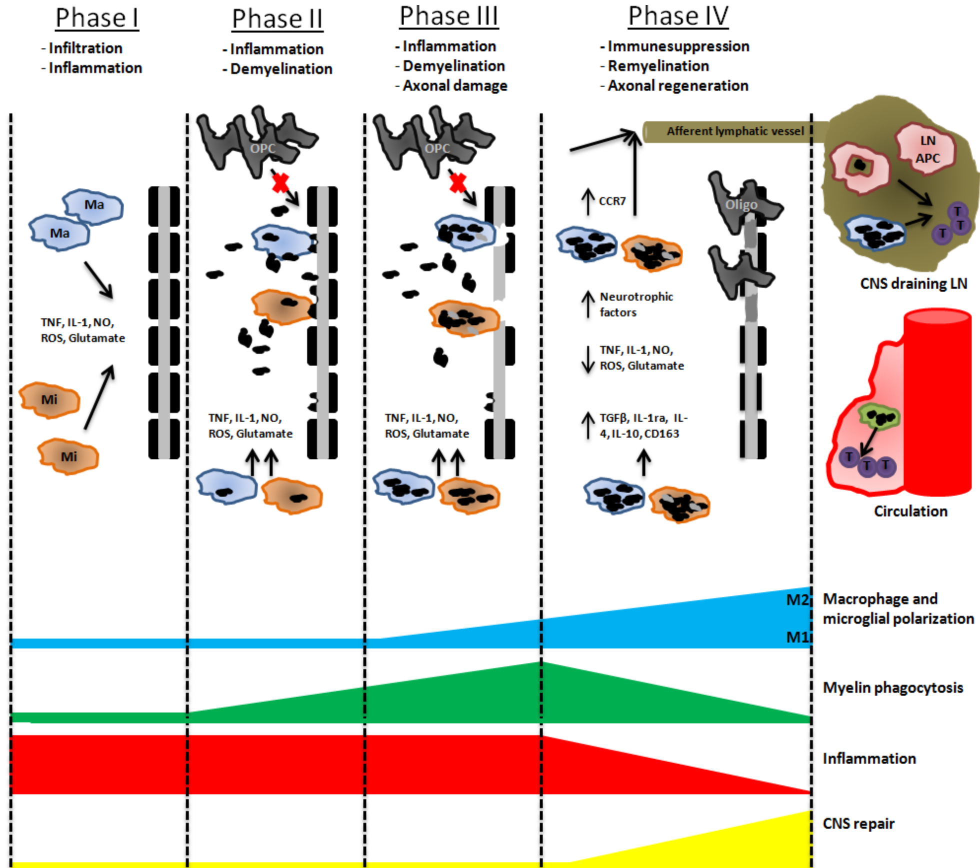

Figure 1.3 (Myelin-phagocytosing) macrophages and microglia in MS pathogenesis. Simplified schematic illustration showing how macrophages (Ma) and microglia (Mi) can aggravate and potentially ameliorate MS. In phase I microglia and infiltrated monocytes (Mo) become activated by local inflammatory mediators in the CNS. Following activation, they secrete inflammatory and toxic mediators (TNF, IL-1, NO, ROS and glutamate), capable of damaging oligodendrocytes and myelinated axons. In phase II myelin damage is apparent. Macrophages and microglia internalize intact myelin and myelin debris, and secrete inflammatory and toxic mediators. OPCs fail to mature and remyelinate nude axons due to the presence of inhibitory myelin debris. Phase III resembles phase II in all aspects, with the exception that axonal damage is apparent in this phase of lesion development. In the final phase (phase IV) myelin-phagocytosing

macrophages and microglia adopt an anti-inflammatory, immunesuppressive phenotype. Whereas the production of pro-inflammatory and toxic mediators is suppressed, anti-inflammatory and neurotrophic factors are induced. OPCs are relieved from the myelin-mediated maturation block due to clearance of myelin debris, resulting in their differentiation into oligodendrocytes (Oligo) and remyelination. Similarly, axonal regeneration is stimulated by the presence of growth factors and the absence of myelin debris. In addition, anti-inflammatory macrophages containing myelin in the perivascular space (PVMs) can influence infiltrating autoreactive T cells. Also, myelin-containing phagocytes in the CNS-draining lymph nodes may modulate autoreactive T cells priming, proliferation and polarization. How brain antigens gain access to CNS draining secondary lymph nodes, either chemotactically in the context of $CCR7^{+}$ phagocytes or as soluble products, remains to be clarified. LN APC: lymph node antigen presenting cell

internalization has been described to increase the expression of CD163, a marker of anti-inflammatory macrophages [572, 573], on macrophages *in vitro* and *in vivo* [378]. The observed functional phenotype of foamy macrophages in MS is in agreement with the previously demonstrated anti-inflammatory nature of lipid-laden macrophages [574, 575]. These studies suggest that both macrophages and microglia can fulfil a neuroprotective role in MS following myelin internalization, as opposed to their detrimental impact on disease progression. By altering their inflammatory phenotype after myelin phagocytosis, microglia and macrophages can for instance suppress demyelination, axonal degeneration and EAE severity [307]. Moreover, as anti-inflammatory macrophages can suppress the proliferation of autoreactive T cell and promote the differentiation of anti-inflammatory Th2 and Tregs [227, 576], myelin-phagocytosing macrophages may exert an immunesuppresive effect during MS pathogenesis. Finally, myelin internalization by macrophages and microglia may directly affect axonal regeneration, as conditioned media from myelin treated macrophages enhances both neuron survival and neurite regeneration of adult dorsal root ganglia [577].

1.3 Aims of the study

MS is a chronic inflammatory, demyelinating, neurodegenerative disorder of the CNS that is marked by a heterogeneous disease course and genetic background. Pathologically, MS is characterized by CNS infiltration of activated autoreactive lymphocytes and macrophages, resulting in an inflammatory microenvironment. Microglia and macrophages typically accumulate in the perivascular spaces and the brain parenchyma near terminal ovoids of transected axons. They are thought to be the primary effector cells in MS and EAE. Effector mechanisms of activated macrophages and microglia include the internalization of myelin, reactivation of autoreactive T cells and the secretion of inflammatory and toxic mediators which negatively influence axonal and myelin integrity. However, macrophages and microglia have also been reported to reduce demyelination and stimulate remyelination and axonal regeneration, indicating a role for these cells in dampening lesion progression and promotion of CNS repair in MS.

Macrophages and microglia are able to adopt divergent phenotypes depending on environmental cues. In MS, macrophages and microglia initially display a pro-inflammatory phenotype. However, upon internalization of myelin, they have been described to obtain anti-inflammatory characteristics. It is far from clear how these myelin-phagocytosing macrophages affect neuroinflammation and what factors in myelin direct this macrophage phenotype switch. We hypothesize that myelin-derived constituents direct macrophages towards a immunomodulatory, neuroprotective phenotype, thereby suppressing neuroinflammation and lesion progression in MS. To test this hypothesis the following aims will be addressed:

Aim I: The impact of myelin-phagocytosing macrophages on autoreactive T cell proliferation

The presence of myelin-containing macrophages in the parenchyma, perivascular spaces and CNS-draining lymph nodes of MS patients and EAE animals suggests that these cells are ideally positioned to exert a profound effect on autoreactive T cell function. Although myelin-loaded macrophages have been demonstrated to reactivate autoreactive lymphocytes upon infiltration, the changes in the functional properties of these cells may alter their

immunomodulatory actions towards T cells. In **chapter II**, we unravel the effect of myelin-phagocytosing macrophages on autoreactive T cell proliferation *in vitro* and *in vivo*. For this purpose, myelin-phagocytosing macrophages are cocultered with MBP- and ovalbumine (OVA)-reactive T cells *in vitro*. By treating MBP- and OVA-immunized animals subcutaneously with myelin, we establish the impact of myelin-phagocytosing macrophages on T cell proliferation *in vivo*.

Aim II: Identification of the pathways underlying the myelin-directed phenotype change in macrophages and their impact on neuroinflammation

The myelin sheath is a fatty insulating layer that surrounds axons. The main constituents of myelin are lipids (70%), like cholesterol and phospholipids, and proteins (30%), such as MBP, PLP, MOG and MAG [578]. In **chapter III and IV** we aim to determine how myelin components skew myelin-phagocytosing macrophages towards an anti-inflammatory phenotype. Microarray analysis was performed to assess the transcriptional events associated with myelin phagocytosis by macrophages. Amongst other pathways, liver-X-receptors (LXRs) and peroxisome proliferator-activated receptor (PPARs) signaling were induced by myelin. These lipid-sensing nuclear receptors are activated by respectively cholesterol derivates and fatty acids, and have recently been described as key regulators of lipid metabolism and inflammation in macrophages [579-581]. Since activation of LXRs and PPARs induces an anti-inflammatory phenotype in macrophages [582-592], a myelin-mediated activation of nuclear receptors may explain the less inflammatory phenotype of macrophages after myelin internalization.

In chapter III, we aim to determine whether myelin-derived cholesterol alters the functional properties of myelin-phagocytosing macrophages through activation of LXRs. For this purpose, wild-type, LXRα-, LXRβ- and LXRαβ-deficient mouse macrophages are treated with myelin, after which the expression and secretion of genes involved in cholesterol efflux and pro-inflammatory and toxic mediators are determined.

Clearance of apoptotic cells by exposure of phosphatidylserine (PS) to macrophages and the subsequent induction of a tolerogenic phenotype is associated with PPAR activation [593]. In **chapter IV,** we determine whether

myelin-derived PS affects the phenotype of myelin-phagocytosing macrophages through activation of PPARs. To do so, macrophages are treated with specific antagonists for PPARα, β/δ and γ, prior to administration of myelin and PS-containing liposomes (PSLs). Subsequently, the concentrations of inflammatory mediators in culture supernatants are assessed. In a next step, the impact of PSL treatment on EAE pathology and severity is determined by evaluating disease symptoms, lymphocyte proliferation, CNS immune cell infiltration and the expression of pro- and anti-inflammatory mediators.

Aim III: The role of collectin sub-family member 12 on myelin-phagocytosing macrophages

Microarray analysis demonstrated an increased expression of collectin sub-family member 12 (COLEC12) on macrophages after internalization of myelin. COLEC12 is structurally related to scavenger receptor A [594]. Furthermore, a recent study demonstrated that COLEC12 facilitates amyloid beta clearance by glial cells and that COLEC12 is expressed in a amyloid beta-dependent fashion [595]. In **chapter V**, we study how myelin induces the expression of COLEC12 on macrophages and what function it fulfils. To determine the involvement of nuclear receptors, macrophages are treated with agonists for LXRs and PPARs. In addition, blocking antibodies for COLEC12 are used to evaluate its role in the internalization of myelin.

Altogether, the results from this study will contribute to a better understanding of the phenotype and role of macrophages in MS. Furthermore, this report will determine a possible link between lipid metabolism and inflammation in macrophages during MS pathogenesis. The identification of myelin-derived lipids capable of dampening inflammation may potentially explain the relapsing-remitting form of MS and holds promise for future therapeutics aimed at modulating neuroinflammation.

2

Myelin-phagocytosing macrophages modulate autoreactive T cell proliferation

Based on:

Myelin-phagocytosing macrophages modulate T cell proliferation

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Abstract

MS is a chronic, inflammatory, demyelinating disease of the central nervous system in which macrophages play a central role. Initially, macrophages where thought to be merely detrimental in MS, however, recent evidence suggests that their functional phenotype is altered following myelin phagocytosis. Macrophages that have phagocytosed myelin may be less inflammatory and may exert beneficial effects. The presence of myelin-containing macrophages in CNS-draining lymph nodes and perivascular spaces of MS patients suggests that these cells are ideally positioned to exert an immuneregulatory role. Therefore we evaluated in this study the effect of myelin-phagocytosing macrophages on lymphocyte reactivity. We demonstrate that myelin-phagocytosing macrophages inhibit TCR-triggered lymphocyte proliferation in an antigen-independent manner. The observed immune suppression is mediated by an increase in NO production by myelin-phagocytosing macrophages upon contact with lymphocytes. Additionally, myelin delivery to primarily $CD169^{+}$ macrophages in popliteal lymph nodes of OVA-immunized animals results in a reduced cognate antigen specific proliferation. In contrast to OVA-immunized animals, lymphocytes from MBP-immunized animals displayed an increased proliferation after stimulation with their cognate antigen, indicating that myelin-phagocytosing macrophages have dual effects depending on the specificity of surrounding lymphocytes.

Collectively our data show that myelin phagocytosis leads to an altered macrophage function that inhibits lymphocyte proliferation. Additionally, results from this study indicate that myelin-phagocytosing macrophages fulfill a dual role *in vivo*. On one hand they aggravate autoimmunity by activating myelin-reactive lymphocytes and on the other hand they suppress lymphocyte reactivity by producing NO.

2.1 Introduction

MS is characterized as a chronic, inflammatory, neurodegenerative disease of the CNS. It is regarded to be an autoimmune disease as activated autoimmune lymphocytes are pivotal in orchestrating the immunopathological processes involved in myelin sheath damage [5, 93, 94, 596].

Pathologically, MS is characterized by CNS infiltration of activated myelin-reactive lymphocytes and macrophages, resulting in an inflammatory microenvironment. Microglia and macrophages typically accumulate in the perivascular spaces and the brain parenchyma near terminal ovoids of transected axons [167]. They are thought to be the primary effector cells in MS and EAE [320-323]. Effector mechanisms of activated macrophages and microglia include the internalization of myelin, and the secretion of inflammatory and toxic mediators which negatively influence axonal and myelin integrity [365, 372-378, 406, 478, 597-600].

In contrast to their apparent detrimental role in MS, increasing evidence suggests an additional neuroprotective role for macrophages. Although two seemingly mutually exclusive processes, various studies have reported such a dual role of monocytes and macrophages in both injury and repair [269, 522, 523]. In neurodegenerative models, remyelination is for instance often correlated with large numbers of macrophages and microglia in an inflammatory microenvironment [525-530]. Furthermore, as contact with CNS myelin debris inhibits oligodendrocyte progenitor maturation *in vitro* [531], and as macrophages have been described to actively phagocytose myelin debris, local clearance of myelin debris in the centre or vicinity of lesions is suggested to be a necessary prerequisite for axonal remyelination following demyelination. This hypothesis is supported by the fact that monocyte depletion and a consequent inability to clear the microenvironment of myelin debris, causes an impairment of oligodendrocyte progenitor differentiation *in vivo* [532, 533]. Finally, recent evidence indicates that monocyte-derived macrophages, peritoneal macrophages, microglia and dendritic cells (DCs) obtain anti-inflammatory characteristics following internalization of myelin [365, 375, 376, 601]. These studies clearly demonstrate that macrophages, besides their apparent role in neurodegeneration, may exert a neuroprotective influence on MS pathogenesis

by clearance of myelin debris and by altering their phenotype following myelin internalization.

Perivascular macrophages, infiltrated macrophages and microglia are ideally positioned to influence infiltrating and infiltrated myelin-reactive lymphocytes. Indeed, CNS reactivation of autoreactive lymphocytes by local antigen presenting cells displaying myelin antigens is thought to initiate and maintain the inflammatory cascade observed in the brain of MS patients [93, 94, 352]. The presence of brain antigen-containing phagocytes in secondary lymph nodes in MS and EAE further emphasizes a possible crucial role of these cells in modulating the immune response during MS and EAE pathogenesis [366-368]. Phenotypical analysis of these macrophages further revealed that in contrast to neuronal antigen containing phagocytes, the majority of myelin-containing APCs express anti-inflammatory mediators. How brain antigens gain excess to CNS draining secondary lymph nodes, either chemotactically in the context of phagocytes or as soluble products, remains to be clarified [365, 369, 370].

In this study we investigated the capacity of myelin-phagocytosing macrophages to influence lymphocyte proliferation. We show that myelin-phagocytosing macrophages inhibit TCR-triggered lymphocyte proliferation in an antigen-independent manner. This process is mediated by an enhanced NO production. Furthermore, we demonstrate that myelin delivery to popliteal lymph nodes of OVA-immunized animals and uptake by primarily CD169^{+} macrophages reduces cognate antigen specific proliferation following restimulation *ex vivo*. The elevated production of NO detected in these lymph node cultures indicates that NO may also mediate the immune suppressive effects *in vivo*. In contrast, myelin delivery to popliteal lymph nodes did increase lymphocyte reactivity in MBP-immunized animals. Thus, myelin-phagocytosing macrophages may play a suppressive role in CNS-draining lymph nodes during MS pathogenesis, depending on the nature of surrounding lymphocytes. Collectively our data provide evidence that myelin phagocytosis leads to an altered macrophage function that modulates lymphocyte responses.

2.2 Materials and methods

2.2.1 Animals

Female Lewis rats, 6-8 weeks of age, were purchased from Harlan Netherlands B.V. (Horst, The Netherlands). Animals were housed in the animal facility of the Biomedical Research Institute of Hasselt University. Experiments were conducted in accordance with institutional guidelines and approved by the local Ethical Committee for Animal Experiments of Hasselt University.

2.2.2 Isolation of peritoneal rat macrophages

Three days prior to macrophage isolation, rats were injected intraperitoneally with 3 ml 3% thioglycolate (Sigma-Aldrich, Bornem, Belgium). Resident peritoneal macrophages were obtained by peritoneal lavage using 10 ml of ice-cold phosphate buffered saline (PBS; Lonza, Vervier, Belgium) supplemented with 5mM ethylenediamine tetraacetic acid (EDTA; VWR, Leuven, Belgium). Peritoneal exudate cells (PECs) were cultured for 2 hours in RPMI 1640 medium. After 2 hours incubation at 37°C with 5% CO_2, non-adherent cells were washed away. Remaining cells were >95% macrophages [602].

2.2.3 Myelin isolation

Myelin was purified from rat brain tissue by means of density-gradient centrifugation, as described previously [603]. Myelin protein concentration was determined by using the BCA protein assay kit (Thermo Fisher Scientific, Erembodegem, Belgium). Lipopolysaccharide (LPS) content was determined using the Chromogenic Limulus Amebocyte Lysate assay kit (Genscript Incorporation, Aachen, Germany). Isolated myelin contained a neglectable amount of endotoxin (<1.8×10^{-3} pg/µg myelin).

Isolated myelin was fluorescently labelled, according to the method of Van der Laan et al. [374]. In short, 10 mg/ml myelin was incubated with 12.5 µg/ml 1,1"-diotadecyl-3,3′,3′,-tetramethylindocarbocyanide perchlorate (DiI; Sigma-Aldrich) for 30 min at 37°C. Next, a myelin phagocytosis assay was performed as previously described [603].

2.2.4 Immunization and *in vivo* myelin treatment

Lewis rats were injected subcutaneously with a 0.1 ml suspension containing 250 µg/ml guinea pig MBP or ovalbumin, 2.5 mg/ml H37RA heat-killed mycobacterium tuberculosis (Difco, Detroit, USA) and 60 µl Complete Freunds adjuvant (Sigma-Aldrich) in both hind paws. Subsequently animals were injected subcutaneously with PBS, 2.6 x 10^6 latex beads (0.8 µm mean particle size, Sigma-Aldrich), 75 µg/animal of isolated myelin or OVA (d-4, 0, 4 and 8 pre- and post-immunization). MBP-immunized rats were weighed and scored daily according to the following neurological scale: 0 = no neurological abnormalities, 0.5 = partial loss of tail tonus, 1 = complete loss of tail tonus, 2 = hind limb paresis, 3 = hind limb paralysis, 4 = moribund, 5 = death.

2.2.5 Generation of antigen-specific lymphocytes

MBP and OVA-specific lymphocytes were obtained 9 days post-immunization by bilateral isolation of the inguinal and popliteal lymph nodes. Single-cell suspensions of harvested lymph nodes were obtained by grinding with a syringe plunger against a 70 µm cell strainer (Bellco Glass Inc., Vineland, USA). To enrich for antigen specific lymphocytes, lymph node cells were restimulated, as described previously [604]. Briefly, lymph node cells were resuspended in stimulation medium: RPMI 1640 medium (Invitrogen, Merelbeke, Belgium) supplemented with 50 U/ml penicillin (Invitrogen), 50 U/ml streptomycin (Invitrogen), 20 µM 2-mercapto-ethanol (Sigma-Aldrich), 1% sodium pyruvate (Invitrogen), 1% MEM non-essential amino acids (Invitrogen), 2% deactivated autologous serum and 33 µg/ml MBP. After 2 days, cells were washed and resuspended in RPMI 1640 medium supplemented with 50 U/ml penicillin, 50 U/ml streptomycin, 20 µM 2-mercapto-ethanol, 10% fetal calf serum (FCS, Hyclone, Erembodegem, Belgium) and 6,5% supernatants of Concanavalin (ConA, Sigma-Aldrich) stimulated spleen cells. Following 2 days, cells were washed and resuspended in RPMI 1640 medium supplemented with 50 U/ml penicillin, 50 U/ml streptomycin, 20 µM 2-mercapto-ethanol and 10% FCS for 3 days.

2.2.6 CFSE-labeling of lymphocytes

A carboxyfluorescein diacetatesuccinimidyl ester (CFSE) stock (10 mM in Dimethylsulfoxide (DMSO), Invitrogen, Merelbeke, Belgium) was diluted in PBS (Biowhittaker™). Antigen-specific lymphocytes were resuspended in PBS supplemented with 0.05% bovine serum albumin (BSA) and 4 µM CFSE (20 x 10^6 cells/ml) for 7 min at 37°C with 5% CO_2. Cells were washed and diluted in 0.5 ml culture medium for 30 min at 37 °C with 5% CO_2 to stabilize the CFSE-labeling. In parallel, to determine macrophage viability following a coculture with lymphocytes, macrophages were labeled with CFSE to distinguish them from unlabeled lymphocytes.

2.2.7 Coculture of macrophages with lymphocytes

Prior to coculture with CFSE labeled lymphocytes, isolated macrophages were seeded in flat-bottem 96-well plates (15 X 10^3 cells/well) in RPMI 1640 medium supplemented with 50 U/ml, 50 U/ml streptomycin and 10% FCS, and treated with 100 µg/ml of isolated myelin for three hours. Excess myelin was removed by washing twice with RPMI 1640 medium at 37°C. Subsequently, stimulation medium containing irradiated thymocytes (15 X 10^4, 3000 rad), CFSE-labeled MBP- or OVA-specific lymphocytes (15 X 10^4) and respectively 10 µg/ml MBP or 10 µg/ml OVA were added. Untreated macrophages were used as a control. To evaluate the involvement of respectively NO, arginase, indoleamine 2,3-dioxygenase (IDO), the phagocytosis process itself, direct cell-cell contact and IFNγ, 1.5 mM N^G-Monomethyl-L-arginine (L-NMMA; VWR), 0.5 mM N^G-Hydroxy-L-arginine (NOHA; VWR), 0.2 mM 1-Methyl-L-tryptophan (1-MT; Sigma Aldrich), latex beads (1:100), 100µg/ml zymosan A (Sigma-Aldrich), transwell inserts (0.4 µm pore size, Sigma-Aldrich) or 10 µg/ml anti-rat IFNγ (Preprotech, London, UK) were tested in the coculture model.

Flow cytometry was used to assess proliferation and cell death of lymphocytes and macrophages after a 4 day coculture. Here, cells were stained with PE-conjugated mouse-anti-rat CD3 (Immunosource, Erembodegem, Belgium) or CD11b (AbD Serotec, Düsseldorf, Germany) and 7 aminoactinomycin D (7AAD, BD Biosciences).

2.2.8 [^{3}H]Thymidine incorporation

Isolated lymph node cells (20 X 10^4) were cultured with MBP (10 µg/ml), OVA (10 µg/ml) or MOG (20 µg/ml). Additionally, 100 µg/ml of isolated myelin was added in some experiments. Following 48hr, 1 µCi [^{3}H]thymidine (Amersham, Buckinghamshire, UK) was added to the culture. Next, cells were harvested with an automatic cell harvester (Pharmacia, Uppsala, Sweden) and uptake of radioactivity was measured in a β-plate liquid scintillation counter (Wallac, Turku, Finland).

2.2.9 Nitrite formation

Coculture supernatants were collected and release of NO was determined using the Griesss reagent system (Promega, Leuven, Belgium), following the manufacturer's instructions. Absorbance was determined by using a microplate reader at 550 nm (Biorad Benchmark).

2.2.10 Histology and immunohistochemistry

Snap-frozen brain and spinal cord material was cut in respectively the coronal and sagittal plane with a Leica CM1900UV cryostat (Leica Microsystems, Wetzlar, Germany) to obtain 10 µm sections. The extent of demyelination and infiltration was determined by staining with Luxol Fast Blue (LFB; Gurr BDH, Poole, England). Briefly, aceton-fixed slides were incubated with LFB for 16hr at 56°C, destained with 0.05% lithium carbonate, and counterstained with cresyl violet (VWR). Analysis was carried out using a Nikon eclipse 80i microscope and NIS Elements BR 3.10 software (Nikon, Tokyo, Japan).
DiI-labeled myelin migration to popliteal and inguinal lymph nodes was determined by immunohistochemistry. Popliteal and inguinal lymph nodes were snap-frozen directly following isolation and cut into 10 µm sections. Following fixation in aceton for 10 min, sections and cells were blocked using 10% goat serum (Millipore, Brussels, Belgium) in PBS. Subsequently, sections and cells were stained with mouse-anti-rat CD169 (1/250 in PBS; Abd Serotec), a marker for macrophages in lymph nodes. As a secondary antibody Alexa fluor 488

$F(ab')_2$ fragment of goat-anti mouse was used (1/500 in PBS; Invitrogen). Control staining was performed by omitting the primary antibody. Nuclear staining was performed using 4,6′-diamidino-2-phenylindole (DAPI; Invitrogen) for 10 min. Autofluorescence was minimalized by using 0.1% Sudan Black in 70% ethanol.

2.2.11 Statistics

Data were statistically analyzed using GraphPad Prism for windows (version 4.03) and are reported as mean ± standard error of the mean (SEM). D'Agostino and Pearson omnibus normality test was used to test normal distribution. An analysis of variances (ANOVA) or two-tailed unpaired student T-test (with Welch's correction if necessary) was used for normally distributed data sets. The Kruskal-Wallis or Mann-Whitney analysis was used for data sets which did not pass normality. *P<0,05, **P<0,01 and ***P<0,001.

2.3 Results

2.2.1 Myelin-laden macrophages inhibit lymphocyte proliferation

Initially we assessed the capacity of peritoneal macrophages to internalize myelin. By culturing macrophages with different concentrations of DiI-labeled myelin for divergent periods of time, it was demonstrated that myelin is internalized in a time- and dose-dependent manner (figure 2.1a). Next, the most optimal macrophage/lymphocyte coculture ratio was determined by using untreated macrophages. At high macrophage/lymphocyte ratio's, lymphocytes demonstrated a reduced viability and proliferation (figure 2.1b and 2.1c). The decline of lymphocyte viability was absent at low macrophage/lymphocyte ratio's (<1/10). To observe differences in lymphocyte proliferation following coculture with myelin-phagocytosing macrophages, subsequent experiments were conducted using a macrophage/lymphocyte ratio of 1/10.
To study whether myelin-phagocytosing macrophages affect antigen-specific lymphocyte proliferation in a different manner compared to untreated macrophages, macrophages or myelin-treated macrophages were cocultured for 4d with MBP- or OVA-reactive lymphocytes, irradiated thymocytes and purified MBP or OVA. Here it was demonstrated that myelin-phagocytosing macrophages inhibit TCR-triggered lymphocyte proliferation more pronounced than untreated macrophages (figure 2.1d and 2.1e). This process was independent of antigen-specificity, since both MBP and OVA-reactive lymphocytes showed the same reduction in proliferation. Proliferation differences were not due to an altered viability of lymphocytes or myelin-phagocytosing macrophages (data not shown).

2.2.2 Inhibition of lymphocyte proliferation is independent of the phagocytosis process or myelin-antigen presentation

To elucidate the mechanisms behind the increased inhibition of lymphocyte proliferation by myelin-phagocytosing macrophages, we assessed whether the phagocytosis process as such is responsible for the observed effects on lymphocyte proliferation. For this purpose, macrophages were loaded for 3 consecutive hours with latex beads or zymosan A prior to coculture with

lymphocytes. Macrophage treatment with beads or zymosan significantly affected their capacity to modulate lymphocyte proliferation compared to control and myelin treated macrophages (figure 2.2a), indicating that the observed increased inhibition of lymphocyte proliferation relies on myelin-specific effects, instead of being induced by the phagocytosis process itself.

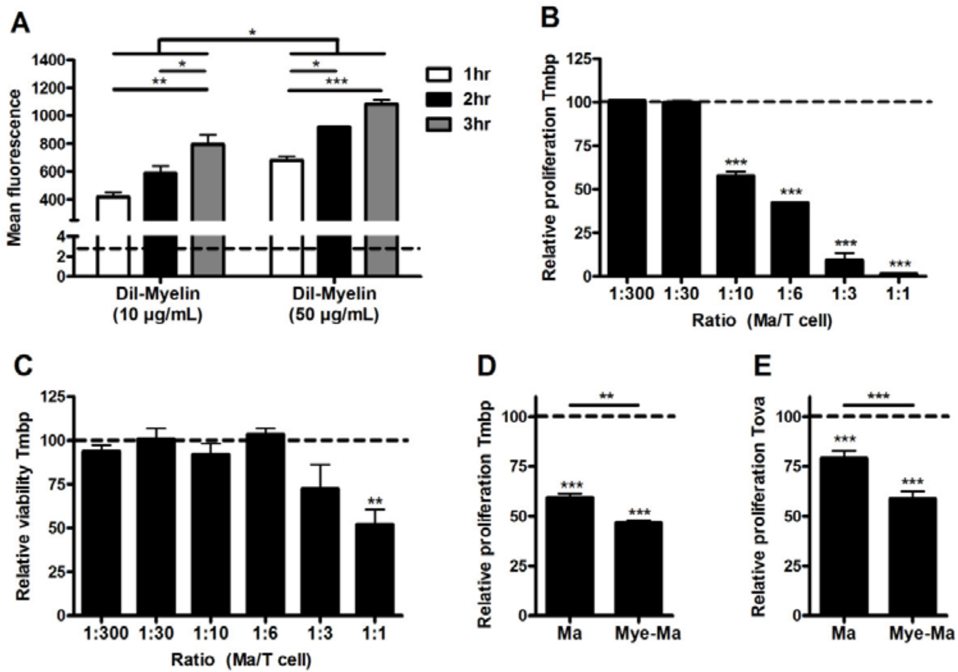

Figure 2.1 Myelin-phagocytosing macrophages inhibit TCR-triggered lymphocyte proliferation in an antigen-independent manner. (a) Mean DiI-flourescence following treatment of macrophages with divergent concentrations of myelin (10 and 50 µg/ml) for several periods of time (1hr; white bars, 2hr; black bars or 3hr; grey bars). The dotted line represents untreated macrophages. Data represent the mean of two independent experiments. (b,c) Lymphocyte proliferation and viability following a 4d coculture with irradiated thymocytes, purified MBP and different concentrations of macrophages. The relative proliferation and viability is defined as the percentage of proliferating or viable cells in experimental cultures divided by values in control lymphocyte cultures without macrophages (dotted line). Data represent the mean of three independent experiments. (d,e) Proliferation of MBP or OVA-reactive lymphocytes following a 4d coculture with irradiated thymocytes, macrophages or myelin-macrophages (macrophage/lymphocyte ratio 1/10) and respectively purified MBP or OVA. Data represent the mean of six (d) and four (e) independent experiments. Dotted line represents the proliferation of control lymphocyte cultures without macrophages. Ma: Macrophages.

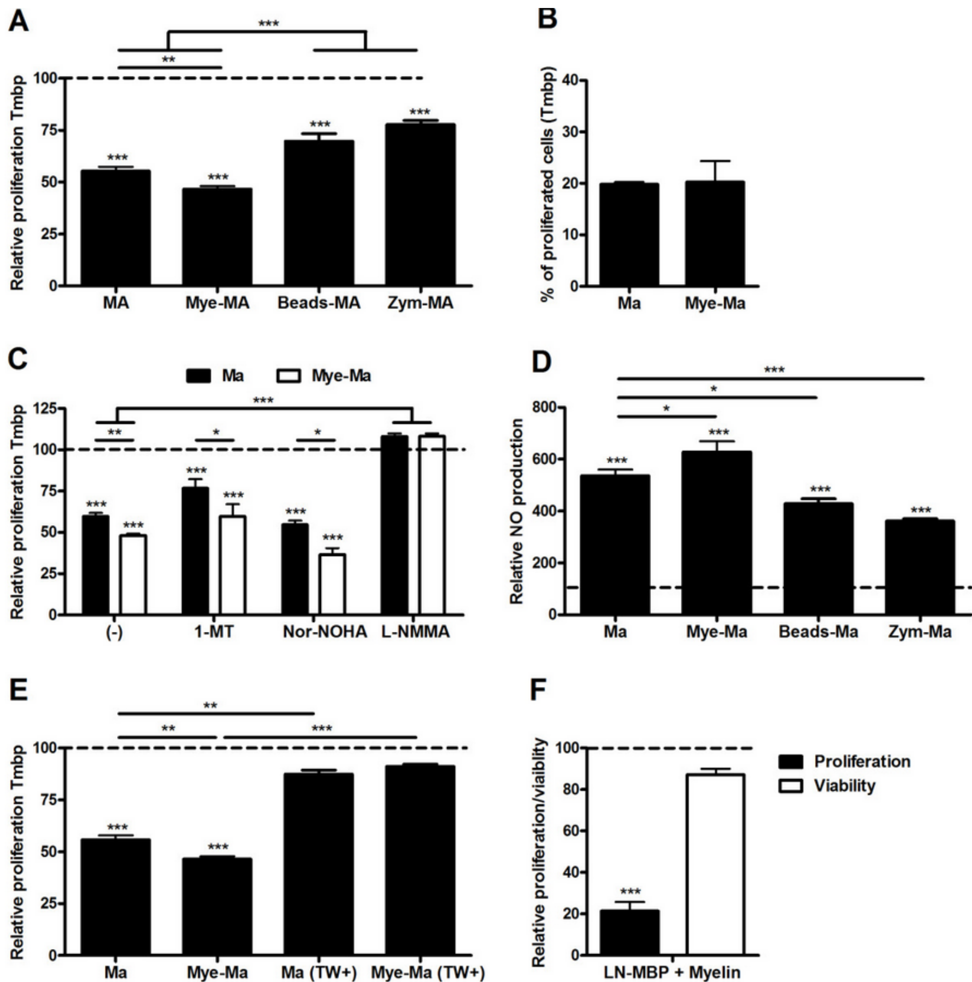

Figure 2.2 Nitric oxide secreted by myelin phagocytosing macrophages inhibits lymphocyte proliferation *in vitro*. (a) Comparison of lymphocyte proliferation following coculture with untreated, myelin-, zymosan- (Zym) or latex bead-treated macrophages. The relative proliferation is defined as the percentage of proliferating cells in experimental cultures divided by values in control lymphocyte cultures without macrophages (dotted line). Data represent the mean of two independent experiments. (b) To determine the capacity of myelin-phagocytosing macrophages to present myelin antigens, MBP-reactive lymphocytes were cocultured with macrophages or myelin-treated macrophages (ratio 1/10) in the absence of thymocytes and MBP. Data represent the mean of two independent experiments. (c) The role of respectively IDO, arginase and NO in the observed inhibition of proliferation, macrophage coculture (black bars) and myelin-treated macrophage coculture (white bars), was determined by addition of an IDO inhibitor (1-MT), an arginase inhibitor (Nor-NOHA) and an iNOS inhibitor (L-NMMA). Dotted line

represents the proliferation of control lymphocyte cultures without macrophages. Data represent four independent experiments. (d) Relative NO concentration in supernatants following a 4d coculture with MBP-reactive lymphocytes. Dotted line represents the NO production of control lymphocyte cultures without macrophages. Data represent the mean of 4 independent experiments. (e) Comparison of lymphocyte proliferation following coculture with untreated and myelin-treated macrophages using transwell inserts. Dotted line represents the proliferation of control lymphocyte cultures without macrophages. Data represent the mean of two independent experiments. (f) *Ex vivo* proliferation (black bar) and viability (white bar) of lymph node cells following a coculture with MBP and myelin. Proliferation was compared to cultures in which no myelin was added. Lymph nodes were isolated 9d-post MBP-immunization. Data represent three independent experiments. Ma: Macrophages, LN: Lymph node, TW: transwell inserts.

Like DCs, macrophages can act as messengers of innate and adaptive immunity by presenting antigen in context of MHC molecules. Myelin-phagocytosing macrophages can therefore be assumed to process endogenous myelin and present it to myelin-reactive lymphocytes, possibly influencing reactivity of these lymphocytes. To determine whether the observed immune suppressive effects are dependent on antigen presentation, untreated macrophages and myelin-treated macrophages were cocultured with MBP-reactive lymphocytes in the absence of MBP and irradiated thymocytes. Myelin-phagocytosing macrophages did not affect lymphocyte proliferation (figure 2.2b), suggesting that, at a macrophage/lymphocyte ratio of 1/10, myelin-phagocytosing macrophages do not influence the proliferation of MBP-reactive lymphocytes. These results are in line with the observed equal inhibition of proliferation of both MBP and OVA-reactive lymphocyte by myelin-phagocytosing macrophages.
Lipid components of myelin, like cholesterol and arachidonic acid-containing phosphatidylcholine, have been reported to directly inhibit proliferation of ConA stimulated lymphocytes [605, 606]. Accordingly, we assessed whether isolated myelin has a direct effect on lymphocyte proliferation. By culturing lymphocytes with increasing concentrations of isolated myelin, in the absence of macrophages, it was established that myelin itself, even at high concentrations, had no significant influence on lymphocyte reactivity (data not shown).
Together, these data indicate that direct effects of myelin on lymphocyte proliferation, myelin-antigen presentation and the phagocytosis process itself are not responsible for the observed effects on lymphocyte proliferation.

2.2.3 Macrophages inhibit lymphocyte proliferation by the production of nitric oxide

Macrophages have been reported to inhibit lymphocyte proliferation *in vitro* by mechanisms involving inducible nitric oxide synthase (iNOS), arginase I or IDO [432, 433, 607-617]. By administrating either an inhibitor of NOS (L-NMMA), arginase (NOR-NOHA) or IDO (1-MT) to the culture, the involvement of these enzymes was evaluated. Here we demonstrated that an increased activity of both IDO and arginase did not account for the increased inhibition of lymphocyte proliferation by myelin-phagocytosing macrophages (figure 2.2c). In contrast, administration of L-NMMA completely abrogated the inhibition of lymphocyte proliferation by both control macrophages and myelin-treated macrophages. Yet again, cocultures of both OVA and MBP-reactive lymphocytes were affected equally (data not shown). The importance of NO was further delineated by assessment of NO levels in the coculture supernatant of MBP-reactive lymphocytes. In contrast to cocultures of lymphocytes with latex beads or zymosan treated macrophages, a significantly increased concentration of NO was observed in the supernatants derived from cocultures with myelin-phagocytosing macrophages, when compared to untreated macrophages (figure 2.2d). This increased NO production by macrophages following myelin internalization was absent in monocultures (data not shown), indicating that the observed increased concentration of NO in the coculture with myelin-treated macrophages was induced by lymphocytes. Lymphocyte-derived IFNγ has previously been described to induce NO production in macrophages [618]. However, in our coculture model no increase in IFNγ in the supernatants of cocultures with myelin-treated macrophages compared to untreated macrophages was found (data not shown). Correspondingly, IFNγ neutralization did neither abrogate the increased inhibition of proliferation or decrease the NO production in cocultures (data not shown). Nonetheless, when transwell inserts were used to restrict direct cell-cell contact, lymphocyte proliferation in cocultures of both untreated or myelin-treated approached control values (figure 2.2e), indicating a role for direct cell-cell contact in the induction of NO.

Noteworthy, when lymph node cells, isolated 9 days post-immunization, were exposed directly to MBP and myelin, an even more pronounced myelin-mediated inhibition of lymphocyte proliferation was observed (figure 2.2f). The latter

indicates that local lymph node phagocytes show a similar immune suppressive response as peritoneal macrophages following myelin ingestion. Differences detected in proliferation were not due to an altered viability of lymphocytes (figure 2.2f).

2.2.4 Myelin modulates lymphocyte proliferation *in vivo*

Since we established that myelin internalization by macrophages alters their capacity to modulate T cell proliferation *in vitro*, we assessed the *in vivo* suppressive capacity of myelin-phagocytosing macrophages. First, to determine whether subcutaneous injected myelin reaches the draining lymph node and is taken up by macrophages, we injected DiI-labeled myelin in the footpad of healthy animals. A notable migration of myelin towards popliteal lymph nodes was observed (figure 2.3a). Immunohistochemical analysis further revealed that myelin was contained primarily in CD169$^+$ macrophages located at the border of the medulla and in the subcapsular sinus (figure 2.3b).

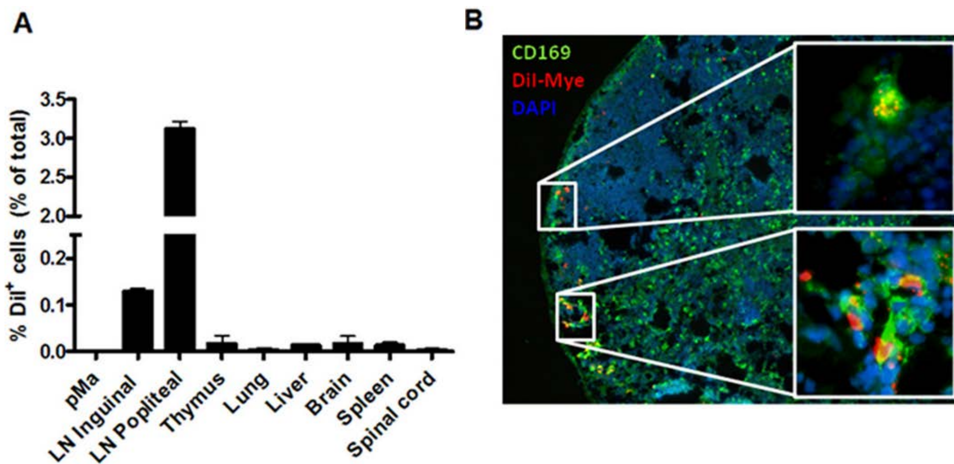

Figure 2.3 Myelin homing to CD169$^+$ macrophages in popliteal lymph nodes. (a) DiI-labeled myelin was injected subcutaneously in the footpad of healthy animals. DiI-flourescene was determined 4d post-injection by flow cytometry. One experiment is shown. (b) Immunohistochemical staining of popliteal lymph nodes 4d post-injection of DiI-labeled myelin. Sections were additionally stained with CD169 and DAPI. pMa: Peritoneal macrophages, LN: Lymph node

Next, OVA-immunized animals were treated subcutaneously in the footpad with myelin (d-4, 0, 4, 8 pre/post-immunization). Recall stimulation *in vitro*, 9d post-immunization, revealed a reduced cognate antigen specific proliferation in animals treated with myelin (figure 2.4a). Interestingly, LPS-stimulated lymph node cultures from myelin-treated animals demonstrated an increased NO production (figure 2.4b). These results demonstrate that myelin is capable of suppressing antigen-specific proliferation *in vivo* and suggest that an increased NO production by these cells is responsible for this effect.

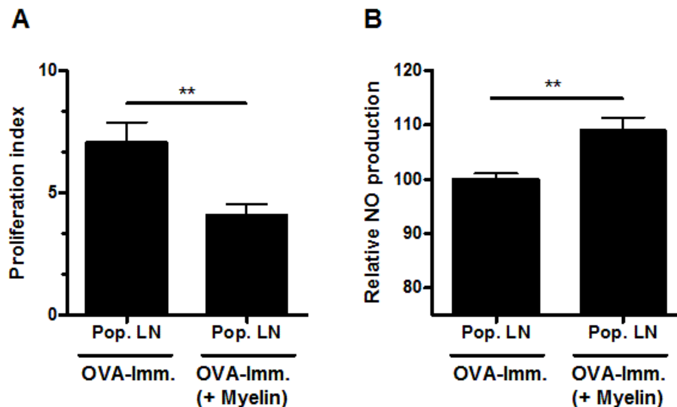

Figure 2.4 Myelin inhibits lymphocyte proliferation in OVA-immunized animals. (a) OVA-immunized animals (N=7) were treated with myelin at day -4, 0, 4 and 8 (or left untreated). Nine days post-immunization, OVA-reactivity of isolated lymph node cultures was assessed. Non-stimulated cultures were used as control. Data represent the mean of seven independent experiments. (b) NO production by LPS-stimulated popliteal lymph node cultures 9d post-immunization. Lymph node cultures were stimulated with LPS for 18hr after which NO production in the supernatant was determined. Data represent the mean of seven independent experiments. Pop. LN: Popliteal lymph node.

In contrast to OVA-immunized animals, MBP-immunized animals revealed an increased reactivity towards MBP and MOG following myelin treatment (figure 2.5a). Moreover, myelin-treated animals demonstrated an earlier onset of paralysis and a more severe neurological score at the peak of disease (figure 2.5b). In concordance, a LFB/cresyl violet staining of brain and spinal cord sections demonstrated increased cellular infiltrates and the presence of demyelinated areas in myelin-treated animals (figure 2.5c). Noteworthy, when MBP-immunized animals were treated with latex beads no overt effect on

disease score and onset was found (data not shown), indicating that the aggravated disease score following myelin treatment is myelin-specific.

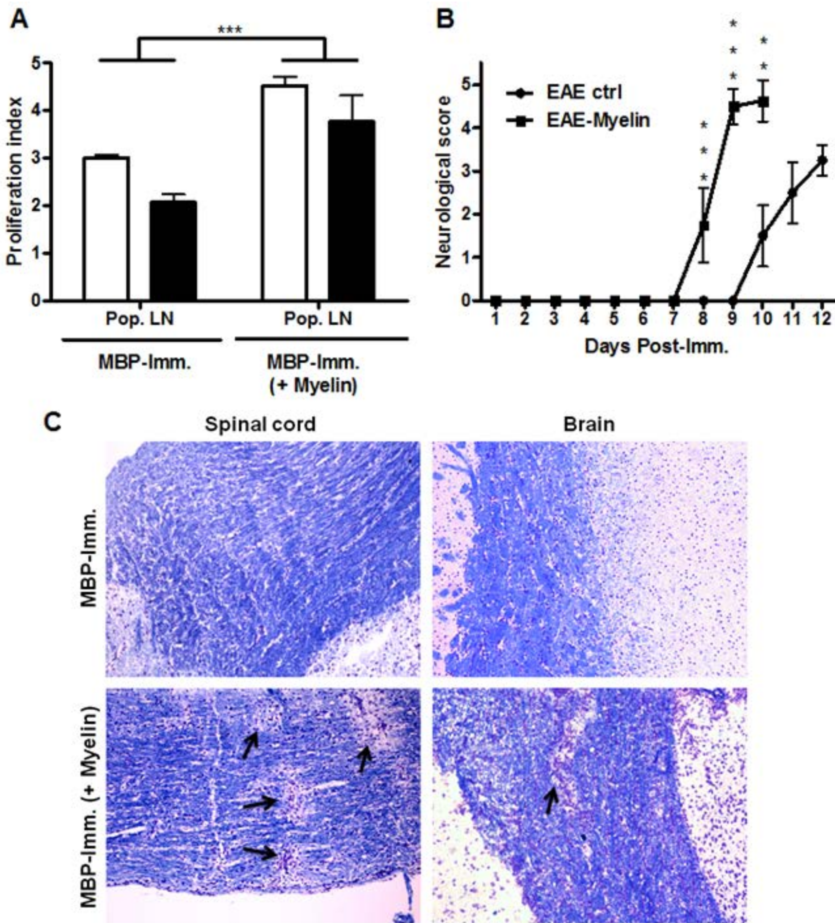

Figure 2.5 MBP-immunized animals demonstrate an aggravated disease course following myelin treatment. (a) MBP-immunized animals were treated with isolated myelin at day -4, 0, 4 and 8 (or left untreated). Ten days post-immunization popliteal lymph node cultures were isolated and MBP (white bars) and MOG (black bars) reactivity was assessed. Non-stimulated cultures were used as control. Data represent the mean of two independent experiments. (b) Neurological score of control (N=5) and myelin-treated (N=5), immunized animals was assessed daily. Due to severity of paralysis, myelin-treated animals were sacrificed at d10. (c) LFB/cresyl violet staining of spinal cord (left) and brain tissue (right) of myelin-treated animals at day 10. Arrows depict demyelinated regions. One experiment is shown. Pop. LN: Popliteal lymph node.

To exclude the possibility that in the restricted area of the lymph node the availability of OVA in OVA-immunized animals is altered by the additional presence of myelin proteins, hereby reducing OVA-specific proliferation, MBP-immunized animals were treated subcutaneously with OVA. As expected, an increased reactivity towards OVA was observed in isolated lymph node cells (figure 2.6a). However, MBP-specific proliferation was unchanged (figure 2.6b), demonstrating that the observed myelin-mediated inhibition of proliferation of lymph nodes cultures from OVA-immunized is independent of a reduced availability of OVA by myelin proteins.

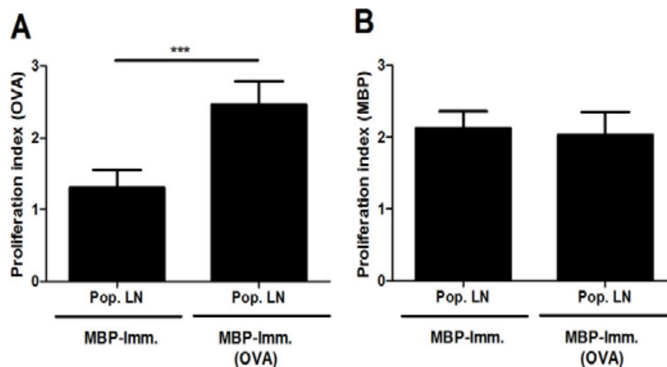

Figure 2.6 OVA does not influence lymphocyte reactivity to MBP in MBP-immunized animals. (a, b) MBP-immunized animals (N=4) were treated with OVA at day -4, 0, 4 and 8 (or left untreated). Nine days post-immunization, OVA- (a) and MBP (b) reactivity of isolated lymph node cultures was assessed. Non-stimulated cultures were used as control. Data represent the mean of four independent experiments. Pop. LN: Popliteal lymph node.

These results indicate that myelin-phagocytosing macrophages have a dual influence on proliferation, depending on the nature of surrounding lymphocytes. On one hand they aggravate autoimmunity by activating myelin-reactive lymphocytes and on the other hand they have the capacity to suppress lymphocyte activation by the secretion of NO.

2.3 Discussion

In this study we have established that macrophages that have phagocytosed myelin modulate the proliferation of autoreactive T cells. The observed inhibition of TCR-triggered lymphocyte proliferation by myelin-phagocytosing macrophages was antigen-independent, as both OVA- and MBP-reactive lymphocytes show an identical reduction in proliferation following coculture with myelin-phagocytosing macrophages *in vitro*. Additionally, when *in vivo* primed lymph node cultures were restimulated directly *in vitro* in the presence of myelin, an even more pronounced immune suppression was observed. These results indicate that both macrophages and lymph node phagocytes obtain immune suppressive properties following myelin internalization.

Macrophages may inhibit proliferation of lymphocytes in various manners, including IDO-mediated depletion of tryptophan, arginase-mediated lowering of L-arginine and lymphocyte CD3ζ expression, and NO-mediated reduction of tyrosine residue phosphorylation in the Jak3/STAT5 pathway and inhibition of caspase activity [432, 433, 607-614]. We demonstrate that the non-selective iNOS inhibitor L-NMMA completely reversed the observed inhibition of proliferation by both control and myelin-treated macrophages while the other pathways were not involved. In line with this, an increased concentration of NO was demonstrated in the coculture supernatant of myelin-phagocytosing macrophages, explaining the observed inhibition of lymphocyte proliferation. Abrogation of direct cell-cell contact restored lymphocyte proliferation in our cocultures. This finding, together with the observed role of NO in the inhibition of lymphocyte proliferation, suggests that direct contact between both cell types is a necessary prerequisite for stimulating NO-mediated inhibition of lymphocyte proliferation by macrophages. On the other hand, NO might, due to extreme short half-life [619], not reach lymphocytes when direct contact is restricted. Future studies should therefore determine the mechanism behind the macrophage- and myelin-phagocytosing macrophage-mediated inhibition of lymphocyte proliferation in our cocultures. Although lymphocyte-derived IFNγ is described to induce NO production by macrophages, we were unable to demonstrate a role for lymphocyte-produced IFNγ in the observed inhibition of lymphocyte proliferation [618].

As we demonstrated an increased, NO-mediated inhibition of lymphocyte proliferation by myelin-phagocytosing macrophages *in vitro*, myelin-rich phagocytes in secondary lymph nodes might fulfill an identical suppressive role *in vivo*. CD169^{+} macrophages in lymph nodes are described to be primarily involved in uptake and relay of viral particles and immune complexes, and activation of follicular B lymphocytes [620-623]. We demonstrate that a subcutaneous injection of myelin in the footpad results in a notable migration of myelin towards CD169^{+} medullary and subcapsular sinus (SCS) macrophages in popliteal lymph nodes. Given the abundance of lipids in myelin, these results are in line with a recent report showing active phagocytosis of lipid-coated silica particles by SCS macrophages [624].

To explore the possible immune suppressive properties of myelin-phagocytosing macrophages *in vivo*, OVA-immunized animals were treated subcutaneously in the footpad with myelin. Restimulated popliteal lymph nodes of myelin-treated animals display reduced OVA-induced proliferation compared to lymphocytes derived from untreated OVA-immunized animals. This effect is independent of interference of myelin proteins on OVA antigen presentation, as lymph node cultures of MBP-immunized animals treated subcutaneously with OVA did not reduce MBP reactivity. These results demonstrate that myelin-phagocytosing macrophages suppress lymphocyte proliferation *in vivo*. In contrast, lymph node cultures derived from MBP-immunized animals that were treated with myelin showed an enhanced proliferative capacity. Although we demonstrated that myelin-treated macrophages are unable to increase proliferation of MBP-reactive lymphocytes *in vitro*, the presence of other myelin-rich antigen-presenting cells, like migrated langerhans cells and local lymph node DCs, might explain the increased reactivity against MBP and MOG. Furthermore, B cells have been described to capture antigen-containing immune complexes from SCS macrophages processes and migrate to the T cell zone to influence antigen presentation [620, 623]. Finally, the discrepancy in literature regarding the skewing of macrophages following myelin internalization suggests that myelin can have divergent effects on macrophage polarization and its APC-like or immune suppressive properties, which may depend on the macrophage origin and local environmental stimuli. Likewise, the nature of surrounding lymphocytes, for example being myelin-protein, non-myelin or myelin-lipid specific, might

determine whether the presence of myelin-phagocytosing macrophages results in stimulation or suppression of lymphocyte activity. Future studies should therefore determine whether lymphocytes surrounding myelin-containing macrophages in CNS draining lymph nodes recognize antigen presented by these cells and are hereby activated.

Interestingly, we demonstrated an increased capacity of lymph nodes cells from myelin treated, OVA-immunized animals to produce NO following LPS stimulation. These results indicate a direct role of macrophage-produced NO in the observed decrease in OVA reactivity in myelin-treated animals, as observed *in vitro*. The importance of NO in the control of inflammation in EAE is supported by studies showing an aggravation or inability to recover following treatment with an iNOS inhibitor in, respectively, the induction or the remission phase of EAE [625, 626]. Likewise, treatment with the NO-donor SIN-1 during the induction phase of EAE ameliorated EAE, which was correlated with a reduced immune cell infiltration and antigen-induced proliferation [431]. Finally, EAE insusceptibility in rat strains like the Piebald Virol Glaxo and the Brown Norway strain is correlated with an increased production of immune suppressive NO following immunization [627, 628]. These results demonstrate that NO displays a disease-mitigating role in EAE by inhibiting lymphocyte proliferation. Based on these and our findings, we suggest that myelin-rich macrophages in the perivascular space and CNS-draining lymph nodes can fulfill a suppressive role in MS by producing NO, hereby silencing autoreactive lymphocytes.

It is unclear which myelin components are responsible for the observed immune suppressive effects. To date, despite the abundance of lipids in myelin, most studies have mainly focused on the role of myelin proteins in neurodegenerative diseases. Interestingly, several lipids present in myelin have been reported to alter macrophage signaling and transcription. Intracellular, lipid sensors like LXR and PPAR, which are respectively activated by cholesterol derivates and non-esterified fatty acids, have recently been described as key regulators of lipid metabolism and inflammation, and may be activated following myelin internalization [579-581]. Similarly, individual lipids present in myelin can alter the macrophage or microglial response by binding to specific receptors and activating or blocking signalling cascades pivotal in inflammation [629-632].

Macrophages can adopt divergent phenotypes based on specific stimuli in their microenvironment [265, 285-288]. Moreover, myelin-phagocytosing macrophages have been described to display divergent phenotypes depending on the location in the lesion, suggesting that they are likely to exert diverse functions depending on their micro-location [365]. By using thioglycolate-elicited PECs, as a representative model for infiltrating monocytes in EAE and MS [633], we established that myelin internalization results in an altered macrophage function, characterized by an increased production of NO. These myelin-phagocytosing macrophages may have dual effects during MS pathogenesis. Whereas NO production can negatively influence neuronal integrity and block axonal conduction locally in the brain parenchyma, we show that NO also suppresses lymphocyte proliferation. Thus, depending on the surrounding cells, myelin-phagocytosing macrophages can be involved in either limiting or promoting autoimmune-mediated demyelination.

Collectively, we demonstrate that myelin phagocytosis leads to an altered macrophage function that inhibits lymphocyte proliferation. The observed immune suppression was mediated by an increased production of NO by myelin-treated macrophages. Additionally, we establish that myelin-phagocytosing macrophages fulfill an ambiguous role *in vivo*. On one hand they aggravate autoimmunity by activating myelin-reactive lymphocytes in secondary lymph nodes and on the other hand they suppress lymphocyte reactivity by producing NO.

3

Myelin-derived lipids modulate macrophage activity by liver X receptor activation

Based on:

Myelin-derived lipids modulate macrophage activity by liver X receptor activation
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Abstract

MS is a chronic, inflammatory, demyelinating disease of the CNS in which macrophages and microglia play a central role. Foamy macrophages and microglia, containing degenerated myelin, are abundantly found in active multiple sclerosis lesions. Recent studies have described an altered macrophage phenotype after myelin internalization. However, it is unclear by which mechanisms myelin affects the phenotype of macrophages and how this phenotype can influence lesion progression.

Here we demonstrate, by using genome wide gene expression analysis, that myelin-phagocytosing macrophages have an enhanced expression of genes involved in migration, phagocytosis and inflammation. Interestingly, myelin internalization also induced the expression of genes involved in LXR signaling and cholesterol efflux. *In vitro* validation shows that myelin-phagocytosing macrophages indeed have an increased capacity to dispose intracellular cholesterol. In addition, myelin suppresses the secretion of the pro-inflammatory mediator IL-6 by macrophages, which was mediated by activation of LXRβ. Our data show that myelin modulates the phenotype of macrophages by nuclear receptor activation, which may subsequently affect lesion progression in demyelinating diseases such as multiple sclerosis.

3.1 Introduction

One of the pathological hallmarks of MS is loss of the nerve-insulating myelin sheath, which contributes to the myriad of symptoms observed in individuals with MS. Infiltrated macrophages and resident microglia are considered to be the primary effector cells in MS and EAE [320-323]. Together with activated autoreactive lymphocytes they orchestrate the immunopathological processes causing demyelination and concomitant axonal degeneration [5, 93, 94, 596]. In addition to the secretion of cytotoxic cytokines or soluble toxic mediators [406, 478, 480, 598-600], microglia and infiltrated macrophages phagocytose and degrade myelin [365, 372, 374-378, 388, 634]. Although presumably detrimental when considering degeneration of intact myelin, clearance of myelin debris has also been reported to be a prerequisite for axonal remyelination [531-533].

Recently, macrophages, microglia and dendritic cells have been described to adopt an altered phenotype following myelin phagocytosis. Nonetheless, the effect myelin has on the inflammatory state of these cells remains controversial. Several studies have reported, for instance, a neuroinflammatory phenotype of macrophages and microglia after myelin internalization, characterized by an increased production of pro-inflammatory and toxic mediators [372-374, 377]. In contrast, other studies describe that monocyte-derived macrophages, peritoneal macrophages, microglia and dendritic cells obtain anti-inflammatory characteristics following internalization of myelin [365, 375, 376, 378, 601].

This study aims to determine the phenotype of myelin-phagocytosing macrophages in a pro-inflammatory environment, similar to which they are exposed to in the parenchyma and perivascular spaces during active demyelination in MS [442, 450, 635]. Microarray analysis discovered 676 differentially regulated genes in myelin-phagocytosing macrophages compared to control macrophages, both treated with IFNγ and IL-1β. Gene ontology and pathway mapping tools demonstrated an overrepresentation of genes in pathways involved in proliferation, chemotaxis, phagocytosis, inflammation, lipid metabolism and LXR signaling. Quantitative PCR validated that several genes involved in lipid metabolism and LXR signaling were differentially regulated in myelin-treated macrophages. These alterations in gene expression have functional consequences as myelin-phagocytosing macrophages showed an

increased efflux of cholesterol. LXR activation has been described to increase the expression of genes involved in lipid metabolism and to suppress inflammation related genes in macrophages. We show that myelin suppresses the macrophage-mediated production of the pro-inflammatory mediator IL-6 by activating the LXRβ-isoform. These results indicate that myelin possesses functional ligands capable of activating LXRs, hereby affecting the phenotype of macrophages.

3.2 Materials and methods

3.2.1 Animals

Wistar rats were purchased from Harlan Netherlands B.V. (Horst, The Netherlands). Wild-type, LXRα-KO, LXRβ-KO and LXRαβ-KO mice have been described previously [636]. Animals were housed in the animal facility of the Biomedical Research Institute of Hasselt University. Experiments were conducted in accordance with institutional guidelines and were approved by the ethical committee for animal experiments of Hasselt University.

3.2.2 Myelin isolation

Myelin was purified from rat and mouse brain tissue by means of density-gradient centrifugation, as described previously [603]. Myelin protein concentration was determined by using the BCA protein assay kit (Thermo Fisher Scientific, Erembodegem, Belgium). Endotoxin content was determined using the Chromogenic Limulus Amebocyte Lysate assay kit (Genscript Incorporation, Aachen, Germany). Isolated myelin contained a neglectable amount of endotoxin ($\leq 1.8 \times 10^{-3}$ pg/µg myelin).

3.2.3 Cell culture

Resident peritoneal macrophages were obtained by peritoneal lavage using ice-cold PBS (Lonza, Vervier, Belgium) supplemented with 5 mM EDTA (VWR, Leuven, Belgium). PECs were cultured for 2 hours in RPMI 1640 medium (Invitrogen, Merelbeke, Belgium). After a 2 hour incubation at 37°C with 5% CO_2, non-adherent cells were washed away. Remaining cells were >95% macrophages [602].

For microarray analysis isolated macrophages were seeded in flat-bottom 12-well plates (1×10^6 cells/ml) in RPMI 1640 medium supplemented with 50 U/ml streptomycin (Invitrogen), 50 U/ml streptomycin (Invitrogen) and 10% FCS (Hyclone, Erembodegem, Belgium), and treated with 100 µg/ml of isolated myelin (n=5) or left untreated (n=5). Following a three day culture, myelin was removed by washing twice with RPMI 1640 medium at 37°C. Subsequently, cells were treated with 100 ng/ml IFNγ and IL-1β (Preprotech, London, UK) for 9

hours. For validation experiments isolated macrophages were treated for 24 or 48 hours with 100 µg/ml of isolated myelin or 10 µM T0901317 (T09; Cayman Chemicals, Huissen, The Netherlands).

3.2.4 RNA isolation

Total RNA was prepared using the RNeasy mini kit (Qiagen, Venlo, The Netherlands), according to the manufacturer's instructions. The RNA concentration and quality was determined with a NanoDrop spectrophotometer (Isogen Life Science, IJsselstein, The Netherlands).

3.2.5 Microarray analysis

RNA was labeled and hybridized to Affymetrix rat 230-2.0 GeneChips (Affymetrix, UK) containing 31000 probe sets which analyze the expression level of over 30000 transcripts and variants from over 28000 well-substantiated rat genes. Hybridized chips were stained, washed and scanned with GeneChip Scanner 3000. All steps were carried out according to the standard Affymetrix protocols.
Raw Affymetrix CEL files from five replicates for each condition were collected. Bioconductor packages running under the R platform were used to process raw data [637]. By using the affy package [638], raw data were pre-processed to obtain robust multichip average (RMA) expression values [639]. Variance-based non-specific filtering was performed using the genefilter package to remove 50% of the probe sets, corresponding to those exhibiting the smallest variations in expression across the samples. Filtered genes that are differentially expressed between the two conditions were identified using unpaired two-sample T test. All data are MIAME compliant and the raw data have been deposited in NCBI's Gene Expression Omnibus [640], accessible through GEO series accession number GSE34811.
The Database for Annotation, Visualization and Integrated Discovery (DAVID, http://david.abcc.ncifcrf.gov/) was used to determine enriched molecular functions/biological processes (ease score <0.01) and KEGG-pathways (ease score <0.1) in both the up- and downregulated gene pool [641]. DAVID utilizes a modified Fisher's exact test to measure the gene enrichment in annotation terms

(EASE score). In parallel, gene-pools were analyzed through the use of Ingenuity Pathway Analysis (IPA, Ingenuity® Systems, www.ingenuity.com). Overrepresented biological functions and canonical pathways with a Fisher exact p-value of <0.02 were considered significant. Overlapping functional categories and related genes in the output of both pathway analysis tools were utilized for further functional characterization.

3.2.6 Quantitative PCR

RNA was converted to cDNA using the reverse transcription system (Promega, Leuven, Belgium). In brief, RNA was supplemented with $MgCl_2$ (25 mM), RTase buffer (10x), DNTP mixture (10 mM); RNasin (20-40 U/µl); AMV RTase (20 U/µl) Oligo(dt) 15 primer and nuclease free water. The reverse transcription reaction was performed on 42°C for 60 minutes, 95°C for 5 minutes, using the iCYCLER (Biorad Benchmark). Quantitative PCR was conducted on a 7500 fast detection system (Applied biosystems, Gaasbeek, Belgium) using universal cycling conditions (10 min 95°C, 40 cycles of 15 s at 95°C and 60 s at 60°C). The PCR reaction consisted of fast SYBR green master mix (Applied biosystems), 10 µM of forward and reverse primers, RNase free water and 12.5 ng template cDNA in a total reaction volume of 10 µl. PCR products were loaded on 1.5% agarose gels to confirm specificity of amplification and the absence of primer dimer formation. Relative quantification of gene expression was accomplished by using the comparative C_t method. Data were normalized to the most stable reference genes, as previously described [642, 643]. In our experimental setup, geNorm identified PGK1 and 18S as the most stable combination of reference genes with an identical M-value of 0.09 (data not shown). Additionally, by analyzing the pairwise variation value, $V_{n/n+1}$, we demonstrated that in our data set two reference genes were sufficient for normalization, since inclusion of an additional reference gene increases the pairwise variation value (data not shown). Primers were chosen according to literature or designed using Primer-Express (http://www.ncbi.nlm.nih.gov/tools/primer-blast). Details of primers used are shown in table 3.1.

Table 3.1 Quantitative PCR primers

Gene symbol	Gene name	Forward and reverse primer
Internal controls		
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	F: ACCACAGTCCATGCCATCAC
		R: TCCACCACCCTGTTGCTGTA
18S	18S subunit ribosomal RNA	F: ACGGACCAGAGCGAAAGCAT
		R: TGTCAATCCTGTCCGTGTCC
CycA	Cyclophilin A	F: TATCTGCACTGCCAAGACTGAGTG
		R: CTTCTTGCTGGTCTTGCCATTCC
ActB	Beta actin	F: TGTCACCAACTGGGACGATA
		R: GGGGTGTTGAAGGTCTCAAA
YWHAZ	Tyrosine 3-monooxygenase/tryptophan	F: GATGAAGCCATTGCTGAACTTG
	5-monooxygenase activation	R: GTCTCCTTGGGTATCCGATGTC
Tbp	TATA box binding protein	F: TGGGATTGTACCACAGCTCCA
		R: CTCATGATGACTGCAGCAAACC
Rpl13A	Ribosomal protein L13A	F: GGATCCCTCCACCCTATGACA
		R: CTGGTACTTCCACCCGACCTC
HMBS	Hydroxymethyl-bilane synthase	F: TCCTGGCTTTACCATTGGAG
		R: TGAATTCCAGGTGAGGGAAC
Pgk1	Phosphoglycerate kinase 1	F: ATGCAAAGACTGGCCAAG
		R: AGCCACAGCCTCAGCATATTTC
Other genes		
RXRα	Retinoid X receptor α	F: ACATGCAGATGGACAAGACG
		R: GGGTTTGAGAGCCCCTTAGA
RXRβ	Retinoid X receptor β	F: GAAGCTCAGGCAAGCACTATGG
		R: TCTCGGCATGAGTAGGTCAGGT
RXRγ	Retinoid X receptor γ	F: GATGGACAGTCATCCCAGCTA
		R: GACCACATTGAGCTGAGAGC
ABCA1	ATP-binding cassette, sub-family A, member 1	F: CCCAGAGCAAAAAGCGACTC
		R: GGTCATCATCACTTTGGTCCTTG
ABCG1	ATP-binding cassette, sub-family G, member 1	F: CAAGACCCTTTTGAAAGGGATCTC
		R: GCCAGAATATTCATGAGTGTGGAC
SCD1	Stearoyl-CoA desaturase 1	F: GATATCCACGACCCCAGCTA
		R: CCCAGGGCACTGATAAGGTA
SCD2	Stearoyl-CoA desaturase 2	F: CCAGAGCGTACCAGCTTTTC
		R: TTACCCACTTCGCAAGCTCT
ApoE	Apolipoprotein E	F: ACTGGGTCGCTTTTGGGATT
		R: CTCCTCCTGCACCTGCTCA

3.2.7 Cholesterol efflux assay

Following isolation, macrophages were seeded in 24-well plates and incubated for 48 hours with 0.5 μCi/ml 1,2- [^{3}H]cholesterol (GE Healthcare, UK). Next, cells were washed and treated with myelin or left untreated. Following 24 hours incubation, cells were washed with PBS, after which RPMI-1640 supplemented with penicillin/streptomycin and 50 μg/ml HDL (VWR) was added for 6 hours. Cholesterol efflux was analyzed using a β-plate liquid scintillation counter (Wallac, Turku, Finland). In addition, cholesterol efflux was determined using

the Amplex Red Cholesterol Assay Kit (Invitrogen), according the manufacturer's instructions.

3.2.8 Nitrite formation and IL-6 production

Culture supernatants of rat or mouse macrophages treated for 24, 48 or 72 hours with 100 µg/ml myelin or 10 µM T09 were collected after 18 hour stimulation with 100 ng/ml LPS (Sigma-Aldrich, Bornem, Belgium) or 100 ng/ml IFNγ/IL-1β (Preprotech). Release of NO and IL-6 was determined using a Griesss reagent system (Promega) and an IL-6 ELISA (R&D systems, Abingdon, UK) respectively.

3.2.9 Statistics

Data were statistically analyzed using GraphPad Prism for windows (version 4.03) and are reported as mean ± SEM. D'Agostino and Pearson omnibus normality test was used to test normal distribution. An ANOVA or two-tailed unpaired student T-test (with Welch's correction if necessary) was used for normally distributed data sets. The Kruskal-Wallis or Mann-Whitney analysis was used for data sets which did not pass normality. *P<0,05, **P<0,01 and ***P<0,001.

3.3 Results

3.3.1 Differentially regulated genes, biological processes and pathways in myelin-phagocytosing macrophages

The transcriptional events, associated with myelin phagocytosis by macrophages in a pro-inflammatory environment, were investigated using Affymetrix rat 230-2.0 GeneChips. Non-phagocytosing macrophages stimulated with IFNγ and IL-1β were used as control cells. The expression levels of individual genes were compared between groups using Bioconductor packages running under the R platform (see methods for details). Differentially expressed genes, their p-values and fold changes are listed in table 3.2 (complete list PMID: 22984598). Employing the cutoffs described in the methods section, the expression of 676 genes was altered, from which 280 genes were upregulated and 396 were downregulated.

To investigate the biological interactions of the genes identified in our screen, differentially expressed genes were further analyzed using pathway analysis software. IPA was used to determine overrepresented biological functions and canonical pathways within the up- and downregulated genes. Respectively 7 and 15 overrepresented canonical pathways were identified in the up- and downregulated gene pool (table 3.3). Canonical pathways in the upregulated gene pool included: aminosugar metabolism ($p=0.0002$, genes: GCK, HEXB, PDE7B, PDE7A, PDE8B and TULP2), peroxisome proliferator-activated receptor (PPAR) signaling ($p=0.004$, genes: FOS, HSP90AB1, PDGFRB, RRAS2 and RXRα), complement system ($p=0.007$, genes: C1QA, CFH and C8A), LXR/retinoid X receptor (RXR) activation ($p=0.009$, genes: ABCG1, APOA1, RXRα and RXRγ) and cyclic adenosine monophosphate (cAMP) mediated signaling ($p=0.01$, genes: CHRM1, HTR6, PDE7B, PDE7A, PDE8B, PKIA and TULP2). Overrepresented pathways in the downregulated gene pool included: p53 signaling ($p=0.0009$, genes: CCND2, CDKN1A, HDAC1, HIPK2, MDM2, MED1 and PIK3C2A), mammalian target of rapamycin (mTOR) signaling ($p=0.005$, genes: AKT1S1, EIF4A2, FNBP1, PDPK1, PIK3C2A, RPS6KA1, RPS6KA5 and STK11), cell cycle checkpoint regulation ($p=0.008$, genes: CCNB1, CDKN1A, MDM2 and RPS6KA1), ciliary neurotrophic factor (CNTF) signaling

Table 3.2 Top 20 up- and downregulated genes in myelin-phagocytosing macrophages

Affy ID	Gene name	Gene symbol	FC	P value
Upregulated genes				
1368810_a_at	Myelin basic protein	MBP	9.12	0.001
1367668_a_at	Stearoyl-CoA desaturase (delta-9-desaturase)	Scd	4.02	0.027
1373098_at	Breast carcinoma amplified sequence 1	BCAS1	3.81	0.007
1368103_at	ATP-binding cassette, sub-family G, member 1	ABCG1	2.40	0.045
1375077_at	N/A	N/A	1.77	0.009
1376652_at	Complement component 1, q subcomponent	C1qa	1.75	0.039
1382153_at	C-type lectin, superfamily member 6	Clescf6	1.64	0.046
1398262_at	Phosphoribosyl pyrophosphate synthetase 2	Prps2	1.63	0.004
1391665_at	Fibroblast growth factor 7	Fgf7	1.53	0.009
1382431_at	ATP-binding cassette, sub-family A, member 1	ABCA1	1.52	0.023
1384534_at	GRAM domain containing 3	GRAMD3	1.48	0.038
1380245_at	N/A	N/A	1.45	0.024
1394673_at	Similar to Myeloid cell surface antigen CD33	LOC687856	1.44	0.002
1370423_at	Guanine nucleotide binding protein, alpha 15	GNA15	1.44	0.029
1373150_at	Catechol-O-methyltransferase domain containing 1	COMTD1	1.44	0.036
1375932_at	Phosphoribosyl pyrophosphate synthetase 2	Prps2	1.43	0.008
1372818_at	Collectin sub-family member 12	Colec12	1.41	0.043
1376155_at	Family with sequence similarity 151, member B	FAM151B	1.41	0.032
1374746_at	Ab1-152	LOC500877	1.41	0.008
Downregulated genes				
1392838_at	Similar to CG13957-PA	RGD1309995	0.47	0.016
1369067_at	Nuclear receptor subfamily 4, group A, member 3	Nr4a3	0.47	0.009
1398846_at	Eukaryotic translation initiation factor 5	EIF5	0.47	0.033
1394935_at	WAS protein family, member 2	Wasf2	0.48	0.019
1369481_at	Tumor necrosis factor superfamily, member 4	TNFSF4	0.49	0.042
1396225_at	Cytoplasmic polyadenylation binding protein 2	CPEB2	0.49	0.011
1376739_at	DEAD (Asp-Glu-Ala-Asp) box polypeptide 24	DDX24	0.51	0.008
1395154_at	Zinc finger CCCH type containing 13	ZC3H13	0.52	0.019
1380144_at	Mps One Binder kinase activator-like 1A/B (yeast)	MOBKL1A/B	0.53	0.015
1395923_at	Nipped-B homolog (Drosophila)	Nipbl	0.53	0.013
1395697_at	Enhancer of zeste homolog 2 (Drosophila)	Ezh2	0.54	0.029
1377151_at	N/A	N/A	0.54	0.011
1381809_at	Ankyrin repeat domain 11	Ankrd11	0.55	0.005
1387391_at	Cyclin-dependent kinase inhibitor 1A (p21, Cip1)	CDKN1A	0.55	0.038
1391701_at	MYST histone acetyltransferase 3	MYST3	0.55	0.013
1375453_at	Hypothetical protein LOC688211	LOC688211	0.56	0.006
1398217_at	Zinc finger and BTB domain containing 41	Zbtb41	0.56	0.033
1380446_at	Myeloid/lymphoid or mixed-lineage leukemia 10	Mllt10	0.56	0.005
1381993_at	Chloride intracellular channel 2	CLIC2	0.57	0.026

(p=0.01, genes: IL6ST, PIK3C2A, RPS6KA1 and RPS6KA5), ras homolog gene family member A (RhoA) signaling (p=0.01, genes: ARHGAP5, GRLF1, MYLPF, PPP1R12A, RDX and ROCK2) and IL-8 signaling (p=0.01, genes: ANGPT2, CCND2, FNBP1, GNAI2, IRAK1, PAK2, PIK3C2A and ROCK2). In concordance, IPA identified significantly overrepresented molecular and cellular functions related to these canonical pathways (table 3.3).

Table 3.3 Overrepresented canonical pathways and biological functions (IPA)

Downregulated gene pool	Upregulated gene pool
Canonical pathways	
p53 Signaling	Aminosugars Metabolism
mTOR Signaling	Thyroid Cancer Signaling
Growth Hormone Signaling	PPAR Signaling
Cell Cycle: G2/M DNA Damage Regulation	Relaxin Signaling
CNTF Signaling	Complement System
Nur77 Signaling in T Lymphocytes	LXR/RXR Activation
FLT3 Signaling in Hematopoietic Progenitor Cells	cAMP-mediated Signaling
RhoA Signaling	
Interleukin-8 Signaling	
Regulation of eIF4 and p70S6K Signaling	
ATM Signaling	
Molecular and cellular functions	
Cellular Development	Carbohydrate Metabolism
Gene Expression	Amino Acid Metabolism
Cell-To-Cell Signaling and Interaction	Cellular Compromise
Cellular Growth and Proliferation	Gene Expression
Cellular Function and Maintenance	Nucleic Acid Metabolism
Protein Synthesis	Small Molecule Biochemistry
Cell Morphology	Cell Cycle
Cell Cycle	Cell Signaling
Cellular Assembly and Organization	Lipid Metabolism
DNA Replication, Recombination, and Repair	Molecular Transport
Cellular Compromise	Antigen Presentation
Amino Acid Metabolism	Cell-To-Cell Signaling and Interaction
Post-Translational Modification	Cellular Assembly and Organization
Small Molecule Biochemistry	Cellular Growth and Proliferation
Cell Death	DNA Replication, Recombination, and Repair
Antigen Presentation	Cellular Development
Carbohydrate Metabolism	Cellular Function and Maintenance
Lipid Metabolism	Cell Morphology
Cell Signaling	Cell Death

For comparison, data were additionally analyzed with DAVID (table 3.4). Like IPA, DAVID identified genes functionally clustered in various categories of KEGG pathways, biological processes and molecular functions. Using the cutoffs described in the methods section, DAVID identified similar enriched pathways and biological processes as IPA.

The 9-fold upregulation of MBP was not due to RNA contamination of myelin, since added myelin contained a negligible amount of RNA (data not shown). Golli-MBP immunoreactivity has been reported in microglia and CNS-infiltrating macrophages in EAE affected animals [644].

Table 3.4 Overrepresented KEGG pathways and biological functions (DAVID)

Downregulated gene pool	**Upregulated gene pool**
KEGG pathways	
p53 Signaling pathways	Lysosome Thyroid Cancer Signaling
Focal adhesion	Calcium signaling pathway
Axon guidance	Complement and coagulation cascades
Leukocyte transendothelial migration	PPAR signaling pathway
Regulation of actin cytoskeleton	
MAPK signaling pathway	
Wnt signaling pathway	
Prion diseases	
Biological processes and molecular functions	
Negative regulation of macromolecule biosynthetic process	Intracellular signaling cascade
Negative regulation of biosynthetic process	Transmission of nerve impulse
Negative regulation of cellular biosynthetic process	Sugar binding
Negative regulation of macromolecule metabolic process	Chemical homeostasis
Regulation of transcription	Tissue morphogenesis
Transcription	Positive regulation of biosynthetic process
Negative regulation of transcription	Neurotransmitter transport
Negative regulation of gene expression	Reverse cholesterol transport
Negative regulation of nucleo-base, -side, -tide and nucleic acid metabolic process	Carbohydrate binding
Negative regulation of nitrogen compound metabolic process	Epithelium development
Tissue morphogenesis	Generation of a signal involved in cell-cell signaling
Positive regulation of transcription	Phagocytosis
Positive regulation of gene expression	

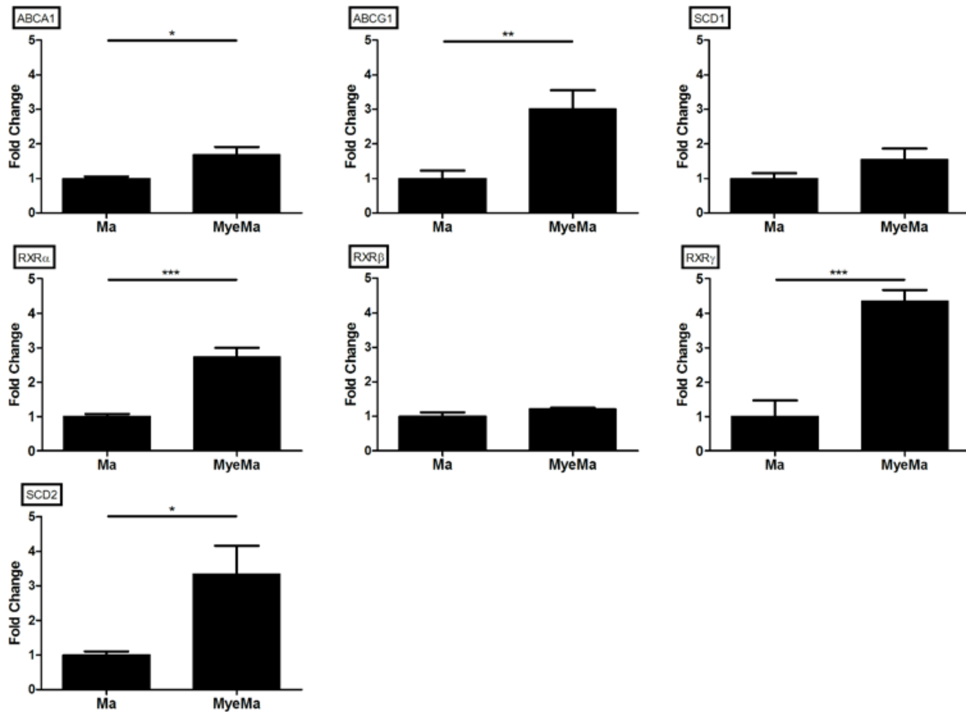

Figure 3.1 Quantitative PCR validation. Comparison of fold changes between IFNγ/IL1β-stimulated untreated (n=5) and myelin treated macrophages (n=5). Relative quantification of gene expression (SCD1/2, ABCA1/G1 and RXRα/β/γ) was accomplished by using the comparative C_t method. Data were normalized to the most stable reference genes, determined by Genorm (18S and PGK1).

3.3.2 QPCR validation of differentially expressed genes

The microarray data demonstrate that there is an overrepresentation of genes in processes like lipid-metabolism, LXR/PPAR signaling and cholesterol efflux in myelin-phagocytosing macrophages. This suggests that myelin activates LXRs and/or PPARs in macrophages, hereby increasing the expression of response genes which are involved in lipid metabolism and cholesterol efflux. To confirm the capacity of myelin to act as an activator of LXR/PPAR signaling, expression of several LXR/PPAR regulated and related genes, like ATP-binding cassette transporter A1/G1 (ABCA1/ABCG1), RXRα/β/γ and stearyl-CoA desaturase 1/2 (SCD1/2), was validated by means of qPCR (figure 3.1). All genes were found to be regulated in a similar manner as in the microarray analysis. Findings were

confirmed by additional qPCR experiments using independent samples (data not shown). These results demonstrate that myelin-derived lipids induce the expression of LXR/PPAR response genes.

3.3.3 Myelin-phagoctyosing macrophages have an increased capacity to dispose intracellular cholesterol

ABCA1 and ABCG1 are pivotal in facilitating reverse cholesterol transport. They mediate the transfer of intracellular cholesterol and phospholipids to lipid-poor apolipoproteins and mature high-density lipoprotein (HDL) [645-649]. As myelin-treated macrophages showed an increased expression of both transporters, we determined whether they are more potent in disposing intracellular cholesterol than control macrophages. As expected, myelin-phagocytosing macrophages display an increased cholesterol efflux when HDL is used as an acceptor (figure 3.2). Similar results were obtained when using the Amplex Red Cholesterol Assay Kit, which measures both free cholesterol and cholesterylesters (data not shown). Collectively, these results show that the increased expression of genes involved in cholesterol metabolism has functional consequences, as myelin-phagocytosing macrophages display an increased capacity to dispose intracellular cholesterol.

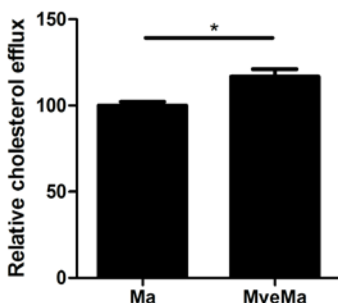

Figure 3.2 Myelin-phagocytosing macrophages have an increased capacity to transfer intracellular cholesterol towards HDL. Macrophages were loaded for 48 hours with 1,2- [^{3}H]cholesterol after which cells were treated with myelin for 24 hours or left untreated. HDL was used as cholesterol acceptor. The relative cholesterol efflux is defined as the amount of transported cholesterol in culture medium of myelin-treated macrophages divided by values in control macrophage cultures. Data represent the mean of four independent experiments.

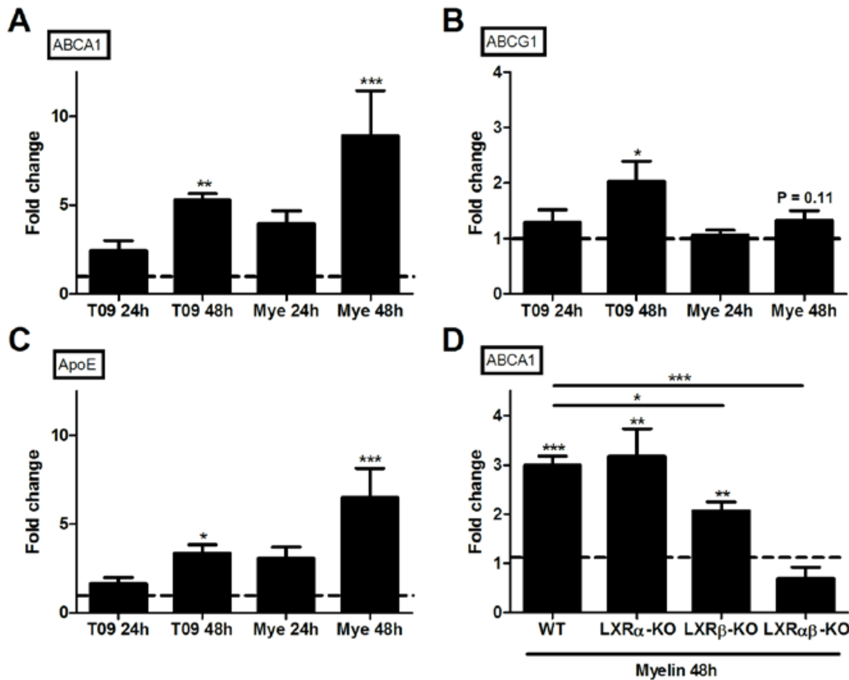

Figure 3.3 Myelin and T09 affect the expression of LXR response genes in a similar manner. (a-c) Comparison of fold changes of LXR response genes between untreated (dotted line) and myelin- or T09-treated macrophages. Macrophages were treated for 24 and 48 hours with 100 μg/ml myelin or 10 μM T09 after which expression of ApoE and ABCA1/G1 was determined. Relative quantification of gene expression was accomplished by using the comparative C_t method. Data were normalized to the most stable reference genes, determined by Genorm (18S and PGK-1). Data represent the mean of four independent experiments. (d) Comparison of fold changes of ABCA1 between untreated (dotted line) and myelin treated wild-type, LXRα-, LXRβ- and LXRαβ-deficient mouse macrophages. Macrophages were treated 48 hours with 100 μg/ml myelin. Data were normalized to the most stable reference genes, determined by Genorm (CycA and HMBS). Data represent the mean of four independent experiments. Mye; Myelin.

3.3.4 Myelin alters the macrophage phenotype by activating the LXRβ isoform

In addition to modulating cholesterol metabolism, LXRs have been described to negatively regulate macrophage inflammatory gene expression [588-592]. Since myelin is a rich source of cholesterol and cholesterol metabolites are natural ligands for LXRs, we evaluated whether myelin affects LXR response gene expression and the secretion of pro-inflammatory mediators in a similar manner

as an LXR ligand. LXR response gene expression was determined after treatment with myelin or a synthetic LXR agonist (T09). We observed that myelin induced apolipoprotein E (ApoE), ABCA1 and ABCG1 expression in macrophages in a similar manner as T09 (figure 3.3a-c), suggesting that myelin contains ligands capable of activating the LXR pathway. To ascertain a myelin-mediated activation of LXRs, LXRα-, LXRβ- and LXRαβ-deficient mouse macrophages were treated with myelin after which ABCA1 gene expression was determined. Here we show that ABCA1 gene induction by myelin is reduced in LXRβ-deficient macrophages, while it is completely absent in LXRαβ-KO macrophages (figure 3d). These results indicate that myelin activates LXRs in macrophages.

To further elucidate the role of LXRs we determined the influence of myelin and T09 on the secretion of inflammatory mediators by macrophages. Both T09 and myelin lowered the LPS or IFNγ/IL-1β induced production of NO and IL-6 to a similar extent (figure 3.4a-d). The reduction in NO and IL-6 production was not due to a reduced viability of myelin- or T09-treated macrophages (data not shown). To determine the role of both the LXRα and LXRβ isoform in the observed effects, LXRα-, LXRβ- and LXRαβ-deficient mouse macrophages were used. We observed that lack of LXRβ partially abolishes the myelin induced suppression of IL-6 secretion, which was not influenced by LXRα depletion (figure 3.4f). However, the reduction of NO production by myelin was not significantly affected in both LXRα-, LXRβ- and LXRαβ macrophages (figure 3.4e), indicating that besides LXRs other pathways are involved in the regulation of the macrophage phenotype after myelin phagocytosis. Collectively, these results indicate that myelin possesses functional ligands capable of activating LXRβ, hereby affecting the inflammatory state of macrophages.

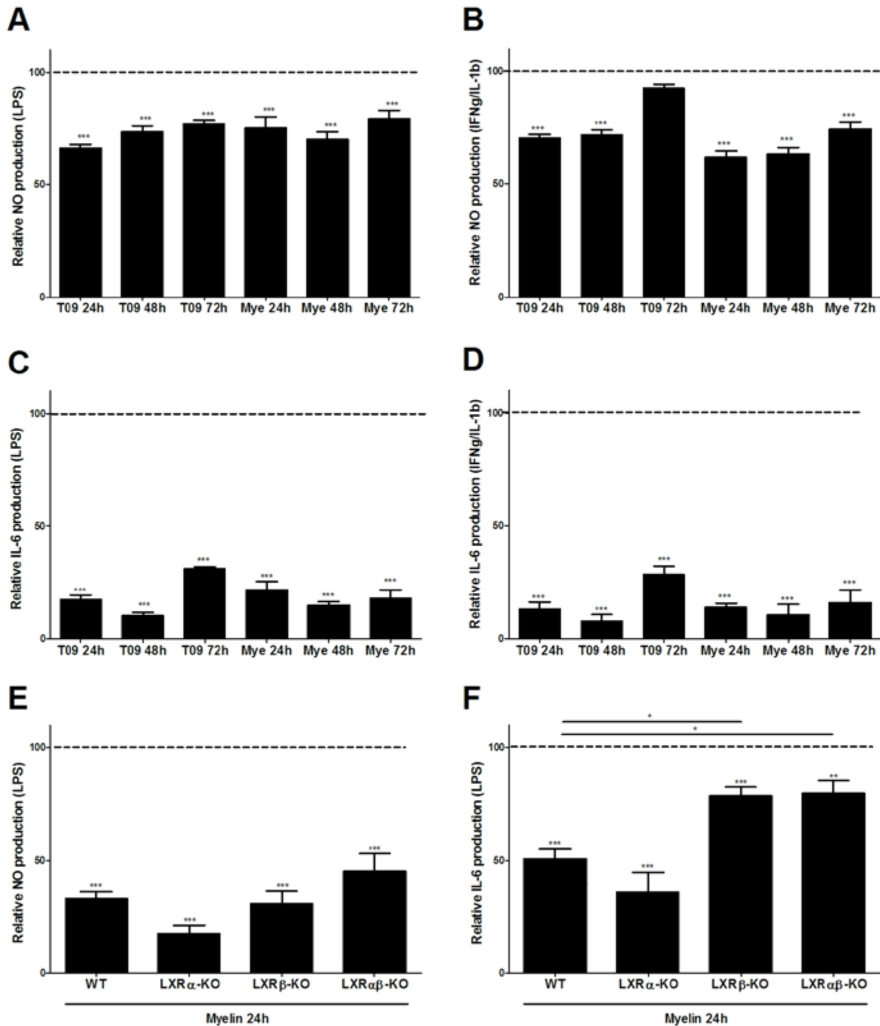

Figure 3.4 Myelin alters the macrophage phenotype by activating the LXRβ isoform. (a-d) Relative NO and IL-6 concentration in supernatants of IFNγ/IL-1β or LPS stimulated myelin- or T09-treated macrophages. The relative NO and IL-6 production is defined as the production of NO/IL-6 in experimental cultures divided by values in stimulated control cultures (dotted line). Data represent the mean of four independent experiments. (e, f) Relative NO and IL-6 concentration in supernatants of LPS stimulated myelin treated wild-type, LXRα-, LXRβ- and LXRαβ-deficient mouse macrophages. Macrophages were treated for 24 hours with myelin prior to stimulation with LPS. Data represent the mean of four independent experiments. Mye; Myelin.

3.4. Discussion

To obtain insight into the influence of myelin internalization on the functional phenotype of macrophages and the mechanisms involved, the gene expression profile of myelin-phagocytosing macrophages was assessed. Microarray analysis revealed that the expression of 676 genes differed significantly. Gene ontology mapping and pathway analysis identified several common enriched pathways related to lipid metabolism, LXR/PPAR signaling and cholesterol efflux.

In addition to the upregulation of pathways related to lipid metabolism, myelin-macrophages showed an overrepresentation of downregulated genes in pathways involved in proliferation, like p53 signaling and cell cycle checkpoint regulation. The reduced expression of p53 target genes, such as MDM2 and CDKN1A (p21) [650-652], and HIPK2, a kinase important for p53-dependent gene transcription [653, 654], suggests that foamy macrophages have a reduced transcriptional activity of p53. Moreover, as p21 regulates cell cycle arrest, these results suggest that myelin has pro-proliferative effects on macrophages.

Chemotaxis plays a pivotal role in the recruitment of monocytes towards the CNS in MS and EAE. Moreover, the presence of myelin-antigen containing phagocytes in CNS draining lymph nodes in MS and EAE suggests that macrophages migrate to lymph nodes after myelin internalization [366-368]. Microarray analysis showed that myelin-phagocytosing macrophages exhibit an overrepresentation of downregulated genes in pathways like mTOR, IL-8 and RhoA signaling, suggesting an altered motility of macrophages after myelin ingestion [655-661]. These results are in line with a recent report showing an aberrant motility of myelin-containing macrophages [370].

In addition to controlling chemotaxis, mTOR and RhoA signaling are reported to influence demyelination, by affecting complement receptor-mediated phagocytosis [656, 662]. Similarly, the upregulated expression of C1q in myelin-treated macrophages may augment their phagocytic capacity [593, 663, 664]. These results indicate that myelin uptake induces a positive feedback loop in macrophages, promoting myelin phagocytosis. Furthermore, alterations in mTOR, complement and cAMP-mediated signaling have been described to modulate the inflammatory properties of macrophages [665-669]. The latter

indicates a complex regulatory network directing the specific phenotype of myelin-phagocytosing macrophages.

Besides affecting cholesterol metabolism, the upregulated expression of GCK and HEXB, genes involved in the aminosugar metabolism pathway, indicates that sphingolipids and hexose structures are also actively metabolized after myelin internalization by macrophages [670, 671]. This is in correspondence with related differentially regulated (non-significant) pathways in the IPA analysis, like sphingolipid (p=0.52), galactose (p=0.11), sucrose (p=0.19), fructose and mannose (p=0.15) metabolism. Interestingly, sphingolipids are described to modulate inflammation and the functional phenotype of macrophages [672, 673], suggesting that the phenotype of macrophages following myelin internalization may also be affected via this pathway.

Intracellular lipid sensors like LXRs, which are activated by cholesterol derivates, have recently been described as key regulators of lipid metabolism and inflammation [579-581]. There are two LXR isoforms termed α and β with considerable sequence homology. Furthermore, they respond to the same endogenous ligands and activate almost identical target genes. However, an important distinction is their tissue distribution. LXRβ is ubiquitously expressed whereas LXRα is highly expressed in the liver and at somewhat lower levels in the adrenal glands, intestine, adipose tissue, macrophages, lung and kidney. Upon activation, LXRs form heterodimers with RXRs and promote transcriptional activation of response genes, like ABCA1, ABCG1 and SCD [674-676]. Both microarray analysis and qPCR demonstrated an increased expression of potential transcriptional partners of LXRs, e.g. RXRα and RXRγ. Additionally, ABCA1, ABCG1 and SCD2 were found to be upregulated in myelin-phagocytosing macrophages. These results suggest that myelin acts as an LXR-RXR heterodimer-selective agonist.

ABCA1 and ABCG1 promote the efflux of cholesterol to respectively APO-AI and HDL. By disposing cellular lipids they prevent lipid accumulation and the concomitant induction of apoptosis and inflammatory responses [677]. In this report we show that myelin-phagocytosing macrophages have an increased efflux of cholesterol to HDL. These results demonstrate that the upregulation of genes involved in cholesterol efflux is functional and suggest that foamy

macrophages protect themselves from the pro-apoptotic and pro-inflammatory effects of intracellular lipid accumulation by promoting cholesterol efflux.

As mentioned earlier, LXRs are cholesterol sensors controlling intracellular and systemic cholesterol homeostasis [678, 679]. However, apart from regulating cholesterol metabolism, they inhibit inflammatory gene expression in macrophages [574, 575, 588-593]. As 25% of the lipid content in myelin consists of cholesterol [578, 680], it is likely that myelin-rich macrophages and microglia in neurodegenerative, demyelinating disorders like MS, display a phenotype which is in part dictated by a myelin-mediated activation of LXRs. In this study we demonstrate that myelin contains ligands capable of activating LXRβ, hereby affecting the expression of LXR response genes like ABCA1 and the secretion of inflammatory mediators like IL-6. Interestingly, LXR activation has been demonstrated to ameliorate EAE by modulating T cell polarization [681-683]. Moreover, an increased expression of LXRβ in peripheral blood mononuclear cells in MS patients was described to counteract T cell proliferation [684]. Our finding that myelin activates LXRs suggests an additional role of these receptors in naturally occurring regulatory mechanisms in macrophages during demyelination.

To date, despite the abundance of lipids in myelin, most studies have mainly focused on the role of myelin proteins in demyelinating diseases. Our data indicate a role for myelin-derived lipids in modulating the metabolic and inflammatory response in macrophages during demyelination. Although myelin-phagocytosing macrophages have a decreased secretion of NO and IL-6, the microarray did not point towards a typical M2 phenotype. These results are in line with a recent report showing that macrophages treated with oxidized phospholipids, so called mox-macrophages, adopt a novel phenotype that differs from conventional M1 and M2 phenotypes [290]. Although both mye- and mox-macrophages induce pathways involved in chemotaxis and phagocytosis, other characteristic genes in mox-macrophages were not differentially expressed in myelin-treated macrophages. The latter indicates that macrophages obtain a specific phenotype following myelin uptake, divergent from M1, M2 and mox-macrophages. Future studies are required to elucidate the importance of lipid metabolism in directing the macrophage phenotype and function, and thereby the influence of lipids in MS lesion pathology.

4

Phosphatidylserine present in myelin alters the inflammatory properties of myelin-phagocytosing macrophages by activating PPARs

Based on:

Phosphatidylserine present in myelin alters the inflammatory phenotype of myelin-phagocytosing macrophages by activating PPARs

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Submitted

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Abstract

Foamy macrophages, containing myelin degradation products, are abundantly found in active MS lesions. Recent studies have described an altered phenotype of macrophages after myelin phagocytosis. However, mechanisms by which myelin affects the phenotype of macrophages and how this phenotype influences lesion progression remain unclear. PS is a phospholipid found in myelin. As apoptotic cell clearance by macrophages via PS induces an immunosuppressive phenotype in macrophages, we investigated the impact of myelin-derived PS on the functional properties of myelin-phagocytosing macrophages. We show that PS present in myelin reduces nitric oxide production by macrophages through activation of PPARβ/δ. Furthermore, uptake of PS by macrophages, after intravenous injection of PSLs, suppresses the production of inflammatory mediators and ameliorates EAE. The protective effect of PSLs in EAE animals is also associated with a reduced immune cell infiltration into the central nervous system and decreased splenic cognate antigen specific proliferation. Interestingly, we find that PPARs are activated in foamy macrophages in active MS lesions, indicating that degraded myelin also activates PPARs in macrophages in the human brain. Collectively, our data show that myelin modulates the phenotype of macrophages via PS-induced PPAR activation, which may subsequently dampen lesion progression in demyelinating diseases such as MS. The immunoregulatory impact of naturally-occurring myelin lipids may hold promise for future MS therapeutics.

4.1 Introduction

MS is characterized by CNS infiltration of activated myelin-reactive lymphocytes and macrophages. Microglia and macrophages typically accumulate in the perivascular spaces and the brain parenchyma near terminal ovoids of transected axons [167]. Effector mechanisms of activated macrophages and microglia include internalization of myelin and secretion of inflammatory and toxic mediators, which negatively influence axonal and myelin integrity [365, 372-378, 406, 478, 597-600].

Macrophages are able to adopt divergent phenotypes depending on environmental cues [265, 285-288]. In MS, macrophages and microglia initially display a pro-inflammatory phenotype [179, 372-374, 377, 422, 570]. However, upon internalization of myelin, they have been described to obtain immunusuppressie and anti-inflammatory characteristics [372-374, 377, 634]. We have previously demonstrated that myelin-derived cholesterol plays a role in directing this typical phenotype of myelin-phagocytosing macrophages by activating the sterol sensing LXRs [685]. Nonetheless, not all myelin-mediated effects on macrophages were induced by LXRs and cholesterol, and it is therefore likely that other myelin components also affect the phenotype of myelin-phagocytosing macrophages.

PS is a phospholipid abundantly found in myelin [578]. One of the hallmarks of apoptosis is the translocation of PS to the outer membrane leaflet, where it serves as an “eat me” signal for phagocytic clearance [686, 687]. Apoptotic cell clearance via PS skews macrophages towards an anti-inflammatory phenotype, similar to myelin-phagocytosing macrophages, hereby suppressing inflammation and maintaining homeostasis [688-691]. Since clearance of apoptotic cells by exposure of PS to macrophages and the subsequent induction of a tolerogenic phenotype has been associated with PPAR activation [593], we determined whether a PS-mediated PPAR activation is involved in directing the phenotype of macrophages during immune-mediated demyelination. Furthermore, we assessed the impact of PSL on neuroinflammation. We demonstrate that myelin-derived PS suppresses the production of the inflammatory mediator NO by myelin-phagocytosing macrophages through activation of PPARβ/δ. Importantly, we provide compelling evidence that PSLs are immunosuppressive in an

experimental MS animal model and that PPAR-responsive genes are markedly upregulated in active demyelinating MS lesions. Taken together, our findings indicate that a PS-mediated change of the macrophage phenotype upon myelin internalization may affect lesion progression in demyelinating diseases such as MS.

4.2 Materials and methods

4.2.1 Animals

Female Dark Agouti rats were purchased from Harlan Netherlands B.V. (Horst, The Netherlands). Animals were housed in the animal facility of the Biomedical Research Institute of Hasselt University. Experiments were conducted in accordance with institutional guidelines and approved by the Ethical Committee for Animal Experiments of Hasselt University.

4.2.2 Myelin isolation

Myelin was purified from rat brain tissue by means of density-gradient centrifugation, as described previously [603]. Myelin protein concentration was determined by using the BCA protein assay kit (Thermo Fisher Scientific, Erembodegem, Belgium). Lipopolysaccharide (LPS) content was determined using the Chromogenic Limulus Amebocyte Lysate assay kit (Genscript Incorporation, Aachen, Germany). Isolated myelin contained a neglectable amount of endotoxin ($<1.8x10^{-3}$ pg/µg myelin). Expression of phosphatidylserine on myelin, PSLs and PCLs was determined by flow cytometry using Fluorescein isothiocyanate (FITC)-labeled Annexin V (Biolegend, Antwerpen, Belgium).

4.2.3 Preparation of liposomes

Liposomes were prepared as described previously [446]. In brief, nitrogen-dried lipid films containing various phospholipids were suspended in PBS and sonicated for 10 min on ice. The liposomes were composed of either phosphatidylcholine (PC; Sigma-Aldrich, Bornem, Belgium) only or a combination of PC and PS (Sigma-Aldrich) at a molar ratio of 7:3. In some experiments, liposomes were fluorescently labeled with DiI (Sigma-Aldrich). For this, liposomes were incubated with DiI for 10 min at 37°C, after which liposomes were centrifuged to remove non-encapsulated DiI. Flow cytometry was used to assess labeling efficacy and the degree of DiI-liposome uptake.

4.2.4 Cell culture

Rat macrophages (NR8383; ATCC, Molsheim, France) were cultured in RPMI 1640 medium (Lonza, Vervier, Belgium) enriched with 10% fetal calf serum (Hyclone, Erenbodegen, Belgium), 50 U/ml penicillin and 50 U/ml streptomycin (Invitrogen, Merelbeke, Belgium). Cells were treated for 24 hours with 100 µg/ml myelin, 250 µg/ml PSLs or 250 µg/ml PC-containing liposomes (PCLs) in 96-well plates (15 x 10^4 cells/well). Subsequently, cells were stimulated with 100 ng/ml LPS (Sigma-Aldrich) for 9 hours for RNA isolation or 18 hours for analysis of culture supernatants. To evaluate the involvement of PPARs, macrophages were pretreated for 2 hours with antagonists for PPARα (GW6471, 10 µM), PPARβ/δ (GSK0660 10 µM) and PPARγ (GW9662, 1 µM) (all from Sigma-Aldrich). Cell viability was determined using a 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay (MTT assay). In short, following LPS stimulation the medium was aspirated and replaced by medium supplemented with 12,5 µl sterile filtered MTT (Sigma-Aldrich). After 4h incubation, the unreacted dye was aspirated and the insoluble formazan crystals were dissolved in 175 µl of a DMSO – glycine solution. Absorbance was measured at 540-550 nm.

4.2.5 Nitrite formation and cytokine concentration

Culture supernatants of macrophages were collected after 18 hour stimulation with LPS. Release of NO was determined using a Griesss reagent system (Promega, Leuven, Belgium). Cytokine concentrations in culture supernatants were determined using a rat TNFα (Ebioscience, Vienna, Austria) and rat IL-6 ELISA (R&D systems, Abingdon, UK).

4.2.6 Induction of EAE and systemic liposome treatment

Rats were immunized subcutaneously at the base of the tail with 140 µg of recombinant human MOG emulsified in incomplete Freund's adjuvant (Sigma-Aldrich) supplemented with 500 µg of heat-inactivated Mycobacterium tuberculosis (Difco, Detroit, USA). Immunized animals were treated daily with PBS, 5 mg/kg PCLs or 5 mg/kg PSLs beginning 5 days post-immunization (dpi) or at disease onset. A total of 400 µl, containing liposomes or PBS, was injected

intravenously in the tail vein. In parallel, to track liposomes in healthy and immunized animals, rats were injected with 5 mg/ml of DiI-labeled liposomes and sacrificed after 24 hours. Immunized rats were weighed and scored daily according to the following neurological scale: 0.5 = partial loss of tail tonus, 1 = complete loss of tail tonus, 2 = hind limb paresis, 3 = hind limb paralysis, 4 = moribund, 5 = death.

4.2.7 [^{3}H]Thymidine incorporation

Cognate antigen specific proliferation of T cells was determined by measuring the amount of [^{3}H]thymidine incorporation. In short, ficoll-separated splenic cells (20 X 10^4, isolated 10 dpi) were cultured in RPMI 1640 medium supplemented with 50 U/ml penicillin, 50 U/ml streptomycin, 20 µM 2-mercapto-ethanol (Sigma-Aldrich), 1% sodium pyruvate (Invitrogen), 1% MEM non-essential amino acids (Invitrogen), 2% deactivated autologous serum and 20 µg/ml MOG. After 48 hours, 1 µCi [^{3}H]thymidine (Amersham, Buckinghamshire, UK) was added to the culture for 18 hours. Next, cells were harvested with an automatic cell harvester (Pharmacia, Uppsala, Sweden) and radioactivity was measured in a β-plate liquid scintillation counter (Wallac, Turku, Finland).

4.2.8 Quantitative PCR

Total RNA from cultures and tissues was prepared using the RNeasy mini kit or RNeasy lipid tissue mini kit (Qiagen, Venlo, The Netherlands), according to the manufacturer's instructions. The RNA concentration and quality was determined with a NanoDrop spectrophotometer (Isogen Life Science, IJsselstein, The Netherlands). RNA was converted to cDNA using the reverse transcription system (Promega) and quantitative PCR was subsequently conducted on a StepOnePlus detection system (Applied biosystems, Gaasbeek, Belgium), as previously described [685]. Relative quantification of gene expression was accomplished by using the comparative C_t method. Data were normalized to the most stable reference genes [642, 643]. Primers were chosen according to literature or designed using Primer-Express (http://www.ncbi.nlm.nih.gov/tools/primer-blast). Details of the primers used are shown in table 4.1.

Table 4.1 Quantitative PCR primers

Gene symbol	Gene name	Forward and reverse primer
Rat primers		
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	F: ACCACAGTCCATGCCATCAC R: TCCACCACCCTGTTGCTGTA
18S	18S subunit ribosomal RNA	F: ACGGACCAGAGCGAAAGCAT R: TGTCAATCCTGTCCGTGTCC
CycA	Cyclophilin A	F: TATCTGCACTGCCAAGACTGAGTG R: CTTCTTGCTGGTCTTGCCATTCC
ActB	Beta actin	F: TGTCACCAACTGGGACGATA R: GGGGTGTTGAAGGTCTCAAA
YWHAZ	Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation	F: GATGAAGCCATTGCTGAACTTG R: GTCTCCTTGGGTATCCGATGTC
Tbp	TATA box binding protein	F: TGGGATTGTACCACAGCTCCA R: CTCATGATGACTGCAGCAAACC
Rpl13A	Ribosomal protein L13A	F: GGATCCCTCCACCCTATGACA R: CTGGTACTTCCACCCGACCTC
HMBS	Hydroxymethyl-bilane synthase	F: TCCTGGCTTTACCATTGGAG R: TGAATTCCAGGTGAGGGAAC
Pgk1	Phosphoglycerate kinase 1	F: ATGCAAAGACTGGCCAAG R: AGCCACAGCCTCAGCATATTTC
iNOS	Inducible nitric oxide synthase 2	F: GCATCCCAAGTACGAGTGGT R: TGTTGTAGCGCTGTGTGTCA
TNFα	Tumor necrosis factor alpha	F: CTTATCTACTCCCAGGTTCTCTTCAA R: GAGACTCCTCCCAGGTACATGG
ARG-1	Arginase 1	F: CAAGCTGGGAATTGGCAAAG R: GGTCCAGTCCATCAACATCAAA
T-bet	T-box transcription factor	F: TCCTGTCTCCAGCCGTTTCT R: CGCTCACTGCTCGGAACTC
GATA-3	GATA binding protein 3	F: ACCACGTCCCGTCCTACTAC R: AGAGATCCGTGCAGCAGAG
RORγt	RAR-related orphan receptor gamma	F: ATCAATGCCAACCGTCCTGG R: TGGAGGTGCTGGAAGTCCTGTAG
Foxp3	Forkhead box P3	F: CCCAGGAAAGACAGCAACCTT R: CTGCTTGGCAGTGCTTGAGAA
Human primers		
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	F: GAGTCAACGGATTTGGTCGT R: GACAAGCTTCCCGTTCTCAG
Pgk1	Phosphoglycerate kinase 1	F: CTGGGCAAGGATGTTCTGTT R: GCATCTTTTCCCTTCCCTTC
CycA	Cyclophilin A	F: AGACTGAGTGGTTGGATGGC R: TCGAGTTGTCCACAGTCAGC
ActB	Beta actin	F: GATCATTGCTCCTCCTGAGC R: AAAGCCATGCCAATCTCATC
YWHAZ	Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation	F: CTTGACATTGTGGACATCGG R: TATTTGTGGGACAGCATGGA
Tbp	TATA box binding protein	F: TATAATCCCAAGCGGTTTGC R: GCTGGAAAACCCAACTTCTG
Rpl13A	Ribosomal protein L13A	F: AAGTTGAAGTACCTGGCTTTCC R: GCCGTCAAACACCTTGAGAC
HMBS	Hydroxymethyl-bilane synthase	F: GAATGAAGTGGACCTGGTTGT R: CTGGTTCCCACCACACTCTT
ADRP	Adipose differentiation related protein	F: TGTGAGATGGCAGAGAACGGT R: CTGCTCACGAGCTGCATCATC
CTP1a	Carnitine palmitoyltransferase 1a	F: CTCAGTGGGAGCGGATGTTTA R: TCGATGGTACACGACGATGTG
PDK4	Pyruvate dehydrogenase kinase isozyme 4	F: CCCGAGAGGTGGAGCATTT R: GCATTTTCTGAACCAAAGTCCAGTA

4.2.8 Immunohistochemistry

Animals were sacrificed in the effector (10 dpi) and chronic phase (30 dpi) of EAE, after which brains, spinal cords and spleens were isolated and snap-frozen. Frozen brain material from MS patients and non-demented controls was obtained from the Netherlands Brain Bank (Amsterdam, Netherlands). Material was sectioned with a Leica CM1900UV cryostat (Leica Microsystems, Wetzlar, Germany) to obtain 7 µm slices. The extent of immune cell infiltration in spinal cord sections was determined using anti-rat CD68 (AbD Serotec) and anti-rat CD3 (AbD Serotec). For tracking of DiI-liposomes, spleen and spinal cord cryosections were stained with CD68 (AbD Serotect) and CD169 (AbD serotec). Additionally, splenic sections were stained with anti-rat Arginase-1 (BD Biosciences, Erembodegem, Belgium). Human brain material was stained with anti-ADRP (Abcam, Cambridge, UK), anti-HLA-DR (LN3 clone, eBioscience) and anti-PLP (Abd Serotec). Alexa fluor secondary antibodies were all purchased from Invitrogen (Merelbeke, Belgium). In short, dried cryosections were fixated in aceton for 10 minutes, after which they were blocked for 30 minutes with 10% normal serum from the same species as the secondary antibody. Sections were incubated overnight with primary antibodies, secondary antibodies were added for 2 hours. Nuclei were visualized using DAPI (Invitrogen). Control stainings were performed by omitting the primary antibody. PBS containing 0.05% Tween 20 (Merck, Darmstadt, Germany) was used for washing and diluting the antibodies. For 3, 3′ diaminobenzidine (DAB) staining, the Dako Envision+ kit (Dako) was used according to manufacturer's instructions. Sections were counterstained with hematoxylin (Merck, Darmstadt, Germany). Analysis was carried out using a Nikon eclipse 80i microscope and NIS Elements BR 3.10 software (Nikon, Tokyo, Japan).

4.2.10 Statistics

Data were statistically analyzed using GraphPad Prism for windows (version 4.03) and are reported as mean ± SEM. D'Agostino and Pearson omnibus normality test was used to test normal distribution. An ANOVA (post-hoc; Tukey) or two-tailed unpaired student T-test was used for normally distributed data sets. The Kruskal-Wallis (Dunns post hoc comparison) or Mann-Whitney

analysis was used for data sets which did not pass normality. EAE scores were analyzed using the Kruskal-Wallis (Dunns post hoc comparison) and Mann-Whitney analysis. An overall effect of treatment was assessed by measuring the area under the curve (AUC). *$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$.

4.3 Results

4.3.1 PS in myelin modulates the macrophages phenotype by activating PPARs

To determine the involvement of PS in modulating the phenotype of macrophages upon myelin uptake, macrophages were incubated with myelin or PSLs. PSLs have been described to mimic the functional effects of apoptotic cell clearance by macrophages [686, 691]. First, the abundance of PS in isolated myelin was determined and compared to that in PSLs and non-PS-containing liposomes (PCLs). Flow cytometric analysis demonstrated that isolated myelin and PSLs contained similar levels of PS (figure 4.1a-c). Subsequently, the capacity of macrophages to internalize liposomes *in vitro* was determined. Like DiI-labeled myelin [634], both DiI-labeled PSLs and PCLs were internalized efficiently by macrophages *in vitro* (figure 4.1d).

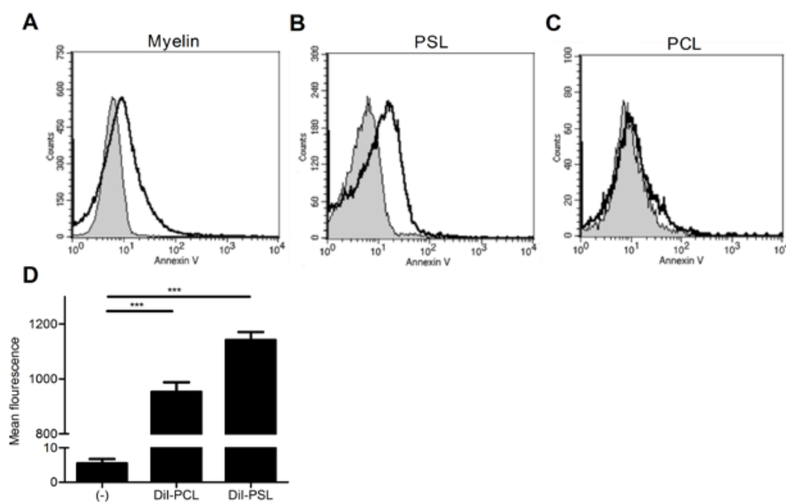


Figure 4.1 Characteristics of myelin and liposomes. (a-c) Annexin V staining of myelin (a), PSLs (b) and PCLs (c). Unstained myelin and liposomes were used as control (grey area). One representative experiment is shown (n=2). (d) Macrophages were treated with DiI-labeled PCLs (DiI-PCL) and PSLs (DiI-PSL) for 1.5 hours. Liposome uptake was assessed by flow cytometry. Data represent the mean ± SEM of two experiments.

Next, we assessed whether PS in myelin affects the production of NO by macrophages through activation of PPARα, β/δ or γ. Macrophages were treated

for 2 hours with specific antagonists for PPARα, β/δ and γ, prior to administration of PSLs or myelin. While PPARα or PPARγ antagonists did not influence the reduction in NO production induced by myelin or PSLs, a PPARβ/δ selective antagonist counteracted the decline in NO production by macrophages induced by both myelin and PSLs (figure 4.2a and 4.2b). In contrast to PSLs, PCLs did not alter NO production by macrophages (figure 4.2d). The decrease in IL-6 production by myelin phagocytosing macrophages was not affected by the antagonists (figure 4.2c). This is in accordance with our previous study in which we demonstrated that suppression of IL-6 production is LXRβ dependent. These results show that PS modulates the inflammatory phenotype of macrophages upon myelin internalization by activating PPARβ/δ.

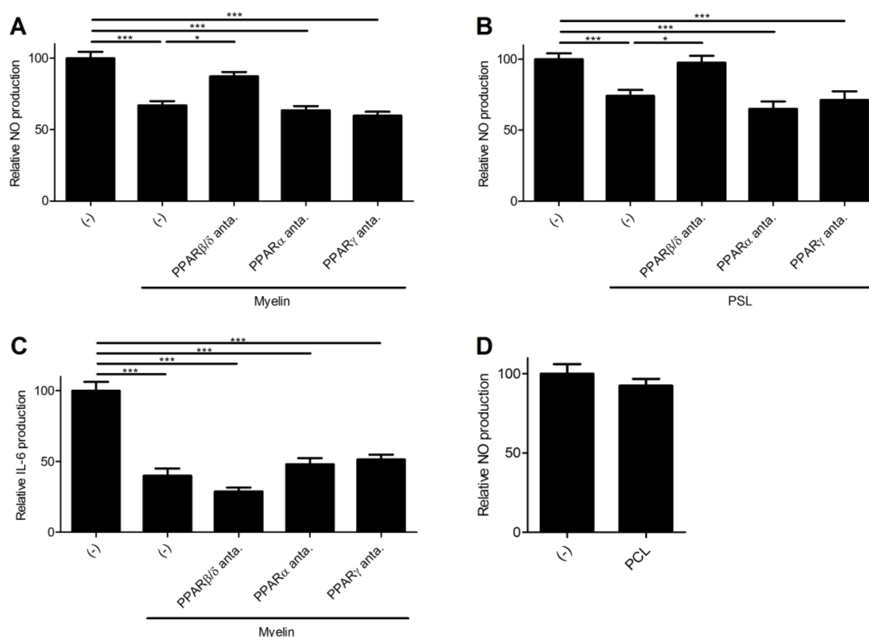


Figure 4.2 Myelin modulates the macrophages phenotype by PPARβ/δ activation. (a, b) Relative NO concentration in supernatants of LPS stimulated control, myelin- or PSL-treated macrophages. Prior to administration of myelin or PSLs, cells were treated with antagonists for PPARα (GW6471), PPARβ/δ (GSK0660) or PPARγ (GW9662). The relative NO production is defined as the production of NO in experimental cultures divided by values in stimulated control cultures. (c) Relative IL-6 concentration in supernatants of LPS stimulated control and myelin-treated macrophages. (d) Relative NO concentration in supernatants of LPS stimulated control and PCL-treated macrophages. Data represent the mean ± SEM of at least six independent experiments.

4.3.2 Systemically administered liposomes home primarily to splenic macrophages and ameliorate EAE

To determine if PS uptake by macrophages influences the pathology and severity of EAE, immunized rats were treated with PBS, PCLs or PSLs. First, the homing properties of liposomes after intravenous administration of DiI-labeled PSLs were determined by flow cytometry and immunohistochemistry. In healthy animals, injected PSLs were primarily retrieved in the spleen and liver (figure 4.3a). Furthermore, immunohistochemical analysis demonstrated that especially splenic CD169^{+} marginal zone and CD68^{+} red pulp macrophages contained liposomes (figure 4.4a and 4.4b). The absence of liposomes in the CNS tissue suggests that liposomes are incapable of crossing an intact BBB.

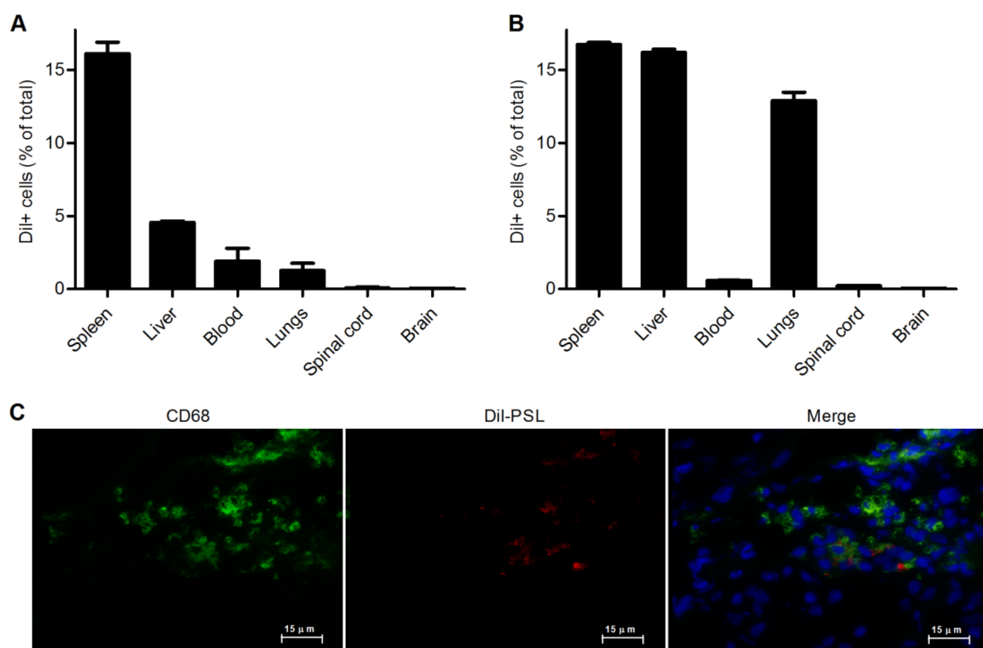


Figure 4.3 Homing properties of liposomes following systemic administration. Healthy (a) and immunized (b) rats were injected with 5 mg/kg DiI-labeled PSLs . After 24 hours animals were sacrificed and the homing properties of liposomes were assessed by flow cytometry. One representative experiment is shown (n=2). (c) Immunized rats were injected with 5 mg/kg DiI-labeled PSLs (DiI-PSL). After 24 hours animals were sacrificed and spinal cord cryosections were stained with CD68 (40x magnification). One representative experiment is shown (n=3).

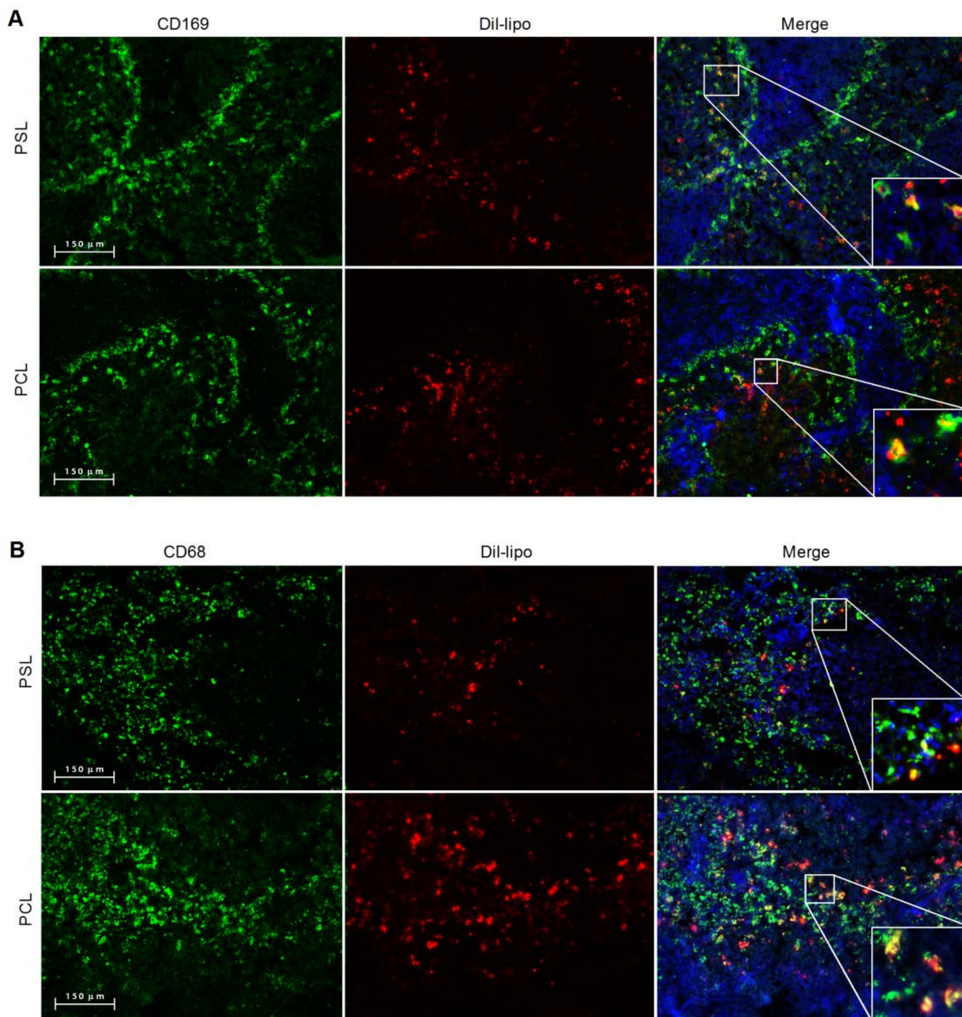


Figure 4.4 Systemically administered liposomes home primarily to splenic marginal zone and red pulp macrophages. (a, b) Healthy rats were injected with 5 mg/kg DiI-labeled PCLs and PSLs. Splenic cryosections were stained with CD169 (a, marginal metallophilic and marginal zone macrophages) and CD68 (b, red pulp macrophages). One representative experiment is shown (20x magnification).

Similar to healthy animals, PSLs homed primarily to the spleen and liver when injected after EAE onset (figure 4.3b). However, in these immunized animals we were also able to detect sporadic CD68^{+} macrophages in the spinal cord that took up DiI-liposomes (figure 4.3c). These results show that PSLs migrate

towards splenic red pulp and marginal zone macrophages after systemic administration, but can also enter the CNS during EAE. Whether liposomes enter the CNS via infiltrating macrophages or freely cross the compromised BBB remains to be clarified.

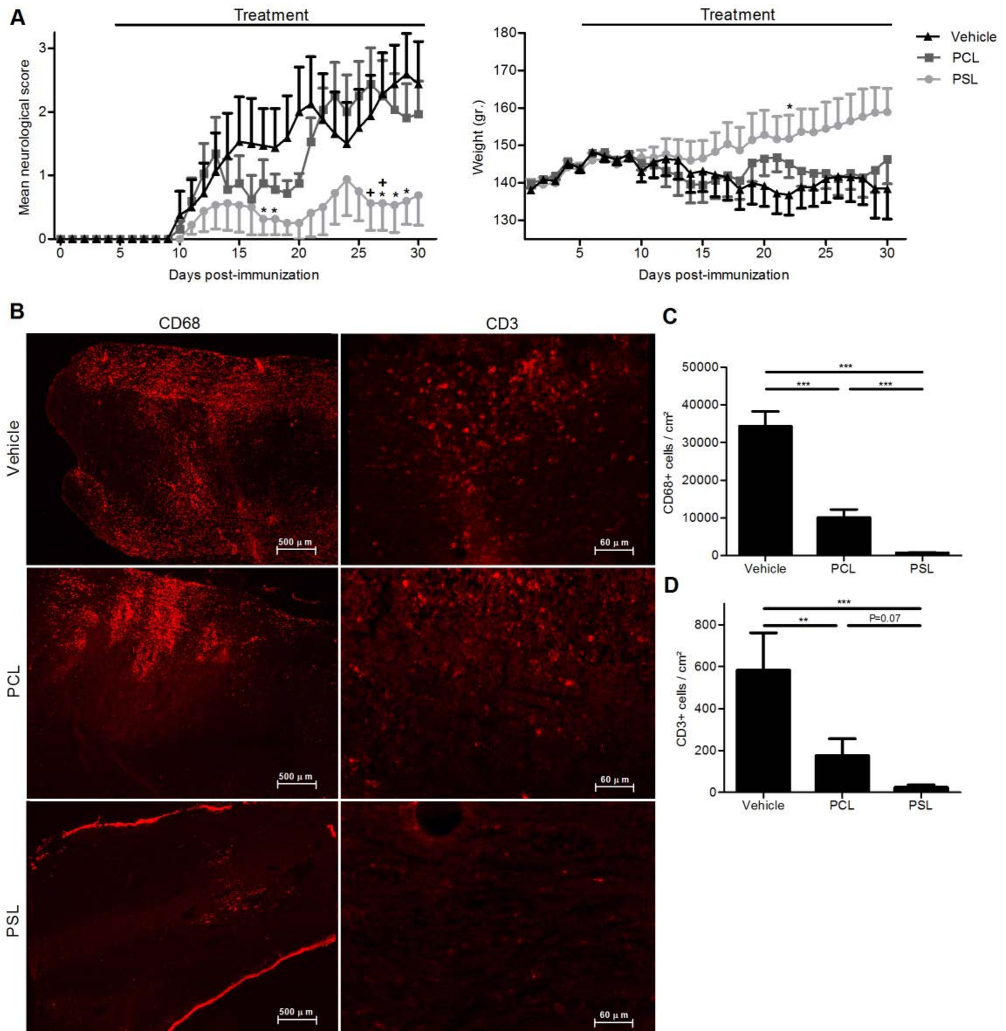


Figure 4.5 Intravenously administered PSLs reduce CNS infiltration of immune cells and ameliorate EAE. (a) MOG-immunized animals were treated daily with PBS (n=8; black), 5 mg/kg PCLs (n=8; dark grey) or 5 mg/kg PSLs (n=8; light grey) starting from day 5. Neurological score and weight were assessed daily. Data represent the mean ± SEM. $^{*}P < 0.05$ (vehicle vs PSL), $^{+}P < 0.05$ (PCL vs. PSL). (b) Spinal cord tissue was isolated 30 dpi and stained with CD3 (20x magnification) and CD68 (4x magnification).

One representative picture is shown. (c, d) Quantification of T cell and macrophage infiltration in spinal cord tissue 30 dpi. Nine cryosections, covering the complete length of the spinal cord, were stained with CD68 (d) and CD3 (e). A 4x magnification was used to determine the amount of immune cell infiltration. Data represent the mean ± SEM of 4 animals.

To assess the effect of PSLs on EAE development, immunized animals were treated daily with PSLs, PCLs or PBS, starting 5 dpi. PSL-treated animals displayed a significantly reduced neurological score compared to PCL- (AUC, PSL: 7.41 ± 4.38 vs PCL: 30.13 ± 6.11, $P < 0.05$, figure 4.5a) and vehicle-treated animals (AUC, PSL: 7.41 ± 4.38 vs vehicle: 34.53 ± 10.80, $P < 0.05$, figure 4.5a). The reduced disease severity in PSL-treated animals was paralleled with decreased numbers of CNS infiltrating macrophages and T cells (figure 4.5c and 4.5d). Although PCL treatment did not significantly affect disease severity, PCL-treated animals did have significantly reduced numbers of CNS infiltrating immune cells, as compared to vehicle treated animals.
To determine the therapeutic potential of PSLs, EAE animals were treated daily with PSLs or PBS, starting one day after disease onset. Similar as in the prophylactic regimen, PSL-treated animals displayed a significantly reduced neurological score compared to vehicle-treated animals (AUC, PSL: 17.5 ± 5.21 vs vehicle: 34.25 ± 3.53, $P < 0.05$, figure 4.6). Collectively, these data show that PSLs attenuate the course of EAE when administered both before and after disease onset.

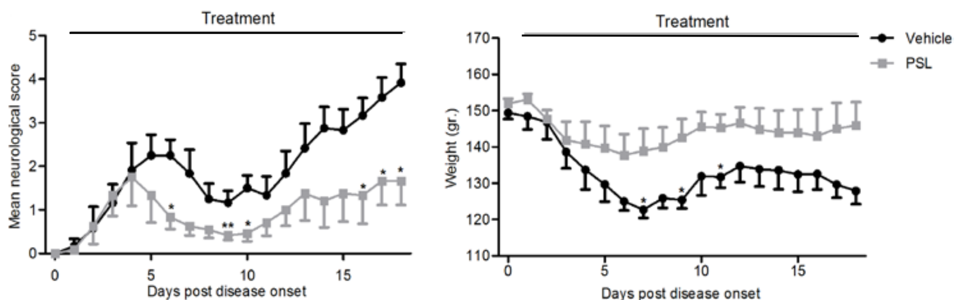


Figure 4.6 PSLs have a therapeutic effect on EAE. (a) MOG-immunized animals were treated daily with PBS (n=6; black) or 5 mg/kg PSLs (n=6; light grey) starting at disease onset. Neurological score and weight was assessed daily. Data represent the mean ± SEM.

4.3.3 PSLs modulate T cell proliferation and the expression of pro- and anti-inflammatory mediators in the spleen

PSLs have been described to modulate T cell differentiation and suppress antigen-specific immune responses *in vivo* [689, 692]. To determine the impact of PSLs on T cell proliferation, cognate antigen specific proliferation of splenic cultures was assessed. Splenic T cells from PSL-treated animals showed a significantly reduced MOG reactivity, compared to both vehicle- and PCL-treated animals (figure 4.7a). In line with this, the mean follicle surface area in the spleen, determined by measuring the marginal metallophilic macrophages-surrounded area, was reduced in animals treated with PSLs (figure 4.7b). Representative images of these measurements are depicted in figure 4.7c. Although PSLs were clearly immunosuppressive *in vivo*, we were unable to detect any differences in splenic gene expression of transcription factors characteristic for divergent T cell subsets; T-bet (Th1), GATA-3 (Th2), RORγt (Th17) and Foxp3 (Treg) (data not shown).

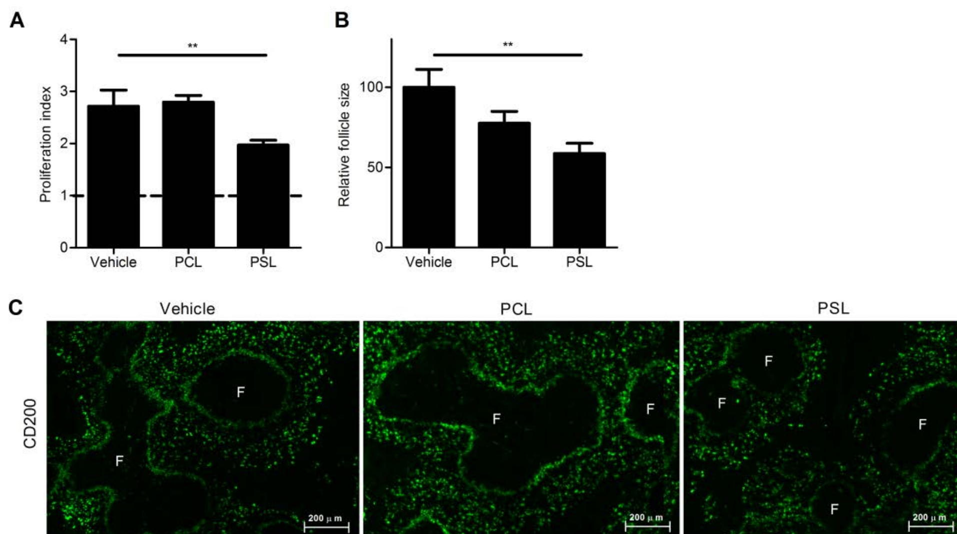


Figure 4.7. PSLs suppress splenic cognate antigen specific proliferation. (a) Cognate antigen specific proliferation (10 dpi) of splenic cultures was assessed by culturing splenic cells from vehicle, PCL and PSL treated animals with MOG. Proliferation was assessed by [3H]thymidine incorporation. Non-stimulated cultures were used as control (dotted line). Data represent the mean ± SEM of four experiments. (b) The size of splenic

follicles was determined using ImageJ software. Three cryosections per animal were stained with CD200 after which the surface area surrounded by the marginal metallophilic macrophages was determined. Five pictures (4x magnification) per section were taken to calculate the mean follicle size. Data represent the mean ± SEM of four animals. (c) Representative images of the CD200 staining. F: follicle.

To further determine the underlying mechanisms by which PSLs modulate EAE pathogenesis, splenic expression of iNOS, TNFα and arginase-1 (ARG-1) was assessed. Whereas iNOS and TNFα are typical inflammatory mediators produced by macrophages, ARG1 is a commonly used marker for alternatively activated macrophages. Reduced iNOS and TNFα mRNA were observed in spleens of PSL-treated animals (figure 4.8a and 4.8b). Furthermore, although splenic ARG1 mRNA expression was unaffected, the total fluorescent intensity of splenic ARG1 expression was significantly increased in animals treated with PSLs, indicating enhanced arginase activity (figure 4.8c). Representative images of these measurements are depicted in figure 4.8d. The altered expression of iNOS, TNFα and ARG1 in PSL-treated animals is in agreement with the ability of PSLs to affect the expression of these mediators by macrophages *in vitro* (figure 4.1b, 4.8e and 4.8f).

These results demonstrate that PSL treatment suppresses T cell proliferation without affecting their polarization. Furthermore, we show that PSLs affect the expression of iNOS, TNFα and ARG1 *in vivo* in a similar manner as PSL treatment of macrophages *in vitro*. These immunosuppressive and anti-inflammatory properties of PSLs likely contribute to the observed reduction in neuroinflammation after PSL treatment.

4.3.4 Myelin-phagocytosing macrophages display increased activation of PPARs in active MS lesions

Our findings show that myelin-derived PS directs the phenotype of myelin-phagocytosing macrophages through activation of PPARβ/δ. To elucidate whether this pathway is also active in MS lesion pathology, we determined PPAR activation in MS lesion tissue by qPCR and immunohistochemistry. The expression of well-defined PPARβ/δ response genes adipose differentiation

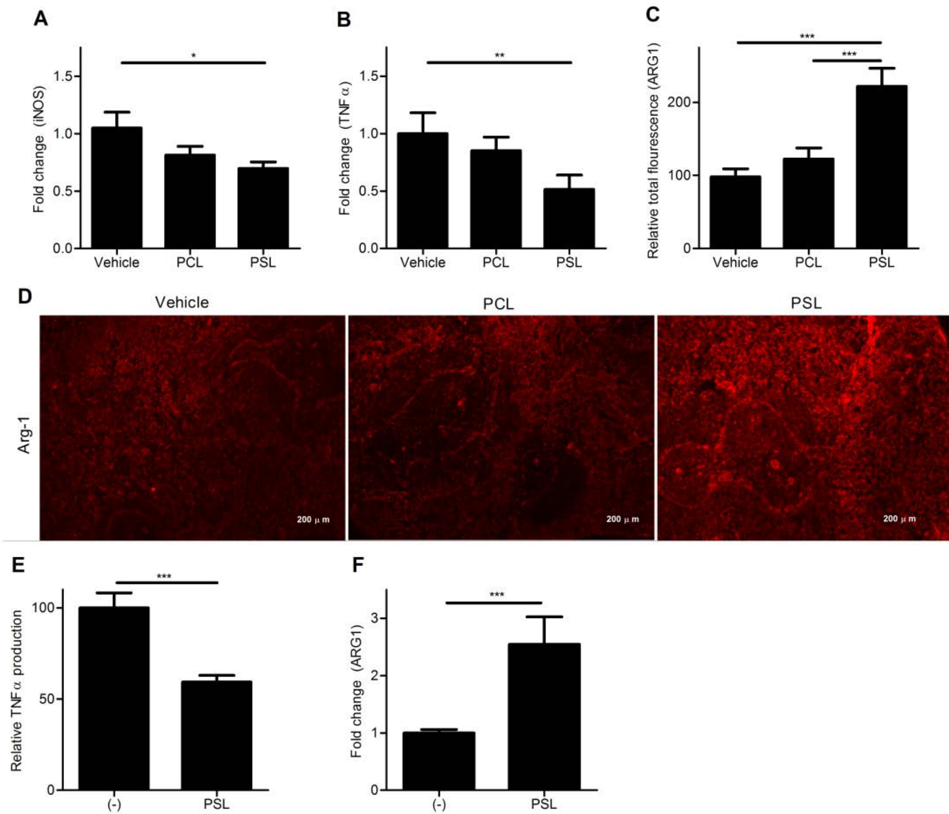
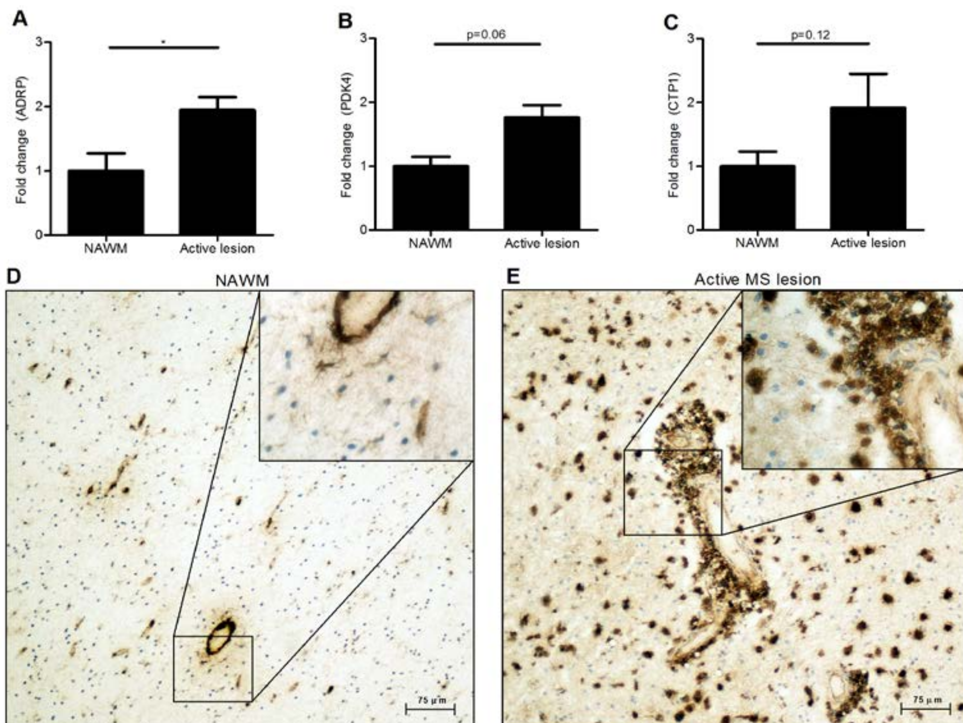


Figure 4.8 PSLs affect splenic expression of TNFα, iNOS and ARG-1. (a, b) Comparison of fold changes between spleens isolated 10 dpi from vehicle, PCL and PSL-treated EAE animals. Relative quantification of iNOS (a) and TNFα (b) was accomplished by using the comparative C_t method. Data were normalized to the most stable reference genes, determined by Genorm (Pgk1 and Rpl13a). Data represent the mean ± SEM of 4 experiments. (c) Spleen cryosections were stained with ARG1 after which the total corrected fluorescence was determined using ImageJ software, as described previously [693]. Three cryosections were stained and 6 pictures (10x magnification) were taken per section. Data represent the mean ± SEM of four animals. (d) Representative images of the ARG1 staining. (e) Relative TNFα concentration in supernatants of LPS stimulated control and PSL-treated macrophages. The relative TNFα production is defined as the production of TNFα in experimental cultures divided by values in stimulated control cultures. Data represent the mean ± SEM of five independent experiments. (f) Comparison of fold changes between LPS stimulated control and PSL-treated macrophages. Relative quantification of ARG-1 gene expression was accomplished by using the comparative C_t method. Data were normalized to the most stable reference genes, determined by Genorm (TBP and HMBS). Data represent the mean ± SEM of three independent experiments.

related protein (ADRP), carnitine palmitoyltransferase I (CPT1a) and pyruvatedehydrogenase kinase isozyme 4 (PDK4) was studied [694-696]. RNA was isolated from regions accommodating lipid-containing macrophages, determined by an Oil red O staining. Expression of all genes tested was increased in active MS lesions, compared to non-demented controls (figure 4.9a-c). In the case of PDK4 and CPT1a this did not reach statistical significance, likely due to small sample size. As the change in ADRP expression was significant, we determined the level of the encoded protein in MS lesions by immunohistochemistry. In agreement with the PCR data, immunohistochemical analysis showed that ADRP was highly abundant in active MS lesions compared to the surrounding normal-appearing white matter (figure 4.9d and 4.9e). Moreover, macrophages containing myelin were intensely stained by anti-ADRP in active MS lesions (figure 4.9f). These data show that myelin-phagocytosing macrophages have activated PPAR signaling, which may skew their phenotype towards an alternatively activated state.



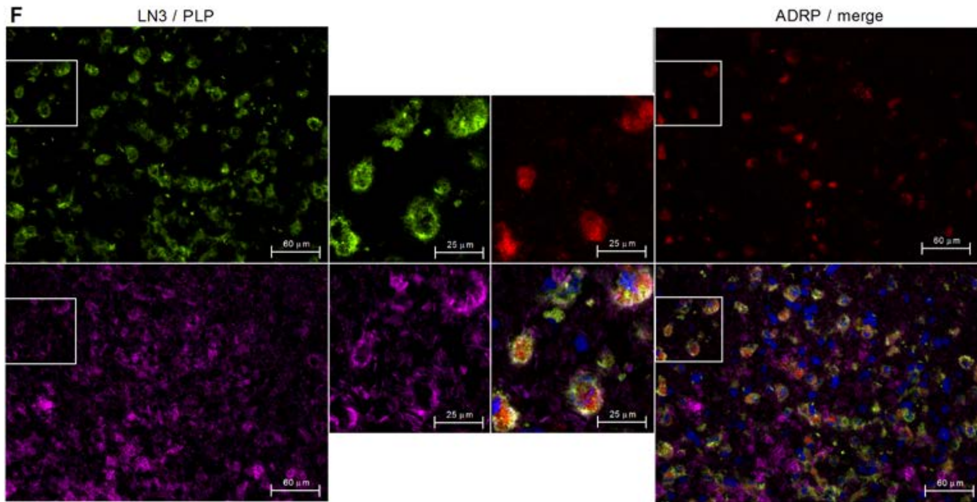


Figure 4.9 PPARs are activated in myelin-phagocytosing macrophages during active MS. (a-c) Comparison of fold changes between non-demented controls (n=3) and active MS lesions (n=3). Relative quantification of ADRP (a), PDK4 (b) and CTP1a (c) was accomplished by using the comparative C_t method. Data were normalized to the most stable reference genes, determined by Genorm (YHWAZ and Rpl13a). (d, e) Normal-appearing WM (NAWM) and an active MS lesion stained for ADRP. One representative picture is shown (20x magnification). (f) Active MS lesion stained with HLA-DR (green top; left corner), ADRP (red; top right corner) and PLP (magenta; bottom left corner). One representative picture is shown (40x magnification).

4.4 Discussion

In this study we aimed to determine whether myelin-derived PS directs the inflammatory phenotype of macrophages and how this phenotype impacts lesion progression in MS. We show that internalization of myelin and PSLs inhibit NO production by macrophages through activation of PPARβ/δ. Furthermore, we demonstrate that PSLs, internalized by splenic macrophages, significantly reduce clinical signs in an experimental MS animal model by suppressing autoaggressive T cells, lowering the expression of inflammatory mediators and inhibiting infiltration of immune cells into the CNS. Interestingly, PPAR-responsive genes and their corresponding proteins were markedly increased in myelin-containing macrophages during active demyelination in MS. These findings suggest that a PS-mediated change of the macrophage phenotype upon myelin internalization induces naturally-occurring regulatory mechanisms through activation of PPARs, which may dampen lesion progression and explain the relapse-remitting nature of MS.

Myelin contains various lipids that may modify the functional properties of macrophages. Recently we demonstrated that myelin-derived cholesterol influences the phenotype of macrophages through activation of LXRs. While the suppressed IL-6 production by myelin-phagocytosing macrophages was LXRβ dependent, the observed reduction in NO production was unaffected in LXR-deficient macrophages. PS is a constituent of myelin and a potent regulator of inflammatory responses. *In vitro*, clearance of apoptotic cells and PSLs skews macrophages towards a tolerogenic phenotype [688, 690, 691, 697-702]. Likewise, myelin internalization induces an anti-inflammatory, immunosuppressive phenotype in macrophages [372-374, 377, 634]. Here we show that both myelin and PSLs lower NO production by macrophages. Moreover, we demonstrate that a PS-mediated activation of PPARβ/δ underlies the effect that PSLs and myelin have on the phenotype of macrophages. This myelin-mediated activation of PPARβ/δ corresponds with the fact that myelin-phagocytosing macrophages have an upregulated expression of genes involved in PPAR signalling [685]. Moreover, these findings are in line with studies showing that the PPARβ/δ is a transcriptional sensor of apoptotic cells and that it regulates the program of

alternative activation in macrophages [579, 583, 584, 587, 593]. Interestingly, in a carrageenan-induced mouse paw edema model it has been shown that PSLs are capable of suppressing inflammation *in vivo* by activating PPARγ [703], indicating that PSLs can affect inflammation via multiple PPAR subtypes.

We demonstrate that systemically administered PSLs, primarily internalized by splenic CD68⁺ red pulp and CD169⁺ marginal zone macrophages, suppress EAE in both prophylactic and therapeutic settings. In line with our findings, a recent study demonstrated that administration of non-encapsulated phosphatidylserine ameliorates EAE when administered at disease onset [704]. In this study it was described that the suppressive effect was mediated by a direct effect of PS on autoaggressive T cell responses. We now provide evidence that PS not only affects T cell responses but also influences macrophage behavior. The PS-mediated change of the macrophage phenotype will contribute to the immunosuppressive capacity of PSLs. *In vivo*, PSLs have been described to promote the resolution of inflammation by modulating macrophage function in a model for inflammatory bone loss and myocardial infarction [699, 700]. As ARG1 activity suppresses antigen-specific T cell responses [607, 608], the increased splenic expression of ARG1 in PSL-treated animals may account for the observed inhibition of splenic T cell proliferation in our model. In addition to the immunosuppressive effects of PSLs, we observed a marked reduction in the numbers of macrophages and T cells infiltrating into the CNS of PSL-treated EAE animals. This indicates that PSLs influence immune cell trafficking towards the CNS, in addition to or as a result of modulating the macrophages phenotype or T cell proliferation. In summary, results from our study indicate that PSLs will affect neuroinflammation by modulating the functional properties of macrophages.

Interestingly, we demonstrate that the expression of PPAR response genes and proteins is upregulated in active MS lesions, especially in myelin-phagocytosing macrophages. All PPAR subtypes have been described to regulate the differentiation of macrophages towards an anti-inflammatory phenotype [582-587]. Moreover, agonists for all PPARs reduce CNS inflammation and demyelination in EAE [705-712]. The importance of PPARβ/δ signaling in maintaining immune-homeostasis and preventing systemic autoimmunity is illustrated by the fact that macrophage-specific PPARβ/δ deficiency delays clearance of apoptotic cells and

increases autoantibody production [593]. Our finding that the expression of PPAR response genes is upregulated in foamy macrophages in active MS lesions indicates that degraded myelin also activates PPARs in macrophages in the human brain. This myelin-mediated PPAR activation may affect lesion progression by inducing an anti-inflammatory environment and by influencing the activity of infiltrating T cells. Moreover, as PPARβ/δ activation enhances the internalization of apoptotic cells [593], myelin-mediated PPAR activation may promote clearance of myelin debris, which inhibits oligodendrocyte precursor maturation and axonal regeneration [531-533, 556, 558], thereby stimulating repair.

We demonstrate a role for PPARβ/δ in directing the phenotype of myelin and PSL treated macrophages. This finding suggests that myelin-derived PS skews myelin-phagocytosing macrophages towards an immunoregulatory phenotype. Since PSLs ameliorate EAE and PPARs are activated in myelin-phagocytosing macrophages in active MS lesions, we hypothesize that myelin uptake by macrophages induces naturally-occurring regulatory mechanisms by PPAR activation. However, the presence of autoantibodies targeting PS in MS may compromise this lipid-mediated protection against neuroinflammation, explaining the absence of recovery in progressive MS [704]. The identification of myelin-derived lipids capable of dampening inflammation holds promise for future intervention strategies aimed at reducing neuroinflammation in disorders like MS.

5

Induction of COLEC12 expression on myelin-phagocytosing macrophages and in MS lesions

Based on:

Induction of COLEC12 expression on myelin-phagocytosing macrophages and in active MS lesions

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In preparation

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Abstract

MS is an inflammatory, demyelinating disease of the CNS in which foamy macrophages, containing myelin degradation products, are abundantly found. Recent studies have described an altered phenotype of macrophages after myelin phagocytosis. However, how this phenotype influences lesion progression remains largely unclear.

In our previous study, microarray analysis demonstrated that internalization of myelin by macrophages induces the expression of collectin sub-family member 12 (COLEC12) mRNA in macrophages. In this report we assessed the function of this receptor and determined its expression level in MS lesions. We show that COLEC12 is highly expressed in foamy-appearing perivascular macrophages and parenchymal astrocytes in active MS lesions. Furthermore, *in vitro* experiments revealed that myelin internalization upregulates the expression of COLEC12 in macrophages but not in microglia. However, COLEC12 did not facilitate myelin clearance by macrophages and microglia. Although the function of COLEC12 remains to be resolved, its increased expression in active MS lesions indicates that it may play a role in lesion progression or resolution in MS.

5.1 Introduction

Macrophage-mediated myelin destruction is considered to be the primary effector mechanism in MS. Nonetheless, especially after internalization of myelin, macrophages have been described to obtain immuneregulatory and anti-inflammatory characteristics [372-374, 377, 634, 685]. However, how this phenotype influences lesion progression remains largely unclear.

In our previous study, microarray analysis demonstrated that internalization of myelin alters the expression of 676 genes in macrophages [685]. COLEC12, also called collectin placenta 1, was significantly upregulated in myelin-phagocytosing-macrophages. COLEC12 is structurally related to SRA due to its collagen-like domain [594], however, it also contains a C-type lectin/carbohydrate recognition domain [713, 714]. Functionally, it has been associated with the binding and internalization of bacteria, yeast and oxidized LDL [713-716]. Furthermore, COLEC12 has been described to recognize carcinoma-associated antigens, possibly via interaction with Lewis^{x} trisaccharide on tumor cells [717, 718], hereby mediating tumor cell-endothelium interactions and possibly tumor spreading [719, 720]. Finally, a recent study has demonstrated that the collagen-like domain of COLEC12 facilitates amyloid beta clearance by glial cells and that COLEC12 is expressed in a amyloid beta-dependent fashion [595]. Together these findings indicate that COLEC12 plays a role in host defense against microorganisms, artherosclerosis, Alzheimer's disease and tumor spreading.

In this report, we determined whether macrophages upregulate COLEC12 following myelin ingestion, if it is involved in the internalization of myelin and in which cell type(s) and at what level it is expressed in MS lesions. We show that COLEC12 is highly expressed in perivascular macrophages and astrocytes in active MS lesions. Furthemore, *in vitro* experiments revealed that myelin uptake enhances the expression of COLEC12 on macrophages but not on microglia. Although the function of COLEC12 remains to be resolved, its increased expression in active MS lesions indicates that it may play a role in lesion progression or resolution in MS.

5.2 Materials and methods

5.2.1 Myelin isolation and labelling

Myelin was purified from mouse brain tissue by means of density-gradient centrifugation, as described previously [603]. Myelin protein concentration was determined by using the BCA protein assay kit (Thermo Fisher Scientific, Erembodegem, Belgium). Endotoxin content was determined using the Chromogenic Limulus Amebocyte Lysate assay kit (Genscript Incorporation, Aachen, Germany). Isolated myelin contained a neglectable amount of endotoxin ($\leq 1.8 \times 10^{-3}$ pg/µg myelin). Myelin was fluorescently labelled, according to the method described by Van der Laan et al. [374]. In short, 10 mg/ml myelin was incubated with 12.5 µg/ml DiI (Sigma-Aldrich) for 30 min at 37°C.

5.2.2 Cell culture

The immortalized mouse macrophage (RAW 264.7; ATCC, Teddington, UK) and microglial (BV-2; ATCC, Teddington, UK) cell lines were used in *in vitro* experiments. Both cell lines were cultured in DMEM (Invitrogen, Merlebeke, België) containing 50 U/ml penicillin (Invitrogen), 50 U/ml streptomycin (Invitrogen) and 10% FCS (Hyclone, Erembodegem, Belgium). The astrocyte cell line (C8-D1a; kindly provided by Maastricht University) was cultured in DMEM (Invitrogen) containing 10% FCS (Hyclone). To determine the effect of myelin and LXR and PPARβ/δ agonists on the expression of COLEC12, cells were treated for 24 hours with 100 µg/ml of isolated myelin, 10 µM T09 (Cayman Chemicals, Huissen, The Netherlands) or 10 µM GW0742 (Sigma). Flow cytometry was used to assess the expression of COLEC12. For this purpose, cells were stained with goat-anti-mouse COLEC12 (R&D systems, Abingdon, UK) or normal goat IgG (R&D systems). Alexa fluor 488 $F(ab')_2$ fragment of rabbit-anti goat (Invitrogen) was used as a secondary antibody.

5.2.3 Myelin phagocytosis assay

To assess the role of COLEC12 in facilitating myelin internalization a myelin phagocytosis assay was performed [721]. Briefly, cells were pretreated for 30 min

with 10 µg/ml of a neutralizing antibody for COLEC12 (R&D systems, Abingdon, UK), after which 100 µg/ml DiI-labeled myelin was added for 1.5 hr. Normal goat IgG (R&D) was used as an isotype control. The amount of myelin phagocytosed was determined by measuring the cellular fluorescence intensity using a FACScalibur (BD Biosciences, Erembodegem, Belgium).

5.2.4 Immunohistochemistry

Frozen brain tissue from active MS lesions was obtained from the Netherlands Brain Bank (Amsterdam, Netherlands). Brain material was cut with a Leica CM1900UV cryostat (Leica Microsystems, Wetzlar, Germany) to obtain 10 µm sections. Slides were fixed for 10 min in acetone and air-dried. For DAB stainings, slides were incubated with goat-anti-human COLEC12 (R&D). After washing, HRP-conjugated rabbit-anti-goat (Dako, Heverlee, Belgium) was added. Subsequently, DAB substrate (Dako) was used to stain slides. Sections were counterstained with hematoxylin (Merck, Darmstadt, Germany). For fluorescent stainings, sections were blocked with 10% serum derived from the same species in which the secondary antibody was raised. Next, slides were incubated with goat-anti-human COLEC12 (R&D) and mouse-anti-human HLA-DR/DP/DQ (HLA-DR/DP/DQ; Dako) or rabbit-anti glial fibrillary acidic protein (GFAP; Dako). Subsequently, slides were stained with Alexa flour secondary antibodies (Invitrogen). Nuclei were visualized using DAPI (Invitrogen). Analysis was carried out using a Nikon eclipse 80i microscope and NIS Elements BR 3.10 software (Nikon, Tokyo, Japan).

5.2.5 Statistics

Data were statistically analyzed using GraphPad Prism for windows (version 4.03) and are reported as mean ± SEM. D'Agostino and Pearson omnibus normality test was used to test normal distribution. An ANOVA or two-tailed unpaired student T-test (with Welch's correction if necessary) was used for normally distributed data sets. The Kruskal-Wallis or Mann-Whitney analysis was used for data sets which did not pass normality. *$P<0.05$, **$P<0.01$ and ***$P<0.001$.

5.3 Results

5.3.1 COLEC12 is upregulated in a LXR- and PPAR-independent manner in myelin-phagocytosing macrophages

Microarray analysis demonstrated that myelin induces the gene expression of COLEC12 in macrophages. To determine whether both macrophages and microglia upregulate the expression of COLEC12 on protein level following myelin internalization, we assessed COLEC12 expression of these cells by flow cytometry. Here we demonstrate that the expression of COLEC12 on macrophages increased after myelin internalization (figure 5.1a). Strikingly, the expression of COLEC12 is unaffected in microglia after myelin internalization (figure 5.1b). While we showed in previous reports that myelin alters the functional properties of macrophages by activating LXRβ and PPARβ/δ, activation of these nuclear receptors by specific agonists did not affect COLEC12 expression (figure 5.1c). Similarly, pro- (IFNγ and LPS) and anti-inflammatory (IL-4) mediators did not affect expression of the receptor on macrophages (figure 5.1d). Collectively these data demonstrate that myelin upregulates COLEC12 expression on macrophages in an LXR- and PPARβ/δ-independent manner.

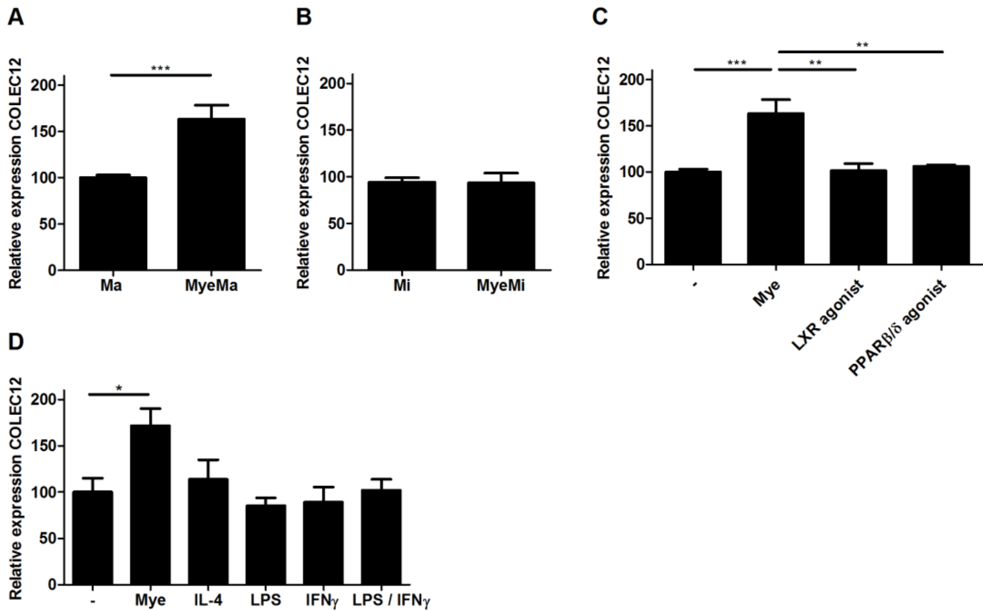

Figure 5.1 COLEC12 is upregulated in a LXR- and PPAR-independent manner in myelin-phagocytosing-macrophages. (a-c) Relative expression of COLEC12 on RAW264.7 (a, c) and BV-2 (b) cells after treatment with myelin, an LXR agonist (T09) and a PPARβ/δ agonist (GW0742). The relative COLEC12 expression is defined as the expression of COLEC12 in experimental cultures divided by values of non-treated cultures (n=5). (d) Relative expression of COLEC12 on RAW264.7 after treatment with pro- (LPS and IFNγ) and anti- (IL-4) inflammatory mediators (n=2).

5.3.2 COLEC12 does not facilitate myelin internalization by macrophages and microglia

COLEC12 facilitates amyloid beta clearance by glial cells. Moreover, as COLEC12 is structurally related to SRA and phagocytosis of myelin by macrophages and microglia is partially mediated by SRA, we determined the role of COLEC12 in the internalization of myelin by macrophages and microglia. By treating macrophages and microglia with a neutralizing antibody for COLEC12, we show that COLEC12 is not involved in the uptake of myelin by macrophages and microglia (figure 5.2a and 5.2b).

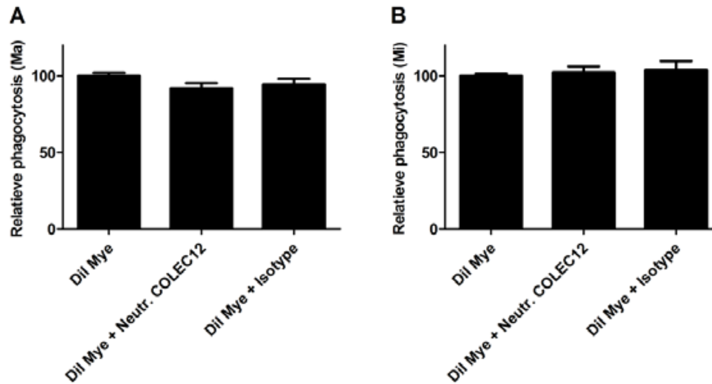

Figure 5.2 COLEC12 is not involved in phagocytosis of myelin. (a, b) Relative internalization of DiI-labeled myelin by RAW264.7 and BV-2 cells. Cells were left untreated or pretreated with a neutralizing antibody for COLEC12 or a matched isotype control, after which cells were treated with DiI-labeled myelin for 1.5 hours. Myelin uptake was assessed by flow cytometry. Data represent the mean of four experiments.

5.3.3 COLEC12 expression is increased in the center and rim of active MS lesion.

Next, we determined the expression of COLEC12 in active MS lesions. In contrast to NAWM (figure 5.3a), in which few cells faintly stained for COLEC12, active MS lesions were packed with COLEC12 positive cells (figure 5.3b). Apart from the increase in COLEC12 positive cells (figure 5.3c), its staining intensity, measured as described previously [722], was also increased in the center and RIM of the lesion (figure 5.3d).

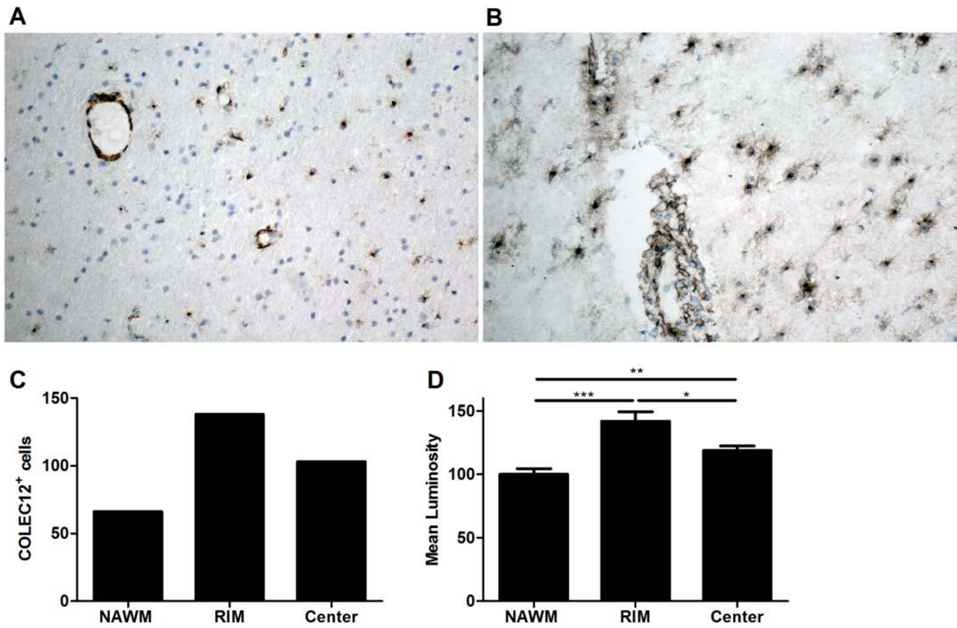

Figure 5.3 COLEC12 expression is increased in the center and rim of active MS lesions. (a, b) Representative pictures of COLEC12-DAB staining of NAWM (a) and the center of an active MS lesion (b, n=2, 20x). (c) Quantity of $COLEC12^{+}$ cells in NAWM and the lesion rim and center. Cryosections were stained for COLEC12, after which 5 pictures (10x magnification) were used to determine the amount of $COLEC12^{+}$ cells. (d) Mean luminosity of COLEC12 in NAWM and the lesion rim and center, measured as described previously [722] (n=2).

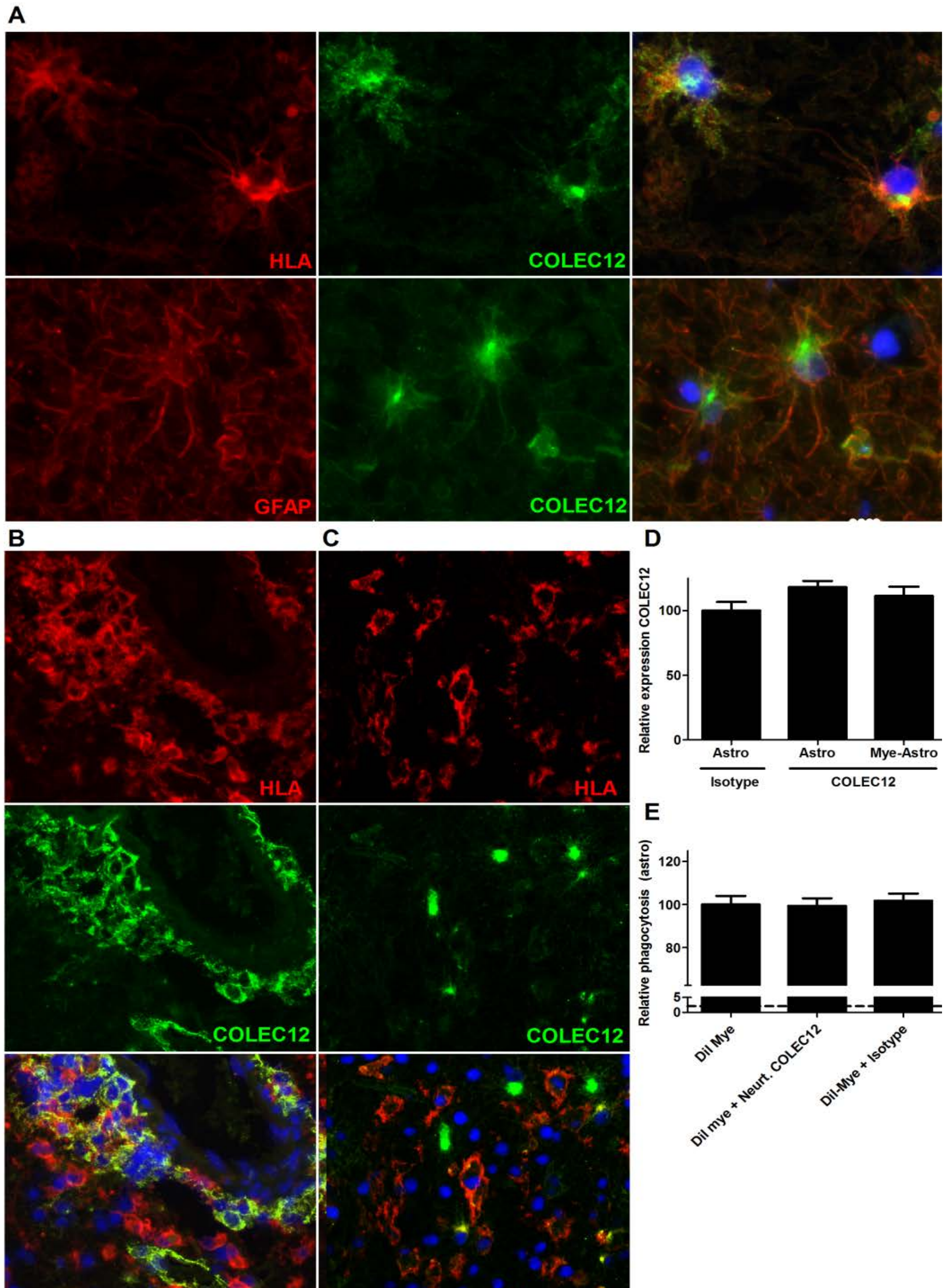

Figure 5.4 COLEC12 is highly expressed on foamy macrophages in perivascular cuffs and parenchymal astrocytes. (a) Fluorescent staining showing colocalization of COLEC12 with astrocytes (GFAP) and MHC class II expressing cells (HLA) in NAWM (40x magnification). One representative picture is shown. (b, c) COLEC12 and HLA expression

in MS tissue (20x magnification). One representative picture is shown. (d) Relative expression of COLEC12 on C8-D1a cells after treatment with myelin. (e) Relative internalization of DiI-labeled myelin by C8-D1a cells. Cells were left untreated or pretreated with a neutralizing antibody for COLEC12 or a matched isotype control, after which cells were treated with DiI-labeled myelin for 1.5 hours. Myelin uptake was assessed by flow cytometry. Dotted line represents untreated macrophages. Data shown are the mean of two experiments.

To determine the cellular source of COLEC12 expression in MS lesions, immunofluorescent stainings were performed. In the NAWM primarily microglia and astrocytes (figure 5.4a) and endothelial cells (figure 5.3a) expressed a low level of COLEC12. Interestingly, perivascular cuffs in MS lesions were packed with HLA-DR$^+$ cells, morphologically resembling foamy macrophages, which stained strongly for COLEC12 (figure 5.4b). In contrast, in the parenchyma HLA-DR$^+$ cells stained only faintly for COLEC12 (figure 5.4c). The observed star-shaped morphology of COLEC12$^+$ cells in the lesion center strongly indicates that also reactive astrocytes increase their expression of COLEC12 in the parenchyma of MS lesions (figure 5.3b). Despite the fact that astrocytes possess membrane-bound compartments enclosing phagocytosed fragments of MBP in acute MS lesions [723], myelin does not increase the expression of COLEC12 on astrocytes nor does COLEC12 facilitate internalization of myelin by astrocytes *in vitro* (figure 5.4d and 5.4e).

5.4 Discussion

In this study we determined the expression of COLEC12 on myelin-phagocytosing macrophages and in active MS lesions, and its role in myelin clearance. We show that COLEC12 is highly expressed in perivascular macrophages and parenchymal astrocytes in active MS lesions. Furthermore, we show that, unlike microglia, only macrophages upregulate COLEC12 on their cell surface after myelin ingestion in vitro. The latter finding underlines the fact that microglia and infiltrating macrophages fulfill cell-specific roles during MS and react differently to environmental cues [724, 725].

Several receptors, like complement-receptor 3, SRA I/II and Fcγ receptors have been described to facilitate myelin clearance by macrophages and microglia [372, 374, 388-392, 395, 396, 398-404]. While COLEC12 resembles SRAs and assists in microglia-mediated amyloid beta clearance [595], we demonstrate that COLEC12 is not involved in the ingestion of myelin by both macrophages and microglia *in vitro*. It would be interesting to assess whether oxidized-myelin is recognized and internalized by COLEC12, since oxLDL has been reported to bind to COLEC12. Furthermore, COLEC12 may be responsible for scavenging of other particles by perivascular macrophages, such as apoptotic cells and pathogens, that have penetrated the perivascular space in the CNS. Notably, perivascular macrophages are known to contribute to the recognition and elimination of pathogens [726, 727].

The expression of several SRAs, like SPα, MARCO and CD36, is enhanced by activation of LXRs or PPARs [728, 729]. We show that the expression of COLEC12 is not regulated by agonists for LXRs and PPARβ/δ. Although we previously reported that myelin alters the functional properties of macrophages through the activation of LXRβ and PPARβ/δ, these data indicate that myelin can alter the phenotype of macrophages through yet other mechanisms. Future studies have to determine how myelin alters the expression level of COLEC12.

Based on the presence of a CRD-domain and ligand specificity, parallels can be drawn between COLEC12 and both DC-SIGN and selectins [730, 731], suggesting it might play a role in cell migration, cell differentiation, antigen-capture and T cell priming [732, 733]. Interestingly, we found that the expression of COLEC12 is upregulated on foamy-appearing macrophages in perivascular cuffs during MS. As perivascular cuffs accommodate lymphocytes during active MS, COLEC12

may play a role in T cell priming, similar to DC-SIGN. Additionally, as myelin-containing phagocytes have been located in the CNS-draining lymphoid organs [366-368], future studies should determine whether COLEC12 may facilitate lymph node directed migration of foamy macrophages.

Astrocytes have long been regarded to merely provide support for neuronal function in heath and disease. However, they also have been described to have the capacity to both promote and inhibit neuroinflammation, demyelination and remyelination [734]. We show that the increased expression of COLEC12 in the lesion center of MS patients colocalizes primarily with astrocytes. Of interest, COLEC12 intensity has been reported to be elevated in amyloid beta positive astrocytes in mice and humans with AD. Moreover, COLEC12 levels increased in neonatal astrocytes treated with amyloid beta *in vitro* [595]. Despite the fact that astrocytes possess membrane-bound compartments enclosing phagocytosed fragments of MBP in acute MS lesions [723], myelin did not increase the expression of COLEC12 on astrocytes nor did COLEC12 facilitate internalization of myelin by astrocytes. Follow-up studies should address whether the increased expression of COLEC12 on astrocytes in MS lesions plays a role in the uptake of other particles, migration, differentiation or plays a role in eliminating pathogens [735, 736].

In summary, this is the first report showing an upregulation of COLEC12 expression in active MS lesions. Whereas foamy-resembling macrophages stained strongly for COLEC12 in perivascular cuffs, the increased COLEC12 expression in the center and rim of the MS lesion colocalized primarily with astrocytes. Future studies should determine the function of COLEC12 on these cells and assess the role it plays during MS pathogenesis.

6

Summary and general discussion

Summary & General discussion

MS is a chronic, inflammatory, neurodegenerative disorder, in which macrophage-mediated myelin destruction is considered to be one the primary effector mechanism. Nonetheless, especially after internalization of myelin, macrophages have been described to obtain a less inflammatory phenotype. However, it is unclear by which mechanisms myelin affects the phenotype of macrophages and how this phenotype can influence lesion progression. In this chapter, the main questions and results of this study are summarized and discussed. Furthermore, some suggestions for future research are presented. A scheme that summarizes and integrates our major findings is depicted in figure 6.1.

Do myelin-phagocytosing macrophages influence T cell reactivity?

Myelin-phagocytosing macrophages are ideally positioned to modulate autoreactive T cell reactivity in MS [314-316, 366-368]. Although reported to reactivate autoreactive lymphocytes upon infiltration [93, 94, 352], the altered phenotype of macrophages upon myelin internalization may change their immunomodulatory capacity [365, 375, 376]. In **chapter II** we show that myelin-phagocytosing macrophages inhibit TCR-triggered lymphocyte proliferation in an antigen-independent manner *in vitro*. This inhibition of lymphocyte proliferation was associated with an increased concentration of NO in the culture supernatant. Both inhibition of NO produced by macrophages and abrogation of direct cell-cell contact restored lymphocyte proliferation. This suggests that direct contact between both cell types is a prerequisite for stimulating NO-mediated inhibition of T cell proliferation by macrophages. How lymphocytes induce NO production by myelin-phagocytosing macrophages is unclear and needs further investigation. On the other hand, NO might, due to its extreme short half-life [619], not reach lymphocytes when direct contact is restricted.

In vivo, myelin-phagocytosing macrophages suppressed the reactivity of non-myelin specific T cells, whereas they boosted the proliferation of myelin-protein reactive T cells and aggravated EAE. This finding is in agreement with the proposed role of macrophages and microglia in the reactivation of myelin-reactive T cells. More importantly, our finding provides compelling evidence that the nature of surrounding lymphocytes, being myelin-protein, non-myelin or

myelin-lipid specific, determines whether myelin-phagocytosing macrophages stimulate or suppress lymphocyte activity. Of interest, neuron-reactive T cells have been reported in MS. As neuron-reactive T cells are not activated by the presentation of myelin proteins, their proliferation is likely more susceptible to a NO-mediated suppression by myelin-phagocytosing macrophages, as compared to myelin-specific T cells [737, 738]. Based on these findings we suggest that myelin-phagocytosing macrophages in the CNS-draining lymph nodes, and possibly in the perivascular space, can fulfill a suppressive role in MS by producing NO, thereby silencing non-myelin specific autoreactive lymphocytes.

Is NO detrimental or beneficial in MS?

There is no doubt that RNS produced by macrophages and microglia have detrimental effects on MS and EAE development. Induced levels of NO and iNOS in MS lesions are associated with macrophages and microglia, and correlate with disease severity [422-427]. Functionally, RNS have been described to affect myelin integrity, damage axons and increase the permeability of the BBB [434-438, 488]. We have demonstrated in **chapter II** that the induced production of NO by myelin-phagocytosing macrophages, upon coculture with T cells, inhibits T cell proliferation. The latter suggests that NO produced by macrophages may suppress neuroinflammation in EAE and MS. The dual role of NO in the control of neuroinflammation is supported by studies showing an aggravation or inability to recover following treatment with an iNOS inhibitor in, respectively, the induction or the remission phase of EAE [625, 626]. Likewise, treatment with the NO-donor SIN-1 during the induction phase of EAE reduced disease severity, which was correlated with a reduced antigen-induced proliferation [431]. Altogether these findings indicate that NO exerts a pleiotrophic role in MS and EAE, depending on the disease stage (early or late phase of disease), the site at which it is produced (parenchyma, perivascular space or in CNS-draining lymph nodes), the nature of surrounding cells (T cells or oligodendrocytes and neurons) and likely the quantity in which it is released.

Are myelin-phagocytosing macrophages anti-inflammatory?

The phenotype of macrophages is dynamic, as they can alter their functional properties in response to changing environmental stimuli [294, 295]. Broadly,

macrophage phenotypes can be classified based on three different homeostatic activities; host defense, wound healing and immune regulation [288]. It is evident that macrophages infiltrating MS lesions confer, at least for a certain period of time, a pro-inflammatory phenotype [179, 422, 570]. However, recent evidence indicates that monocyte-derived macrophages, peritoneal macrophages, microglia and DCs obtain less inflammatory characteristics following internalization of myelin [365, 375, 376, 601]. In support of the latter, we demonstrate in **chapter III and IV** that LPS or IFNγ/IL-1β-stimulated myelin-treated macrophages adopt a less inflammatory phenotype, as compared to stimulated control macrophages. Myelin-treated macrophages secreted significantly lower amounts of IL-6 and NO. Interestingly, in **chapter II,** we show that myelin-phagocytosing macrophages are also immunosuppressive in the vicinity of activated T cells. These findings indicate that macrophages adopt a phenotype that may dampen neuroinflammation after myelin internalization. This less-inflammatory, immunoregulatory phenotype may suppress lesion development and create an environment susceptible for repair in MS. In other words, the macrophage phenotype changes after internalization of myelin may reflect an attempt of the innate immune system to restore CNS homeostasis and stimulate repair mechanism in MS.

The observed phenotype of myelin-phagocytosing macrophages differs from the well described M1, M2a, M2b and M2c phenotypes. Based on the reduced secretion of inflammatory mediators, myelin-phagocytosing macrophages can be classified as M2a macrophages (wound healing macrophages). However, their capacity to suppress lymphocyte proliferation suggests that they also adopt characteristics of M2c macrophages (immunoregulatory macrophages). These findings indicate that myelin-exposed macrophages commit to a unique phenotype. Also for the recently described M2d (tumor-associated macrophages), M4 (CXCL4-induced macrophages) and Mox (oxidized phospholipid-induced macrophages) macrophages specific phenotypes have been described, indicating that a whole range of macrophage phenotypes exists [289-293]. Moreover, while in monocultures myelin-phagocytosing macrophages demonstrated a reduced secretion of the typical M1 marker NO, an increase in the production of the M1 marker NO by myelin-treated macrophages was found in T cell cocultures. The latter indicates that myelin-phagocytosing macrophages

can adopt a spectrum of phenotypes depending on soluble mediators and cells in their vicinity. Notably, as the diversity in gene expression among different populations of macrophages is considerable [739], divergent macrophage subsets are likely to respond differently to myelin internalization. The latter and differences in the experimental setup may explain why the microarray did not detect an altered expression of inflammatory markers, previously demonstrated to be affected by myelin in macrophages [365, 375, 376, 601]. Collectively, while macrophages may possess a intrinsic anti-inflammatory profile after myelin engulfment, environmental stimuli may affect their phenotype.

Are myelin-derived lipids involved in altering the functional properties of myelin-phagocytosing macrophages?

To obtain insight into the influence of myelin internalization on the functional properties of macrophages and the mechanisms involved, the gene expression profile of myelin-phagocytosing macrophages was assessed (**chapter III**). Microarray analysis revealed an overrepresentation of several common enriched pathways related to lipid metabolism, LXR/PPAR signaling and cholesterol efflux.

LXRs and PPARs, which are activated by respectively cholesterol derivates and fatty acids (present in phospholipids), have recently been described as key regulators of lipid metabolism and inflammation [579-581]. By using LXRα-, LXRβ- and LXRαβ-deficient macrophages and antagonists for PPARα, PPARβ/δ and PPARγ, we established in **chapter III and IV** that myelin-derived cholesterol and phosphatidylserine direct the inflammatory and metabolic phenotype of myelin-phagocytosing macrophages through activation of LXRβ and PPARβ/δ, respectively. Myelin-mediated activation of these nuclear receptors resulted in a reduced secretion of NO and IL-6, and an induced expression of lipid efflux transporters. These findings are in line with the abundance of these lipids in myelin [578, 680]. Moreover, synthetic LXR and PPARβ/δ agonists, PS and cellular cholesterol accumulation have been reported to induce a less-inflammatory, tolerogenic phenotype in macrophages and microglia [574, 584, 588, 591, 593, 691, 698-700, 702], similar to myelin-phagocytosing macrophages and microglia.

Divergent functions for LXRα and LXRβ in cholesterol and bile acid metabolism, glucose homeostasis, energy utilization and fat storage have been reported [678, 740, 741]. With respect to the specific role of LXRβ in dampening IL-6 production by

myelin-phagocytosing macrophages, our data signify an additional functional difference for LXRα and LXRβ in directing the inflammatory properties of (myelin-phagocytosing) macrophages. Remarkably, although both LXR subtypes can repress the expression of inflammatory genes in astrocytes in a similar manner, LXRα and LXRβ undergo differential SUMOylation in these cells [742]. SUMOylation is a post-translational modification involved in transcriptional regulation. Whether this aberrant SUMOylation of LXRs plays a role in our findings remains to be clarified.

Future studies should also determine how myelin-derived PS and cholesterol activate PPARβ/δ and LXRs in macrophages. As cholesterol as such is not a ligand for LXR, the formation of oxidized derivatives of cholesterol may activate LXRs after myelin engulfment by macrophages [743]. Furthermore, the potential accumulation of cellular desmosterol levels following myelin engulfment can contribute to LXR activation in myelin-phagocytosing macrophages [574, 649]. Similarly, whether myelin-derived PS activates PPARβ/δ by binding to its receptor(s) on the membrane or exerts its effect upon internalization and cellular accumulation remains to be clarified. In regard of the latter, it would be interesting to evaluate the role of fatty acids attached to the glycerol backbone of PS in activating PPARβ/δ [704, 744-746]. Moreover, desmosterol accumulation in foamy macrophages has been reported to induce the expression of genes involved in the metabolism of fatty acids, such as SCD1/2. In agreement with this hypothesis, in **chapter III** we observed a significantly increased expression of SCD2 in myelin-phagocytosing macrophages. This suggests that desmosterol accumulation in myelin-exposed macrophages may indirectly activate PPARβ/δ by inducing the generation of fatty acids.

Collectively, our data show that myelin-derived lipids play a crucial role in directing the inflammatory and metabolic properties of macrophages upon myelin uptake through the activation of PPARβ/δ and LXRs. However, underlying molecular mechanisms of the myelin-mediated activation of these nuclear receptors remain elusive.

Do myelin and myelin-derived lipids influence EAE pathology and severity?

In **chapter II, III and IV**, we demonstrate that macrophages obtain a less-inflammatory and immunoregulatory phenotype after internalization of myelin. These findings suggest that myelin may suppress neuroinflammation *in vivo*. However, when we subcutaneously administered myelin debris, which was taken up by macrophages in lymph nodes, EAE animals displayed an earlier onset of paralysis and a more severe neurological score at the peak of disease (**chapter II**). This aggravated disease severity was associated with an increased reactivity of myelin-reactive T cells. These findings suggest that, despite their capacity to dampen T cell proliferation, myelin-phagocytosing macrophages promote the proliferation of myelin-reactive T cells by presentation of myelin antigens, thereby aggravating EAE severity. Interestingly, another study has reported that intravenously administered MOG peptide-loaded myelin-laden macrophages ameliorate EAE as compared to unpulsed myelin-laden macrophages, which did not affect EAE severity [747]. With respect to these findings, apoptotic cells have been described to have divergent effects on inflammation depending on the route of administration. Whereas intravenous administration results in homing of apoptotic cells to splenic macrophages and leads to immunosuppression or tolerance [748, 749], subcutaneously injected apoptotic cells are internalized by lymph node macrophages and are often immunogenic [749, 750]. It would be interesting to determine the outcome on neuroinflammation and EAE severity when myelin debris is injected intravenously and taken up by splenic macrophages.

We have established that myelin-derived lipids, like cholesterol and PS, play a crucial role in directing the inflammatory properties of macrophages upon myelin internalization. Strikingly, in **chapter IV,** we show that myelin-associated PS, primarily internalized by macrophages, suppresses the proliferation of MOG-reactive T cells and ameliorates EAE. Liposomes were used to specifically target PS to macrophages. These findings support the hypothesis that myelin-phagocytosing macrophages suppress T cell proliferation and dampen neuroinflammation. In line with our results, PSLs have been described to promote the resolution of inflammation by modulating macrophage function in a model for inflammatory bone loss and myocardial infarction [699, 700]. Moreover,

non-encapsulated phosphatidylserine has recently been reported to suppress autoaggressive T cell responses [704]. In addition to a dampened T cell proliferation, PSL-treated EAE animals displayed a marked reduction in the numbers of macrophages and T cells infiltrating into the CNS, which indicates that PSLs influence immune cell trafficking towards the CNS, in addition to or as a result of modulating macrophage and T cell activity. Remarkably, we found that PSLs also reduced EAE severity in a therapeutic setting, suggesting that apart from being immunomodulatory they may also promote remyelination and axonal regeneration. Future studies should determine whether anti-inflammatory and neurotrophic factors produced by PS-stimulated macrophages may affect CNS repair processes. Moreover, as PPARβ/δ activation induces the capacity of macrophages to clear apoptotic cells, a PS-mediated activation of PPARβ/δ may promote internalization of inhibitory myelin debris, thereby stimulating CNS repair processes [531-533, 556, 558, 593]. In regard of the latter, follow-up studies should assess the role of PPARβ/δ in the immunosuppressive and neuroprotective effects of PSL on EAE. Finally, it would be interesting to evaluate the role of cholesterol-loaded macrophages on demyelination, remyelination and inflammation, and determine the specific impact of both LXRα and LXRβ activation in macrophages on EAE pathogenesis. Interestingly, LXR activation has already been demonstrated to ameliorate EAE by modulating T cell polarization [681-683], however our data indicate that macrophages likely also contribute to the observed neuroprotective effect of LXR agonists on inflammation and disease severity.

What is the role of nuclear receptors in MS?

In **chapter IV** we demonstrate that the expression of PPARβ/δ response genes and proteins is upregulated in active MS lesions, especially in myelin-phagocytosing macrophages. These findings indicate that degraded myelin also activates PPARβ/δ in macrophages in the human brain. This myelin-mediated PPAR activation may affect lesion progression by inducing an anti-inflammatory environment that influences the activity of infiltrating T cells and enhances the clearance of myelin debris [531-533, 556, 558, 593]. In support of an immunomodulatory and neuroprotective role of PPARβ/δ in MS, PPARβ/δ agonists inhibit inflammation in a carrageenan-induced paw edema model and ameliorate EAE

severity [706, 751, 752]. The importance of PPARβ/δ signaling in maintaining immune-homeostasis and preventing systemic autoimmunity is illustrated by the fact that macrophage-specific PPARβ/δ deficiency delays clearance of apoptotic cells and increases autoantibody production [593]. Furthermore, in a focal cerebral ischemia model, PPARβ/δ plays an important role in the control of central inflammation, as indicated by an increased infarct size, and an increase in the level of IFNγ in PPARβ/δ knockout mice compared to wild-type mice [753]. These studies support the hypothesis that myelin-mediated activation of PPARβ/δ in macrophages can suppress lesion progression. Future studies should also determine whether LXRs are activated in active MS lesions. Interestingly, preliminary data show that LXR response genes are upregulated in the CNS of EAE animals and MS patients. Whether this enhanced expression is present in myelin-phagocytosing macrophages remains to be clarified. Similar to PPARβ/δ, activation of LXRs has been demonstrated to ameliorate EAE [681-683], indicating that a myelin-mediated activation of LXRs in macrophages may dampen neuroinflammation in MS.

Our data argue that myelin-derived lipids are capable of dampening macrophage-mediated inflammation by nuclear receptor activation, which can potentially explain the relapse-remitting nature of early MS. However, why is this naturally-occurring regulatory mechanism absent or imperfect in progressive forms of MS in which CNS repair is rare? Does ongoing CNS inflammation and degeneration predominate the immunosuppressive and regenerative capacity of nuclear receptor activation in progressive MS? Is the myelin-mediated activation of nuclear receptors blocked by the presence of inhibitory molecules? For instance, lipid-reactive antibodies could obstruct the otherwise neuroprotective impact of myelin-derived lipids on macrophages [704, 754-756]. Alternatively, age-dependent alterations in macrophages or nuclear receptors may underlie the reduced capacity of the CNS to regenerate in progressive MS. In support of the latter, young monocytes are more efficient in clearing inhibitory myelin debris than senescent monocytes and thereby better capable of stimulating remyelination [194, 195]. Furthermore, the mRNA and protein expression, and DNA-binding capacity of PPARs show an age-dependent decline in rats [757]. Answering these questions will increase our understanding concerning the role of nuclear receptors in macrophages during chronic MS and can potentially result in the

development of new treatments directed at the progressive phase of the disease.

What is the role of COLEC12 on macrophages and in MS lesions?

Microarray analysis demonstrated an increased expression of COLEC12 on macrophages after myelin internalization (**chapter III**). COLEC12 is structurally related to SRA, which is one of the cardinal receptors involved in myelin phagocytosis [388-390]. Despite this resemblance, COLEC12 did not facilitate myelin clearance by both macrophages and microglia, as discussed in **chapter V**. In addition, although the phenotype of myelin-phagocytosing macrophages is directed by LXRβ and PPARβ/δ (**chapter III and IV**), agonists for both nuclear receptors were unable to induce its expression. Interestingly, COLEC12 expression is increased in the center and rim of active MS lesions, especially in perivascular macrophages and parenchymal astrocytes. Although the expression of COLEC12 seems to be associated with amyloid beta in astrocytes [595], its expression on astrocytes was not affected by myelin and did not contribute to myelin clearance [723].

Apart from its collagen-like domain, COLEC12 also contains a C-type CRD domain [713, 714]. Concerning the CRD domain, parallels can be drawn between COLEC12 and both DC-SIGN and selectins [717, 718, 730, 731], suggesting that COLEC12 might play a role in cell migration, cell differentiation and T cell priming [732, 733]. As perivascular cuffs accommodate both lymphocytes and macrophages and myelin-phagocytosing phagocytes are located in the CNS-draining lymphoid organs in MS, future studies should determine the involvement of COLEC12 in regulating cell migration and T cell reactivity. In addition, it would be appealing to demonstrate how myelin alters the expression of COLEC12 on macrophages upon internalization. Finally, follow-up studies should address whether the increased expression of COLEC12 on astrocytes in MS lesions plays a role in the uptake of other particles, migration, differentiation or plays a role in eliminating pathogens [735, 736].

What are the implications of findings in this report for the development of new MS drugs?

Although there are currently several medications approved by the FDA for the treatment of MS, none of them provide a cure. Modulation of macrophage function holds promise for future MS therapeutics, as they play a crucial role in lesion progression and resolution in MS. In this thesis, I have demonstrated that myelin-associated lipids direct macrophages towards an immunoregulatory and less-inflammatory phenotype through activation of LXRβ and PPARβ/δ, respectively. This naturally-occurring regulatory mechanism may be harnessed for future MS therapeutics. For instance, delivery of PS, cholesterol metabolites or LXRβ / PPARβ/δ agonists to systemic monocytes may dampen neuroinflammation and halt lesion development. As we and others have demonstrated that liposomes naturally target cells of the mononuclear phagocytic system [758], especially macrophages, loading liposomes with these agonists or lipid (metabolites) may offer an efficient way of targeting macrophages and altering their functional properties. Interestingly, oral administration of liposomes is feasible when the pH-stability and mucus-penetrating capacity of liposomes are enhanced [759].

While PPARα and PPARγ agonists have been repeatedly suggested as a treatment option for neurological diseases [760-762], the potential use of PPARβ/δ selective drugs for treating neurological disorders is a relatively new concept. However, it should be noted that PPARδ activation has been reported to promote intestinal tumor growth, raising concerns about the use of PPARβ/δ agonists in humans [763]. Considering LXR agonists as a potential drug for MS, LXR activation is associated with stimulation of lipogenesis resulting in increased plasma TG levels and hepatic steatosis [764, 765]. These side-effects have been associated with the selective activation of LXRα, suggesting that LXRβ-specific agonists may be potential candidates for MS therapeutics. Interestingly, we demonstrated that myelin altered the functional properties through activation of LXRβ. Identification of the underlying molecular mechanism of this myelin-mediated activation of LXRβ may result in the discovery of a new natural LXRβ agonist capable of ameliorating MS.

Apart from the activation of PPARs by liposomes, nutrition may affect the activation of this nuclear receptor and MS pathogenesis. For instance, as

polyunsaturated fatty acids and their respective derivatives can serve as PPAR agonists [766], consumption of vegetables, sea food and fish oil, which are rich in unsaturated fatty acids (omega-3), may dampen lesion progression in MS by directing macrophages to an immunoregulatory and less-inflammatory phenotype [767]. This hypothesis is supported by the fact that consumption of saturated animal fat is directly related to the frequency of MS [768, 769]. In contrast to PPARs, dietary activation of LXRs is less obvious, as oxysterols are natural agonists for LXRs. Oxysterols are primarily found in a cholesterol-high diet, which is appreciated to promote artherosclerosis and coronary heart disease.
Collectively, our findings indicate that liposomal or dietary activation of LXRβ and PPARβ/δ holds promise for future intervention strategies aimed at reducing neuroinflammation in MS.

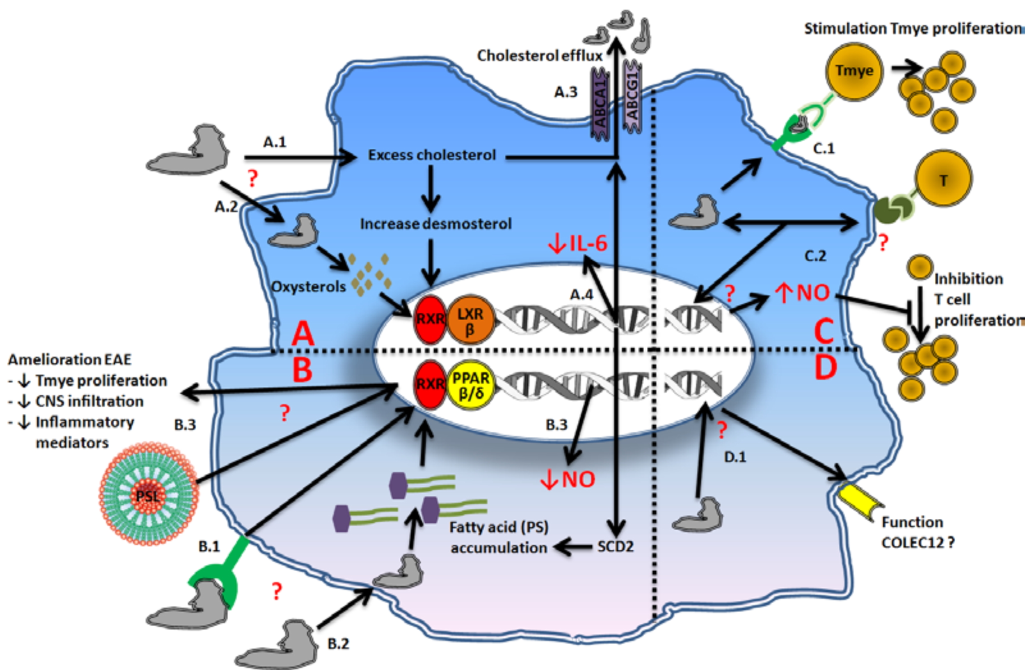

Figure 6.1 Summary of findings and remaining questions from this thesis. (A and B) In chapter 3 and 4, we have demonstrated that myelin-derived cholesterol and PS direct the inflammatory (A.4 and B.3) and metabolic (A.3) phenotype of macrophages upon myelin internalization through activation of LXRβ and PPARβ/δ. How myelin is internalized and activates LXRβ and PPARβ/δ remains to be clarified (A.1, A.3, B.1 and B.2). Furthermore, we found that uptake of PS by macrophages, after intravenous

injection of PSLs, suppresses the production of inflammatory mediators, inhibits infiltration of immune cells into the CNS, dampens proliferation of myelin-reactive T cells and ameliorates EAE (B.3). These findings indicate that myelin-derived lipids play a crucial role in directing the phenotype of macrophages upon myelin internalization, thereby likely affecting lesion progression in MS. (C) In chapter 2, we demonstrated that myelin-phagocytosing macrophages inhibit T cell proliferation by the production of NO (C.2). While we showed that the induced production of NO by macrophages is dependent on direct contact between T cells and macrophages, the underlying mechanism remain elusive. In vivo, myelin-phagocytosing macrophages stimulated and suppressed the proliferation of myelin-reactive and ovalbumine-reactive T cells (C.1 and C.2), respectively. This indicates that myelin-phagocytosing macrophages are immunosuppressive, depending on the nature of surrounding cells. (D) We have shown in chapter 5 that myelin upregulates the expression of COLEC12 on macrophages but not on microglia. Furthermore, we found that COLEC12 is highly expressed in perivascular macrophages and parenchymal astrocytes in active MS lesions. How myelin induces the expression of COLEC12 on macrophages and what function it fulfills on these cells is unclear (D.1).

Final conclusion

The relapsing-remitting nature of MS suggests the presence of naturally-occurring regulatory mechanisms in the disease, which may be harnessed for future intervention strategies aimed at reducing neuroinflammation and stimulation CNS repair. In this thesis we show that myelin internalization by macrophages induces a less inflammatory, immunesuppressive phenotype in macrophages, indicating that myelin-phagocytosing macrophages may contribute to the remission phase observed in relapse-remitting MS patients. By affecting autoreactive T cell function, either in lymphoid organs or locally in the CNS, they can dampen the inflammatory burden in MS-affected individuals. Moreover, the suppressed production of inflammatory mediators by myelin-phagocytosing macrophages can repress demyelination and axonal degeneration, and may create an environment more susceptible for repair.

To date, despite the abundance of lipids in myelin, most studies have mainly focused on the role of myelin proteins in demyelinating diseases. Our data indicate a role for myelin-derived lipids in modulating the metabolic and inflammatory response in macrophages during demyelination. By modulating the macrophage phenotype through activation of LXRβ and PPARβ/δ, myelin-derived

lipids may affect lesion progression in MS. This unrecognized link between demyelination, lipid metabolism and macrophage-mediated inflammation during MS pathogenesis holds promise for future MS therapeutics.

7

Nederlandse samenvatting

Multiple sclerose (MS) is een chronische auto-immuunziekte van het centrale zenuwstelsel (CZS). Wereldwijd lijden meer dan 2 miljoen mensen aan MS en de incidentie is 1:1000 in Europa en Noord-Amerika. Het is de meest voorkomende oorzaak van niet-traumatische neurologische invaliditeit bij jong volwassenen. Daarnaast worden vrouwen vaker getroffen dan mannen. Waar bij de meerderheid van de patiënten in het begin nog herstel plaatsvindt, ondervinden alle patiënten na verloop van tijd progressieve achteruitgang. Volgens de meest gangbare hypothese wordt MS veroorzaakt door een ontsporing van het eigen immuunsysteem, dat ons normaliter beschermt tegen indringers. Tijdens MS infiltreren bepaalde immuuncellen, zoals autoreactieve T-cellen en macrofagen, het CZS. Lokaal in de hersenen creëren deze immuuncellen een chronische ontstekingsreactie en vallen ze de isolerende myelineschede rond de zenuwuitlopers aan, waardoor de communicatie tussen zenuwcellen wordt verstoord. Dit leidt tot symptomen zoals spierzwakte, sensorische en visuele uitval, cognitieve problemen en vermoeidheid. Ondanks dat de oorzaak nog onbekend is, lijken zowel erfelijke als omgevingsfactoren een rol te spelen in de totstandkoming van MS.

In het CZS van MS-patiënten zijn op meerdere plaatsen harde plekken zichtbaar (multiple = meervoudig; sclero = hard), zogenaamde plaques of lesies, die geïnfiltreerde immuuncellen bevatten. Diverse studies hebben laten zien dat macrofagen de belangrijkste immuuncellen zijn die aanwezig zijn in MS-lesies. Macrofagen zijn witte bloedcellen betrokken bij de opruiming van dode of beschadigde lichaamseigen cellen en ziekteverwekkers. In MS vertonen macrofagen een geactiveerd fenotype (totaal van alle waarneembare eigenschappen) en bevatten grote hoeveelheden myeline, wat wijst op actieve betrokkenheid van deze cellen in de afbraak van de myelineschede (demyelinisatie). Daarnaast is bekend dat macrofagen schadelijke mediatoren produceren die een additionele negatieve invloed hebben op de integriteit van myeline en die zenuwuitlopers kunnen beschadigen. Tot voor kort werd er daarom ook gedacht dat macrofagen enkel schadelijk zijn in MS. Echter, recente bevindingen tonen aan dat in bepaalde omstandigheden macrofagen een minder schadelijk en mogelijk zelfs een beschermend fenotype kunnen aannemen.

Het fenotype van macrofagen blijkt belangrijk te zijn voor de mate waarin ze schadelijke of juist beschermende factoren produceren. Zo zijn *klassiek*

geactiveerde macrofagen (M1-macrofagen) erop gericht schadelijke ziekteverwekkers te verwijderen. Deze M1 macrofagen produceren aanzienlijke hoeveelheden ontstekingsmediatoren, welke indringers zoals bacteriën en virussen kunnen doden. Ondanks dat er weinig bekend is over het precieze fenotype van macrofagen in MS-lesies, is het duidelijk dat ze tenminste voor een bepaalde tijd een M1-achtig fenotype aannemen. Alhoewel dit fenotype noodzakelijk is voor het bestrijden van ziekteverwekkers, heeft het een schadelijke uitwerking op de progressie van MS, zoals vermeld in de vorige alinea. In tegenstelling tot M1-macrofagen, spelen *alternatief geactiveerde macrofagen* (M2-macrofagen) een rol bij allergie, herstelmechanismen en regulatie van het immuunsysteem. Uit recente aanwijzingen blijkt dat macrofagen een M2-gelijkend fenotype kunnen aannemen in MS-lesies, vooral nadat ze myeline hebben opgenomen. Op basis van deze studies kan men suggereren dat macrofagen die myeline opnemen (myeline-fagocyterende macrofagen) mogelijk een rol spelen in natuurlijk voorkomende herstelmechanismen in MS. Het doel van deze thesis is het **ophelderen van de rol van myeline-fagocyterende macrofagen in MS**. Daarnaast zal worden bepaald **hoe myeline het fenotype van macrofagen na opname verandert**.

Wat is het effect van myeline-fagocyterende macrofagen op autoreactieve T-cellen?

Myeline-fagocyterende macrofagen bevinden zich tijdens MS in het CZS en CZS-drainerende lymfeknopen. Om deze reden zijn ze ideaal gesitueerd om CZS-infiltrerende T-cellen te beïnvloeden. T-cellen zijn net als macrofagen betrokken bij het afweersysteem van ons lichaam. In tegenstelling tot macrofagen hebben T-cellen een receptor die met hoge specifiteit reageert op componenten van ziekteverwekkers. Voorgaande studies hebben al aangetoond dat deze macrofagen, door myeline-eiwitten te presenteren op hun oppervlak, myelin-reactieve T-cellen kunnen activeren en laten vermenigvuldigen. Echter, de beschreven verandering in de functionele eigenschappen van macrofagen na myeline opname zou mogelijk hun capaciteit om T-cellen te beïnvloeden kunnen wijzigen. In **hoofdstuk II** werd daarom het effect van myeline-fagocyterende macrofagen op de activiteit van T-cellen bestudeerd. Onze resultaten wijzen erop dat myeline-fagocyterende macrofagen de deling van niet-myeline

reactieve T-cellen remmen. Daarnaast tonen we aan dat een verhoogde stikstofoxide (NO)-productie door myeline-fagocyterende macrofagen verantwoordelijk is voor de remming van de T-cel activiteit. Onze bevindingen laten zien dat de aard van de omringende T-cellen bepaalt of myeline-fagocyterende macrofagen de activiteit van T-cellen stimuleert dan wel onderdrukt. Met andere woorden, myeline-specifieke T-cellen kunnen worden geactiveerd, terwijl niet-myeline specifieke T-cellen in MS geremd kunnen worden.

Is NO goed of slecht in MS?

Van NO is bekend dat het schadelijk effecten heeft in MS en EAE. Verhoogde niveaus van NO en het enzym verantwoordelijk voor de productie van NO (iNOS) worden teruggevonden in macrofagen aanwezig in MS-lesies en hangen samen met de ernst van verlammingsverschijnselen. Het is beschreven dat NO de myelineschede en de onderliggende zenuwcellen kan beschadigen. Daarnaast kan het de doorlaatbaarheid van de bloed-hersen barrière (de grens tussen bloedvaten en het CZS) verhogen en zodoende de infiltratie van schadelijke immuuncellen naar het CZS vergemakkelijken. Onze resultaten tonen echter aan dat de verhoogde productie van NO door myeline-fagocyterende macrofagen de activatie van T-cellen remt, waardoor ontstekingsreacties in MS en EAE onderdrukt kunnen worden. Eerder is al aangetoond dat NO een protectieve functie heeft in de inductie fase van EAE. Het lijkt er dus op dat NO een dubbele rol in MS en EAE speelt, afhankelijk van waar en wanneer in het ziekteproces het wordt aangemaakt, hoe de omringende cellen erop reageren en waarschijnlijk in welke hoeveelheden het wordt aangemaakt.

Wat is de rol van myeline-lipiden op het fenotype van myeline-fagocyterende macrofagen?

Om beter inzicht te krijgen in het fenotype van macrofagen na opname van myeline en om de onderliggende mechanismen betrokken bij de totstandkoming van dit fenotype op te helderen werd in **hoofdstuk III** een microarray uitgevoerd. Een microarray kan een patroon van genexpressie zichtbaar maken door de mate van expressie van 28000 genen tegelijkertijd te meten. De microarrayanalyse toonde aan dat genen die betrokken zijn bij

lipidenmetabolisme oververtegenwoordigd zijn in myeline-fagocyterende macrofagen. Lipiden zijn vetten of vetachtige stoffen die het lichaam nodig heeft als energiebron of als bouwstof voor lichaamscellen. Zo werd een verhoogde activiteit van *lever-X-receptoren* (LXRs) en *peroxisoom proliferator-activated-receptoren* (PPARs) waargenomen.

Myeline is een vetachtige stof bestaande uit eiwitten en lipiden. Ondanks de overvloed aan lipiden in myeline hebben tot op heden de meeste studies zich voornamelijk gericht op de rol van myeline-eiwitten in MS. Zo werd aangetoond dat T-cellen in MS voornamelijk gericht zijn tegen myeline-specifieke eiwitten. Daarnaast is het meest gebruikte diermodel voor MS gebaseerd op de activatie en vermenigvuldiging van myeline-eiwit specifieke T-cellen. Onze resultaten laten echter zien dat PPARs en LXRs worden geactiveerd in macrofagen na opname van myeline, hetgeen duidt op een rol voor myeline-lipiden in MS. LXRs en PPARs zijn receptoren die geactiveerd worden door een ophoping van respectievelijk cholesterol en vetzuur-afgeleiden in de cel. Na activatie zullen deze receptoren de expressie verhogen van diverse genen die betrokken zijn in het metabolisme van deze lipiden. Recent is echter ook aangetoond dat wanneer LXRs and PPARs geactiveerd worden in macrofagen, deze macrofagen een beschermend M2 fenotype aannemen. In **hoofdstuk III en IV** laten we zien dat myeline opname door macrofagen leidt tot een activatie van de LXRβ en PPARβ/δ subtypes en resulteert in een verminderde productie van ontstekingsmediatoren. Daarnaast laten we in **hoofdstuk IV** zien dat fosfatidylserine, een fosfolipide aanwezig in myeline, PPARβ/δ activeert en zo een beschermend M2-fenotype in myeline-fagocyterende macrofagen induceert. Onze bevindingen wijzen erop dat myeline-afgeleide lipiden een belangrijke rol spelen in de aanname van een minder schadelijk fenotype van macrofagen.

Wat is het effect van myeline-lipiden op de ernst van EAE?

Een recente studie heeft laten zien dat M2-macrofagen de verlammingsverschijnselen in EAE verminderen. Deze bevinding suggereert dat myeline-fagocyterende macrofagen ook een beschermende invloed kunnen hebben op EAE. Desondanks zagen we in **hoofdstuk II** een verergering van de ziekteverschijnselen wanneer EAE dieren onderhuids met myeline werden behandeld, hetgeen leidt tot opname van myeline door macrofagen. De met

myeline behandelde dieren vertoonden een verhoogde activiteit van myeline-reactieve T-cellen, wat de verergering van ziekteverschijnselen in deze dieren kan verklaren. In **hoofdstuk IV** werd daarom het effect van fosfatidylserine-fagocyterende macrofagen op het verloop van EAE bepaald. Zoals vermeld in de vorige sectie induceert myeline-afgeleid fosfatidylserine een beschermend M2-fenotype in macrofagen. In hoofdstuk IV leveren we overtuigend bewijs dat fosfatidylserine-fagocyterende macrofagen verlammingsverschijnselen onderdrukken. Fosfatidylserine behandeling had zowel een preventief als therapeutisch effect op het verloop van EAE. Behandelde dieren vertoonden een verminderde activiteit van myelin-specifieke T-cellen, weinig tot geen migratie van immuuncellen naar het CZS en een lagere expressie van ontstekingsmediatoren. Onze resultaten zijn in overeenstemming met studies die een beschermende en helende rol van fosfatidylserine-fagocyterende macrofagen hebben laten zien in diermodellen voor botverlies en hartinfarct.

Worden PPARs and LXR geactiveerd in humane MS-lesies?

Gebruikmakend van dierlijke cellen hebben we aangetoond dat macrofagen na opname van myeline een beschermend fenotype aannemen door de activatie van LXRs and PPARs. Dit suggereert dat myeline-afgeleide lipiden mogelijk een rol spelen in natuurlijk voorkomende herstel mechanismen tijdens demyelinisatie in MS. Echter, extrapolatie van dierstudies naar mensen is in het verleden soms problematisch gebleken. Waar bepaalde mediatoren beschermend waren in dierlijke ziektemodellen, blijken deze soms geen effect of zelfs een verergering in mensen te bewerkstelligen. In **hoofdstuk IV** tonen we aan dat genen en eiwitten, waarvan bekend is dat ze worden gereguleerd door PPARβ/δ, verhoogd tot expressie komen in MS-lesies. De verhoogde expressie van deze PPARβ/δ-gereguleerde genen en eiwitten lijkt daarbij voornamelijk gesitueerd te zijn in myeline-fagocyterende macrofagen. Deze bevinding wijst erop dat PPARβ/δ ook wordt geactiveerd in het humane CZS tijdens MS. Zoals beschreven in de vorige alinea's zou de activatie van PPARβ/δ in macrofagen na opname van myeline lesieprogressie in MS patiënten kunnen beïnvloeden. Preliminaire resultaten laten zien dat LXR-gereguleerde genen tevens verhoogd tot expressie komen in MS-lesies. Of deze expressie is geassocieerd met myeline-fagocyterende macrofagen moet nog worden bepaald.

Wat is de rol van COLEC12 op myeline-fagocyterende macrofagen en in MS-lesies?

De microarray analyse in **hoofdstuk III** toonde een verhoogde expressie van COLEC12 op macrofagen na myeline opname. COLEC12 is een receptor gelokaliseerd op het celmembraan. Structureel gezien vertoond het overeenkomsten met scavenger receptor A, een receptor met een bewezen rol in de opname van myeline. Daarnaast is het bekend dat COLEC12 betrokken is bij de binding en opname van bacteriën, schimmels en geoxideerde vormen van low density lipoproteïnes. Wij laten in **hoofdstuk V** echter zien dat COLEC12 niet betrokken is bij de opname van myeline door macrofagen. Daarnaast tonen we aan dat COLEC12 een hoge expressie vertoont in MS-lesies en dit vooral in macrofagen gesitueerd in de ruimte rond de bloedvaten en astrocyten. Toekomstige studies moeten bepalen welk effect de verhoogde expressie van COLEC12 op myeline-fagocyterende macrofagen en in MS-lesies heeft op ziekteprogressie.

Wat zijn de implicaties van de bevindingen in dit proefschrift voor de ontwikkeling van nieuwe medicijnen voor MS?

Op dit moment zijn er verschillende medicaties op de markt die symptomen van MS patiënten verlichten. Deze medicijnen kunnen MS echter niet genezen. Aangezien macrofagen een belangrijke rol spelen in MS, biedt medicatie die het fenotype van macrofagen verandert een goed perspectief voor toekomstige MS-therapieën. In dit proefschrift heb ik aangetoond dat myeline-afgeleide lipiden (fosfatidylserine en cholesterol) LXRβ en PPARβ/δ activeren in macrofagen nadat ze myeline hebben opgenomen. Deze activatie leidt tot de totstandkoming van een beschermend fenotype in macrofagen. Dit natuurlijk voorkomende mechanisme kan worden aangewend voor toekomstige therapieën gericht tegen MS. Het feit dat we in **hoofdstuk IV** hebben aangetoond dat liposomen (kunstmatig gesynthetiseerde deeltjes die bestaat uit een door een lipiden-membraan omgeven inhoud) specifiek door macrofagen worden opgenomen, zouden deze benut kunnen worden worden om fosfatidylserine, cholesterol of andere activators van LXRβ en PPARβ/δ in macrofagen te krijgen. Daarnaast is het beschreven dat liposomen zodanig kunnen worden aangepast zo dat ze kunnen fungeren als orale medicatie.

Naast de activatie van PPARβ/δ door liposomen kunnen bepaalde voedingsbestanddelen LXRs en PPARs activeren en zodoende mogelijk MS-symptomen verminderen. Zo is bijvoorbeeld beschreven dat onverzadigde vetzuren PPARs kunnen activeren. Groenten, zeevruchten en visolie bevatten deze onverzadigde vetzuren en een dieet rijk aan deze producten zou MS kunnen verlichten. In tegenstelling tot PPARs ligt activatie van LXRs door voeding minder voor de hand, omdat cholesterol-afgeleiden natuurlijke activators zijn voor LXRs. Deze cholesterol-afgeleiden worden in het lichaam aangemaakt na consumptie van voeding met een hoog cholesterolgehalte. Van een dieet met een hoog cholesterolgehalte is echter bekend dat het ontwikkeling van hart- en vaatziektes kan bevorderen door het verhogen van het triglyceridengehalte in het bloed. Daarnaast kan LXR-activatie de functie van de lever aantasten. Deze effecten worden toegeschreven aan de specifieke activatie van het LXRα-subtype. Dit suggereert dat medicaties die enkel LXRβ activeren kunnen worden aangewend om MS te bestrijden.
Samengevat, onze bevindingen wijzen erop dat activatie van LXRβ en PPARβ/δ in de toekomst mogelijk kan worden aangewend als interventiestrategie gericht op het terugdringen van ontstekingsreacties in MS.

Eindconclusie

In de meerderheid van MS-patiënten vindt er in the initiële fase nog herstel van symptomen plaats. Dit suggereert dat er natuurlijk voorkomende regulerende mechanismen aanwezig zijn tijdens MS die de ziekte onder controle houden en die mogelijk kunnen worden aangewend voor toekomstige behandelingen. Resultaten van dit proefschrift tonen aan dat macrofagen een beschermend fenotype aannemen na opname van myeline. Dit fenotype blijkt onder andere tot stand te komen door activatie van LXRs and PPARs door myeline-afgeleide lipiden. Door de vermenigvuldiging van autoreactieve T-cellen te remmen, zouden myeline-fagocyterende macrofagen de ontstekingslast in MS patiënten kunnen onderdrukken. Bovendien kan de verlaagde productie van ontstekingsmediatoren door myeline-fagocyterende macrofagen demyelinisatie en schade aan zenuwcellen tegengaan. Ontwikkeling van medicaties die de aanname van dit fenotype in macrofagen bevorderen zouden in toekomst

mogelijk kunnen worden aangewend om MS te bestrijden.

Reference List

1. Finger,S. A happy state of mind: a history of mild elation, denial of disability, optimism, and laughing in multiple sclerosis. *Arch. Neurol.* **55**, 241-250 (1998).
2. Charcot J.M. Histologie de la sclerose en plaque. 41, 554-566. 1868. Gazette des Hopitaux.
3. Humphries,C. Progressive multiple sclerosis: The treatment gap. *Nature* **484**, S10 (2012).
4. Noseworthy,J.H., Lucchinetti,C., Rodriguez,M., & Weinshenker,B.G. Multiple sclerosis. *N. Engl. J. Med.* **343**, 938-952 (2000).
5. Hellings,N., Raus,J., & Stinissen,P. Insights into the immunopathogenesis of multiple sclerosis. *Immunol. Res.* **25**, 27-51 (2002).
6. Pugliatti,M. *et al.* The epidemiology of multiple sclerosis in Europe. *Eur. J. Neurol.* **13**, 700-722 (2006).
7. Hafler,D.A. Multiple sclerosis. *J. Clin. Invest* **113**, 788-794 (2004).
8. Hemmer,B., Archelos,J.J., & Hartung,H.P. New concepts in the immunopathogenesis of multiple sclerosis. *Nat. Rev. Neurosci.* **3**, 291-301 (2002).
9. Pugliatti,M., Sotgiu,S., & Rosati,G. The worldwide prevalence of multiple sclerosis. *Clin. Neurol. Neurosurg.* **104**, 182-191 (2002).
10. Navikas,V. & Link,H. Review: cytokines and the pathogenesis of multiple sclerosis. *J. Neurosci. Res.* **45**, 322-333 (1996).
11. Goldenberg,M.M. Multiple sclerosis review. *P. T.* **37**, 175-184 (2012).
12. Lassmann,H., Bruck,W., & Lucchinetti,C.F. The immunopathology of multiple sclerosis: an overview. *Brain Pathol.* **17**, 210-218 (2007).
13. Lublin,F.D. & Reingold,S.C. Defining the clinical course of multiple sclerosis: results of an international survey. National Multiple Sclerosis Society (USA) Advisory Committee on Clinical Trials of New Agents in Multiple Sclerosis. *Neurology* **46**, 907-911 (1996).
14. McDonald,W.I. *et al.* Recommended diagnostic criteria for multiple sclerosis: guidelines from the International Panel on the diagnosis of multiple sclerosis. *Ann. Neurol.* **50**, 121-127 (2001).
15. Polman,C.H. *et al.* Diagnostic criteria for multiple sclerosis: 2005 revisions to the "McDonald Criteria". *Ann. Neurol.* **58**, 840-846 (2005).
16. Polman,C.H. *et al.* Diagnostic criteria for multiple sclerosis: 2010 revisions to the McDonald criteria. *Ann. Neurol.* **69**, 292-302 (2011).
17. Inglese,M., Grossman,R.I., & Filippi,M. Magnetic resonance imaging monitoring of multiple sclerosis lesion evolution. *J. Neuroimaging* **15**, 22S-29S (2005).
18. Awad,A. *et al.* Analyses of cerebrospinal fluid in the diagnosis and monitoring of multiple sclerosis. *J. Neuroimmunol.* **219**, 1-7 (2010).
19. Correale,J. & de los Milagros Bassani Molinas Oligoclonal bands and antibody responses in multiple sclerosis. *J. Neurol.* **249**, 375-389 (2002).
20. Bourahoui,A. *et al.* CSF isoelectrofocusing in a large cohort of MS and other neurological diseases. *Eur. J. Neurol.* **11**, 525-529 (2004).
21. Paolino,E. *et al.* A prospective study on the predictive value of CSF oligoclonal bands and MRI in acute isolated neurological syndromes for subsequent progression to multiple sclerosis. *J. Neurol. Neurosurg. Psychiatry* **60**, 572-575 (1996).
22. Miller,J.R., Burke,A.M., & Bever,C.T. Occurrence of oligoclonal bands in multiple sclerosis and other CNS diseases. *Ann. Neurol.* **13**, 53-58 (1983).
23. Palace,J. Making the diagnosis of multiple sclerosis. *J. Neurol. Neurosurg. Psychiatry* **71 Suppl 2**, ii3-ii8 (2001).
24. Furlan,R., Cuomo,C., & Martino,G. Animal models of multiple sclerosis. *Methods Mol. Biol.* **549**, 157-173 (2009).

25. Gold,R., Linington,C., & Lassmann,H. Understanding pathogenesis and therapy of multiple sclerosis via animal models: 70 years of merits and culprits in experimental autoimmune encephalomyelitis research. *Brain* **129**, 1953-1971 (2006).
26. Baxter,A.G. The origin and application of experimental autoimmune encephalomyelitis. *Nat. Rev. Immunol.* **7**, 904-912 (2007).
27. Rivers,T.M., Sprunt,D.H., & Berry,G.P. OBSERVATIONS ON ATTEMPTS TO PRODUCE ACUTE DISSEMINATED ENCEPHALOMYELITIS IN MONKEYS. *J. Exp. Med.* **58**, 39-53 (1933).
28. Lipton,H.L. & Jelachich,M.L. Molecular pathogenesis of Theiler's murine encephalomyelitis virus-induced demyelinating disease in mice. *Intervirology* **40**, 143-152 (1997).
29. Goverman,J. *et al.* Transgenic mice that express a myelin basic protein-specific T cell receptor develop spontaneous autoimmunity. *Cell* **72**, 551-560 (1993).
30. Sriram,S. & Steiner,I. Experimental allergic encephalomyelitis: a misleading model of multiple sclerosis. *Ann. Neurol.* **58**, 939-945 (2005).
31. Comabella,M. & Martin,R. Genomics in multiple sclerosis--current state and future directions. *J. Neuroimmunol.* **187**, 1-8 (2007).
32. Furlan,R. *et al.* Intrathecal delivery of IFN-gamma protects C57BL/6 mice from chronic-progressive experimental autoimmune encephalomyelitis by increasing apoptosis of central nervous system-infiltrating lymphocytes. *J. Immunol.* **167**, 1821-1829 (2001).
33. Panitch,H.S., Hirsch,R.L., Haley,A.S., & Johnson,K.P. Exacerbations of multiple sclerosis in patients treated with gamma interferon. *Lancet* **1**, 893-895 (1987).
34. Selmaj,K.W. & Raine,C.S. Experimental autoimmune encephalomyelitis: immunotherapy with anti-tumor necrosis factor antibodies and soluble tumor necrosis factor receptors. *Neurology* **45**, S44-S49 (1995).
35. TNF neutralization in MS: results of a randomized, placebo-controlled multicenter study. The Lenercept Multiple Sclerosis Study Group and The University of British Columbia MS/MRI Analysis Group. *Neurology* **53**, 457-465 (1999).
36. Steinman,L. & Zamvil,S.S. How to successfully apply animal studies in experimental allergic encephalomyelitis to research on multiple sclerosis. *Ann. Neurol.* **60**, 12-21 (2006).
37. Dyment,D.A., Ebers,G.C., & Sadovnick,A.D. Genetics of multiple sclerosis. *Lancet Neurol.* **3**, 104-110 (2004).
38. Sadovnick,A.D. & Macleod,P.M. The familial nature of multiple sclerosis: empiric recurrence risks for first, second-, and third-degree relatives of patients. *Neurology* **31**, 1039-1041 (1981).
39. Sadovnick,A.D., Ebers,G.C., Dyment,D.A., & Risch,N.J. Evidence for genetic basis of multiple sclerosis. The Canadian Collaborative Study Group. *Lancet* **347**, 1728-1730 (1996).
40. Jersild,C., Svejgaard,A., & Fog,T. HL-A antigens and multiple sclerosis. *Lancet* **1**, 1240-1241 (1972).
41. Olerup,O. & Hillert,J. HLA class II-associated genetic susceptibility in multiple sclerosis: a critical evaluation. *Tissue Antigens* **38**, 1-15 (1991).
42. Gourraud,P.A., Harbo,H.F., Hauser,S.L., & Baranzini,S.E. The genetics of multiple sclerosis: an up-to-date review. *Immunol. Rev.* **248**, 87-103 (2012).
43. Imrell,K., Greiner,E., Hillert,J., & Masterman,T. HLA-DRB115 and cerebrospinal-fluid-specific oligoclonal immunoglobulin G bands lower age at attainment of important disease milestones in multiple sclerosis. *J. Neuroimmunol.* **210**, 128-130 (2009).
44. Sawcer,S. *et al.* Genetic risk and a primary role for cell-mediated immune mechanisms in multiple sclerosis. *Nature* **476**, 214-219 (2011).
45. Hafler,D.A. *et al.* Risk alleles for multiple sclerosis identified by a genomewide study. *N. Engl. J. Med.* **357**, 851-862 (2007).

46. Patsopoulos,N.A. *et al.* Genome-wide meta-analysis identifies novel multiple sclerosis susceptibility loci. *Ann. Neurol.* **70**, 897-912 (2011).
47. Cree,B.A. *et al.* A major histocompatibility Class I locus contributes to multiple sclerosis susceptibility independently from HLA-DRB1*15:01. *PLoS One* **5**, e11296 (2010).
48. Baranzini,S.E. *et al.* Genome, epigenome and RNA sequences of monozygotic twins discordant for multiple sclerosis. *Nature* **464**, 1351-1356 (2010).
49. Marrie,R.A. Environmental risk factors in multiple sclerosis aetiology. *Lancet Neurol.* **3**, 709-718 (2004).
50. Gale,C.R. & Martyn,C.N. Migrant studies in multiple sclerosis. *Prog. Neurobiol.* **47**, 425-448 (1995).
51. Kurtzke,J.F. MS epidemiology world wide. One view of current status. *Acta Neurol. Scand. Suppl* **161**, 23-33 (1995).
52. Nieves,J., Cosman,F., Herbert,J., Shen,V., & Lindsay,R. High prevalence of vitamin D deficiency and reduced bone mass in multiple sclerosis. *Neurology* **44**, 1687-1692 (1994).
53. Mowry,E.M. *et al.* Vitamin D status is associated with relapse rate in pediatric-onset multiple sclerosis. *Ann. Neurol.* **67**, 618-624 (2010).
54. Mowry,E.M. *et al.* Vitamin D status predicts new brain magnetic resonance imaging activity in multiple sclerosis. *Ann. Neurol.* **72**, 234-240 (2012).
55. Simpson S Jr *et al.* Higher 25-hydroxyvitamin D is associated with lower relapse risk in multiple sclerosis. *Ann. Neurol.* **68**, 193-203 (2010).
56. Munger,K.L. *et al.* Vitamin D intake and incidence of multiple sclerosis. *Neurology* **62**, 60-65 (2004).
57. Cantorna,M.T. Vitamin D and its role in immunology: multiple sclerosis, and inflammatory bowel disease. *Prog. Biophys. Mol. Biol.* **92**, 60-64 (2006).
58. Milo,R. & Kahana,E. Multiple sclerosis: geoepidemiology, genetics and the environment. *Autoimmun. Rev.* **9**, A387-A394 (2010).
59. Willer,C.J. *et al.* Timing of birth and risk of multiple sclerosis: population based study. *BMJ* **330**, 120 (2005).
60. Fleming,J. & Fabry,Z. The hygiene hypothesis and multiple sclerosis. *Ann. Neurol.* **61**, 85-89 (2007).
61. Fleming,J.O. & Cook,T.D. Multiple sclerosis and the hygiene hypothesis. *Neurology* **67**, 2085-2086 (2006).
62. Riccio,P. The molecular basis of nutritional intervention in multiple sclerosis: a narrative review. *Complement Ther. Med.* **19**, 228-237 (2011).
63. Schwarz,S. & Leweling,H. Multiple sclerosis and nutrition. *Mult. Scler.* **11**, 24-32 (2005).
64. Piccio,L., Stark,J.L., & Cross,A.H. Chronic calorie restriction attenuates experimental autoimmune encephalomyelitis. *J. Leukoc. Biol.* **84**, 940-948 (2008).
65. Swank,R.L. & Goodwin,J. Review of MS patient survival on a Swank low saturated fat diet. *Nutrition* **19**, 161-162 (2003).
66. Correale,J., Fiol,M., & Gilmore,W. The risk of relapses in multiple sclerosis during systemic infections. *Neurology* **67**, 652-659 (2006).
67. Buljevac,D. *et al.* Prospective study on the relationship between infections and multiple sclerosis exacerbations. *Brain* **125**, 952-960 (2002).
68. Alotaibi,S., Kennedy,J., Tellier,R., Stephens,D., & Banwell,B. Epstein-Barr virus in pediatric multiple sclerosis. *JAMA* **291**, 1875-1879 (2004).
69. Pohl,D. *et al.* High seroprevalence of Epstein-Barr virus in children with multiple sclerosis. *Neurology* **67**, 2063-2065 (2006).
70. Lunemann,J.D. *et al.* Broadened and elevated humoral immune response to EBNA1 in pediatric multiple sclerosis. *Neurology* **71**, 1033-1035 (2008).
71. Levin,L.I., Munger,K.L., O'Reilly,E.J., Falk,K.I., & Ascherio,A. Primary infection with the Epstein-Barr virus and risk of multiple sclerosis. *Ann. Neurol.* **67**, 824-830 (2010).

72. Lindberg,C., Andersen,O., Vahlne,A., Dalton,M., & Runmarker,B. Epidemiological investigation of the association between infectious mononucleosis and multiple sclerosis. *Neuroepidemiology* **10**, 62-65 (1991).
73. Thacker,E.L., Mirzaei,F., & Ascherio,A. Infectious mononucleosis and risk for multiple sclerosis: a meta-analysis. *Ann. Neurol.* **59**, 499-503 (2006).
74. DeLorenze,G.N. *et al.* Epstein-Barr virus and multiple sclerosis: evidence of association from a prospective study with long-term follow-up. *Arch. Neurol.* **63**, 839-844 (2006).
75. Levin,L.I. *et al.* Temporal relationship between elevation of epstein-barr virus antibody titers and initial onset of neurological symptoms in multiple sclerosis. *JAMA* **293**, 2496-2500 (2005).
76. Serafini,B. *et al.* Dysregulated Epstein-Barr virus infection in the multiple sclerosis brain. *J. Exp. Med.* **204**, 2899-2912 (2007).
77. Lunemann,J.D. *et al.* Increased frequency and broadened specificity of latent EBV nuclear antigen-1-specific T cells in multiple sclerosis. *Brain* **129**, 1493-1506 (2006).
78. Lunemann,J.D. *et al.* EBNA1-specific T cells from patients with multiple sclerosis cross react with myelin antigens and co-produce IFN-gamma and IL-2. *J. Exp. Med.* **205**, 1763-1773 (2008).
79. Kakalacheva,K., Munz,C., & Lunemann,J.D. Viral triggers of multiple sclerosis. *Biochim. Biophys. Acta* **1812**, 132-140 (2011).
80. Libbey,J.E. & Fujinami,R.S. Potential triggers of MS. *Results Probl. Cell Differ.* **51**, 21-42 (2010).
81. Bjartmar,C., Wujek,J.R., & Trapp,B.D. Axonal loss in the pathology of MS: consequences for understanding the progressive phase of the disease. *J. Neurol. Sci.* **206**, 165-171 (2003).
82. Dutta,R. & Trapp,B.D. Mechanisms of neuronal dysfunction and degeneration in multiple sclerosis. *Prog. Neurobiol.* **93**, 1-12 (2011).
83. Dutta,R. & Trapp,B.D. Pathogenesis of axonal and neuronal damage in multiple sclerosis. *Neurology* **68**, S22-S31 (2007).
84. Jingwu,Z. *et al.* Myelin basic protein-specific T lymphocytes in multiple sclerosis and controls: precursor frequency, fine specificity, and cytotoxicity. *Ann. Neurol.* **32**, 330-338 (1992).
85. Van der Aa,A., Hellings,N., Bernard,C.C., Raus,J., & Stinissen,P. Functional properties of myelin oligodendrocyte glycoprotein-reactive T cells in multiple sclerosis patients and controls. *J. Neuroimmunol.* **137**, 164-176 (2003).
86. Markovic-Plese,S., Cortese,I., Wandinger,K.P., McFarland,H.F., & Martin,R. CD4+. *J. Clin. Invest* **108**, 1185-1194 (2001).
87. Bielekova,B. *et al.* Expansion and functional relevance of high-avidity myelin-specific CD4+ T cells in multiple sclerosis. *J. Immunol.* **172**, 3893-3904 (2004).
88. Scholz,C., Patton,K.T., Anderson,D.E., Freeman,G.J., & Hafler,D.A. Expansion of autoreactive T cells in multiple sclerosis is independent of exogenous B7 costimulation. *J. Immunol.* **160**, 1532-1538 (1998).
89. Zhang,J. *et al.* Increased frequency of interleukin 2-responsive T cells specific for myelin basic protein and proteolipid protein in peripheral blood and cerebrospinal fluid of patients with multiple sclerosis. *J. Exp. Med.* **179**, 973-984 (1994).
90. Trapp,B.D. & Nave,K.A. Multiple sclerosis: an immune or neurodegenerative disorder? *Annu. Rev. Neurosci.* **31**, 247-269 (2008).
91. Stys,P.K., Zamponi,G.W., van,M.J., & Geurts,J.J. Will the real multiple sclerosis please stand up? *Nat. Rev. Neurosci.* **13**, 507-514 (2012).
92. Hellings,N., Raus,J., & Stinissen,P. Insights into the immunopathogenesis of multiple sclerosis. *Immunol. Res.* **25**, 27-51 (2002).
93. McFarland,H.F. & Martin,R. Multiple sclerosis: a complicated picture of autoimmunity. *Nat. Immunol.* **8**, 913-919 (2007).
94. Goverman,J. Autoimmune T cell responses in the central nervous system. *Nat. Rev. Immunol.* **9**, 393-407 (2009).

95. Becher,B., Bechmann,I., & Greter,M. Antigen presentation in autoimmunity and CNS inflammation: how T lymphocytes recognize the brain. *J. Mol. Med. (Berl)* **84**, 532-543 (2006).
96. Greter,M. *et al.* Dendritic cells permit immune invasion of the CNS in an animal model of multiple sclerosis. *Nat. Med.* **11**, 328-334 (2005).
97. McMahon,E.J., Bailey,S.L., Castenada,C.V., Waldner,H., & Miller,S.D. Epitope spreading initiates in the CNS in two mouse models of multiple sclerosis. *Nat. Med.* **11**, 335-339 (2005).
98. Katz-Levy,Y. *et al.* Endogenous presentation of self myelin epitopes by CNS-resident APCs in Theiler's virus-infected mice. *J. Clin. Invest* **104**, 599-610 (1999).
99. Kaushansky,N., Eisenstein,M., Zilkha-Falb,R., & Ben-Nun,A. The myelin-associated oligodendrocytic basic protein (MOBP) as a relevant primary target autoantigen in multiple sclerosis. *Autoimmun. Rev.* **9**, 233-236 (2010).
100. Grau-Lopez,L. *et al.* Myelin peptides in multiple sclerosis. *Autoimmun. Rev.* **8**, 650-653 (2009).
101. Weissert,R. *et al.* High immunogenicity of intracellular myelin oligodendrocyte glycoprotein epitopes. *J. Immunol.* **169**, 548-556 (2002).
102. Huseby,E.S., Huseby,P.G., Shah,S., Smith,R., & Stadinski,B.D. Pathogenic CD8 T cells in multiple sclerosis and its experimental models. *Front Immunol.* **3**, 64 (2012).
103. Krakowski,M. & Owens,T. Interferon-gamma confers resistance to experimental allergic encephalomyelitis. *Eur. J. Immunol.* **26**, 1641-1646 (1996).
104. Okuda,Y. *et al.* IL-6-deficient mice are resistant to the induction of experimental autoimmune encephalomyelitis provoked by myelin oligodendrocyte glycoprotein. *Int. Immunol.* **10**, 703-708 (1998).
105. Sean,R.D. *et al.* Challenging cytokine redundancy: inflammatory cell movement and clinical course of experimental autoimmune encephalomyelitis are normal in lymphotoxin-deficient, but not tumor necrosis factor-deficient, mice. *J. Exp. Med.* **187**, 1517-1528 (1998).
106. Cua,D.J. *et al.* Interleukin-23 rather than interleukin-12 is the critical cytokine for autoimmune inflammation of the brain. *Nature* **421**, 744-748 (2003).
107. Deshpande,P., King,I.L., & Segal,B.M. IL-12 driven upregulation of P-selectin ligand on myelin-specific T cells is a critical step in an animal model of autoimmune demyelination. *J. Neuroimmunol.* **173**, 35-44 (2006).
108. Leonard,J.P., Waldburger,K.E., & Goldman,S.J. Prevention of experimental autoimmune encephalomyelitis by antibodies against interleukin 12. *J. Exp. Med.* **181**, 381-386 (1995).
109. Ichikawa,M. *et al.* Anti-IL-12 antibody prevents the development and progression of multiple sclerosis-like relapsing--remitting demyelinating disease in NOD mice induced with myelin oligodendrocyte glycoprotein peptide. *J. Neuroimmunol.* **102**, 56-66 (2000).
110. Constantinescu,C.S. *et al.* Antibodies against IL-12 prevent superantigen-induced and spontaneous relapses of experimental autoimmune encephalomyelitis. *J. Immunol.* **161**, 5097-5104 (1998).
111. Segal,B.M., Dwyer,B.K., & Shevach,E.M. An interleukin (IL)-10/IL-12 immunoregulatory circuit controls susceptibility to autoimmune disease. *J. Exp. Med.* **187**, 537-546 (1998).
112. Pelfrey,C.M. *et al.* Quantification of self-recognition in multiple sclerosis by single-cell analysis of cytokine production. *J. Immunol.* **165**, 1641-1651 (2000).
113. Merrill,J.E. Proinflammatory and antiinflammatory cytokines in multiple sclerosis and central nervous system acquired immunodeficiency syndrome. *J. Immunother. (1991.)* **12**, 167-170 (1992).
114. Ferber,I.A. *et al.* Mice with a disrupted IFN-gamma gene are susceptible to the induction of experimental autoimmune encephalomyelitis (EAE). *J. Immunol.* **156**, 5-7 (1996).

115. Becher,B., Durell,B.G., & Noelle,R.J. Experimental autoimmune encephalitis and inflammation in the absence of interleukin-12. *J. Clin. Invest* **110**, 493-497 (2002).
116. Harrington,L.E. *et al.* Interleukin 17-producing CD4+ effector T cells develop via a lineage distinct from the T helper type 1 and 2 lineages. *Nat. Immunol.* **6**, 1123-1132 (2005).
117. Langrish,C.L. *et al.* IL-23 drives a pathogenic T cell population that induces autoimmune inflammation. *J. Exp. Med.* **201**, 233-240 (2005).
118. Matusevicius,D. *et al.* Interleukin-17 mRNA expression in blood and CSF mononuclear cells is augmented in multiple sclerosis. *Mult. Scler.* **5**, 101-104 (1999).
119. Lock,C. *et al.* Gene-microarray analysis of multiple sclerosis lesions yields new targets validated in autoimmune encephalomyelitis. *Nat. Med.* **8**, 500-508 (2002).
120. Hofstetter,H.H. *et al.* Therapeutic efficacy of IL-17 neutralization in murine experimental autoimmune encephalomyelitis. *Cell Immunol.* **237**, 123-130 (2005).
121. Gold,R. & Luhder,F. Interleukin-17--extended features of a key player in multiple sclerosis. *Am. J. Pathol.* **172**, 8-10 (2008).
122. Komiyama,Y. *et al.* IL-17 plays an important role in the development of experimental autoimmune encephalomyelitis. *J. Immunol.* **177**, 566-573 (2006).
123. Kroenke,M.A. & Segal,B.M. IL-23 modulated myelin-specific T cells induce EAE via an IFNgamma driven, IL-17 independent pathway. *Brain Behav. Immun.* **25**, 932-937 (2011).
124. Kebir,H. *et al.* Human TH17 lymphocytes promote blood-brain barrier disruption and central nervous system inflammation. *Nat. Med.* **13**, 1173-1175 (2007).
125. Waite,J.C. & Skokos,D. Th17 response and inflammatory autoimmune diseases. *Int. J. Inflam.* **2012**, 819467 (2012).
126. O'Connor,R.A. *et al.* Cutting edge: Th1 cells facilitate the entry of Th17 cells to the central nervous system during experimental autoimmune encephalomyelitis. *J. Immunol.* **181**, 3750-3754 (2008).
127. Stromnes,I.M., Cerretti,L.M., Liggitt,D., Harris,R.A., & Goverman,J.M. Differential regulation of central nervous system autoimmunity by T(H)1 and T(H)17 cells. *Nat. Med.* **14**, 337-342 (2008).
128. Yang,Y. *et al.* T-bet is essential for encephalitogenicity of both Th1 and Th17 cells. *J. Exp. Med.* **206**, 1549-1564 (2009).
129. Kroenke,M.A., Carlson,T.J., Andjelkovic,A.V., & Segal,B.M. IL-12- and IL-23-modulated T cells induce distinct types of EAE based on histology, CNS chemokine profile, and response to cytokine inhibition. *J. Exp. Med.* **205**, 1535-1541 (2008).
130. Pollinger,B. *et al.* Spontaneous relapsing-remitting EAE in the SJL/J mouse: MOG-reactive transgenic T cells recruit endogenous MOG-specific B cells. *J. Exp. Med.* **206**, 1303-1316 (2009).
131. Haak,S. *et al.* IL-17A and IL-17F do not contribute vitally to autoimmune neuro-inflammation in mice. *J. Clin. Invest* **119**, 61-69 (2009).
132. Babbe,H. *et al.* Clonal expansions of CD8(+) T cells dominate the T cell infiltrate in active multiple sclerosis lesions as shown by micromanipulation and single cell polymerase chain reaction. *J. Exp. Med.* **192**, 393-404 (2000).
133. Serafini,B. *et al.* Dendritic cells in multiple sclerosis lesions: maturation stage, myelin uptake, and interaction with proliferating T cells. *J. Neuropathol. Exp. Neurol.* **65**, 124-141 (2006).
134. Neumann,H., Medana,I.M., Bauer,J., & Lassmann,H. Cytotoxic T lymphocytes in autoimmune and degenerative CNS diseases. *Trends Neurosci.* **25**, 313-319 (2002).

135. Wong,G.H., Bartlett,P.F., Clark-Lewis,I., Battye,F., & Schrader,J.W. Inducible expression of H-2 and Ia antigens on brain cells. *Nature* **310**, 688-691 (1984).
136. Neumann,H., Schmidt,H., Cavalie,A., Jenne,D., & Wekerle,H. Major histocompatibility complex (MHC) class I gene expression in single neurons of the central nervous system: differential regulation by interferon (IFN)-gamma and tumor necrosis factor (TNF)-alpha. *J. Exp. Med.* **185**, 305-316 (1997).
137. Neumann,H., Cavalie,A., Jenne,D.E., & Wekerle,H. Induction of MHC class I genes in neurons. *Science* **269**, 549-552 (1995).
138. Junker,A. *et al.* Multiple sclerosis: T-cell receptor expression in distinct brain regions. *Brain* **130**, 2789-2799 (2007).
139. Jacobsen,M. *et al.* Oligoclonal expansion of memory CD8+ T cells in cerebrospinal fluid from multiple sclerosis patients. *Brain* **125**, 538-550 (2002).
140. Bitsch,A., Schuchardt,J., Bunkowski,S., Kuhlmann,T., & Bruck,W. Acute axonal injury in multiple sclerosis. Correlation with demyelination and inflammation. *Brain* **123 (Pt 6)**, 1174-1183 (2000).
141. Huseby,E.S. *et al.* A pathogenic role for myelin-specific CD8(+) T cells in a model for multiple sclerosis. *J. Exp. Med.* **194**, 669-676 (2001).
142. Sun,D. *et al.* Myelin antigen-specific CD8+ T cells are encephalitogenic and produce severe disease in C57BL/6 mice. *J. Immunol.* **166**, 7579-7587 (2001).
143. Ford,M.L. & Evavold,B.D. Specificity, magnitude, and kinetics of MOG-specific CD8+ T cell responses during experimental autoimmune encephalomyelitis. *Eur. J. Immunol.* **35**, 76-85 (2005).
144. Brisebois,M., Zehntner,S.P., Estrada,J., Owens,T., & Fournier,S. A pathogenic role for CD8+ T cells in a spontaneous model of demyelinating disease. *J. Immunol.* **177**, 2403-2411 (2006).
145. Ji,Q., Perchellet,A., & Goverman,J.M. Viral infection triggers central nervous system autoimmunity via activation of CD8+ T cells expressing dual TCRs. *Nat. Immunol.* **11**, 628-634 (2010).
146. Olsson,J.E. & Link,H. Immunoglobulin abnormalities in multiple sclerosis. Relation to clinical parameters: exacerbations and remissions. *Arch. Neurol.* **28**, 392-399 (1973).
147. Avasarala,J.R., Cross,A.H., & Trotter,J.L. Oligoclonal band number as a marker for prognosis in multiple sclerosis. *Arch. Neurol.* **58**, 2044-2045 (2001).
148. Genain,C.P., Cannella,B., Hauser,S.L., & Raine,C.S. Identification of autoantibodies associated with myelin damage in multiple sclerosis. *Nat. Med.* **5**, 170-175 (1999).
149. Lucchinetti,C.F., Bruck,W., Rodriguez,M., & Lassmann,H. Distinct patterns of multiple sclerosis pathology indicates heterogeneity on pathogenesis. *Brain Pathol.* **6**, 259-274 (1996).
150. Baranzini,S.E. *et al.* B cell repertoire diversity and clonal expansion in multiple sclerosis brain lesions. *J. Immunol.* **163**, 5133-5144 (1999).
151. Colombo,M. *et al.* Accumulation of clonally related B lymphocytes in the cerebrospinal fluid of multiple sclerosis patients. *J. Immunol.* **164**, 2782-2789 (2000).
152. Qin,Y. *et al.* Clonal expansion and somatic hypermutation of V(H) genes of B cells from cerebrospinal fluid in multiple sclerosis. *J. Clin. Invest* **102**, 1045-1050 (1998).
153. Magliozzi,R. *et al.* Meningeal B-cell follicles in secondary progressive multiple sclerosis associate with early onset of disease and severe cortical pathology. *Brain* **130**, 1089-1104 (2007).
154. Serafini,B., Rosicarelli,B., Magliozzi,R., Stigliano,E., & Aloisi,F. Detection of ectopic B-cell follicles with germinal centers in the meninges of patients with secondary progressive multiple sclerosis. *Brain Pathol.* **14**, 164-174 (2004).
155. Hauser,S.L. *et al.* B-cell depletion with rituximab in relapsing-remitting multiple sclerosis. *N. Engl. J. Med.* **358**, 676-688 (2008).

156. Viglietta,V., Baecher-Allan,C., Weiner,H.L., & Hafler,D.A. Loss of functional suppression by CD4+CD25+ regulatory T cells in patients with multiple sclerosis. *J. Exp. Med.* **199**, 971-979 (2004).
157. Haas,J. *et al.* Reduced suppressive effect of CD4+CD25high regulatory T cells on the T cell immune response against myelin oligodendrocyte glycoprotein in patients with multiple sclerosis. *Eur. J. Immunol.* **35**, 3343-3352 (2005).
158. Venken,K. *et al.* Secondary progressive in contrast to relapsing-remitting multiple sclerosis patients show a normal CD4+CD25+ regulatory T-cell function and FOXP3 expression. *J. Neurosci. Res.* **83**, 1432-1446 (2006).
159. Huan,J. *et al.* Decreased FOXP3 levels in multiple sclerosis patients. *J. Neurosci. Res.* **81**, 45-52 (2005).
160. Venken,K. *et al.* Compromised CD4+ CD25(high) regulatory T-cell function in patients with relapsing-remitting multiple sclerosis is correlated with a reduced frequency of FOXP3-positive cells and reduced FOXP3 expression at the single-cell level. *Immunology* **123**, 79-89 (2008).
161. Zhang,X. *et al.* IL-10 is involved in the suppression of experimental autoimmune encephalomyelitis by CD25+CD4+ regulatory T cells. *Int. Immunol.* **16**, 249-256 (2004).
162. Kohm,A.P., Carpentier,P.A., Anger,H.A., & Miller,S.D. Cutting edge: CD4+CD25+ regulatory T cells suppress antigen-specific autoreactive immune responses and central nervous system inflammation during active experimental autoimmune encephalomyelitis. *J. Immunol.* **169**, 4712-4716 (2002).
163. Antel,J.P., Bania,M.B., Reder,A., & Cashman,N. Activated suppressor cell dysfunction in progressive multiple sclerosis. *J. Immunol.* **137**, 137-141 (1986).
164. Killestein,J. *et al.* Cytokine producing CD8+ T cells are correlated to MRI features of tissue destruction in MS. *J. Neuroimmunol.* **142**, 141-148 (2003).
165. Tennakoon,D.K. *et al.* Therapeutic induction of regulatory, cytotoxic CD8+ T cells in multiple sclerosis. *J. Immunol.* **176**, 7119-7129 (2006).
166. Hemmer,B., Nessler,S., Zhou,D., Kieseier,B., & Hartung,H.P. Immunopathogenesis and immunotherapy of multiple sclerosis. *Nat. Clin. Pract. Neurol.* **2**, 201-211 (2006).
167. Trapp,B.D. *et al.* Axonal transection in the lesions of multiple sclerosis. *N. Engl. J. Med.* **338**, 278-285 (1998).
168. Ferguson,B., Matyszak,M.K., Esiri,M.M., & Perry,V.H. Axonal damage in acute multiple sclerosis lesions. *Brain* **120 (Pt 3)**, 393-399 (1997).
169. Kornek,B. *et al.* Multiple sclerosis and chronic autoimmune encephalomyelitis: a comparative quantitative study of axonal injury in active, inactive, and remyelinated lesions. *Am. J. Pathol.* **157**, 267-276 (2000).
170. Popescu,B.F. & Lucchinetti,C.F. Pathology of demyelinating diseases. *Annu. Rev. Pathol.* **7**, 185-217 (2012).
171. Lassmann,H., van,H.J., & Mahad,D. Progressive multiple sclerosis: pathology and pathogenesis. *Nat. Rev. Neurol.* **8**, 647-656 (2012).
172. Frischer,J.M. *et al.* The relation between inflammation and neurodegeneration in multiple sclerosis brains. *Brain* **132**, 1175-1189 (2009).
173. Kutzelnigg,A. *et al.* Cortical demyelination and diffuse white matter injury in multiple sclerosis. *Brain* **128**, 2705-2712 (2005).
174. Lassmann,H. Review: the architecture of inflammatory demyelinating lesions: implications for studies on pathogenesis. *Neuropathol. Appl. Neurobiol.* **37**, 698-710 (2011).
175. Prineas,J.W. *et al.* Multiple sclerosis. Oligodendrocyte proliferation and differentiation in fresh lesions. *Lab Invest* **61**, 489-503 (1989).
176. Raine,C.S., Scheinberg,L., & Waltz,J.M. Multiple sclerosis. Oligodendrocyte survival and proliferation in an active established lesion. *Lab Invest* **45**, 534-546 (1981).
177. Prineas,J.W. *et al.* Multiple sclerosis. Pathology of recurrent lesions. *Brain* **116 (Pt 3)**, 681-693 (1993).

178. Bramow,S. *et al.* Demyelination versus remyelination in progressive multiple sclerosis. *Brain* **133**, 2983-2998 (2010).
179. Bruck,W. *et al.* Monocyte/macrophage differentiation in early multiple sclerosis lesions. *Ann. Neurol.* **38**, 788-796 (1995).
180. Bruck,W. *et al.* Oligodendrocytes in the early course of multiple sclerosis. *Ann. Neurol.* **35**, 65-73 (1994).
181. van der Goes,A. *et al.* Determination of the sequential degradation of myelin proteins by macrophages. *J. Neuroimmunol.* **161**, 12-20 (2005).
182. Kappos,L. *et al.* Predictive value of gadolinium-enhanced magnetic resonance imaging for relapse rate and changes in disability or impairment in multiple sclerosis: a meta-analysis. Gadolinium MRI Meta-analysis Group. *Lancet* **353**, 964-969 (1999).
183. Ingle,G.T., Sastre-Garriga,J., Miller,D.H., & Thompson,A.J. Is inflammation important in early PPMS? a longitudinal MRI study. *J. Neurol. Neurosurg. Psychiatry* **76**, 1255-1258 (2005).
184. Mews,I., Bergmann,M., Bunkowski,S., Gullotta,F., & Bruck,W. Oligodendrocyte and axon pathology in clinically silent multiple sclerosis lesions. *Mult. Scler.* **4**, 55-62 (1998).
185. Kornek,B. *et al.* Multiple sclerosis and chronic autoimmune encephalomyelitis: a comparative quantitative study of axonal injury in active, inactive, and remyelinated lesions. *Am. J. Pathol.* **157**, 267-276 (2000).
186. Prineas,J.W., Barnard,R.O., Kwon,E.E., Sharer,L.R., & Cho,E.S. Multiple sclerosis: remyelination of nascent lesions. *Ann. Neurol.* **33**, 137-151 (1993).
187. Raine,C.S. & Wu,E. Multiple sclerosis: remyelination in acute lesions. *J. Neuropathol. Exp. Neurol.* **52**, 199-204 (1993).
188. Lassmann,H., Bruck,W., Lucchinetti,C., & Rodriguez,M. Remyelination in multiple sclerosis. *Mult. Scler.* **3**, 133-136 (1997).
189. Patrikios,P. *et al.* Remyelination is extensive in a subset of multiple sclerosis patients. *Brain* **129**, 3165-3172 (2006).
190. Keirstead,H.S. & Blakemore,W.F. The role of oligodendrocytes and oligodendrocyte progenitors in CNS remyelination. *Adv. Exp. Med. Biol.* **468**, 183-197 (1999).
191. Lucchinetti,C.F., Noseworthy,J.H., & Rodriguez,M. Promotion of endogenous remyelination in multiple sclerosis. *Mult. Scler.* **3**, 71-75 (1997).
192. Franklin,R.J. & Ffrench-Constant,C. Remyelination in the CNS: from biology to therapy. *Nat. Rev. Neurosci.* **9**, 839-855 (2008).
193. Chari,D.M., Crang,A.J., & Blakemore,W.F. Decline in rate of colonization of oligodendrocyte progenitor cell (OPC)-depleted tissue by adult OPCs with age. *J. Neuropathol. Exp. Neurol.* **62**, 908-916 (2003).
194. Shields,S.A., Gilson,J.M., Blakemore,W.F., & Franklin,R.J. Remyelination occurs as extensively but more slowly in old rats compared to young rats following gliotoxin-induced CNS demyelination. *Glia* **28**, 77-83 (1999).
195. Ruckh,J.M. *et al.* Rejuvenation of regeneration in the aging central nervous system. *Cell Stem Cell* **10**, 96-103 (2012).
196. Li,W.W., Penderis,J., Zhao,C., Schumacher,M., & Franklin,R.J. Females remyelinate more efficiently than males following demyelination in the aged but not young adult CNS. *Exp. Neurol.* **202**, 250-254 (2006).
197. Bieber,A.J., Ure,D.R., & Rodriguez,M. Genetically dominant spinal cord repair in a murine model of chronic progressive multiple sclerosis. *J. Neuropathol. Exp. Neurol.* **64**, 46-57 (2005).
198. Mason,J.L. *et al.* Oligodendrocytes and progenitors become progressively depleted within chronically demyelinated lesions. *Am. J. Pathol.* **164**, 1673-1682 (2004).
199. Williams,A. *et al.* Semaphorin 3A and 3F: key players in myelin repair in multiple sclerosis? *Brain* **130**, 2554-2565 (2007).
200. Kuhlmann,T. *et al.* Differentiation block of oligodendroglial progenitor cells as a cause for remyelination failure in chronic multiple sclerosis. *Brain* **131**, 1749-1758 (2008).

201. Wolswijk,G. Chronic stage multiple sclerosis lesions contain a relatively quiescent population of oligodendrocyte precursor cells. *J. Neurosci.* **18**, 601-609 (1998).
202. Peterson,J.W., Bo,L., Mork,S., Chang,A., & Trapp,B.D. Transected neurites, apoptotic neurons, and reduced inflammation in cortical multiple sclerosis lesions. *Ann. Neurol.* **50**, 389-400 (2001).
203. Cifelli,A. *et al.* Thalamic neurodegeneration in multiple sclerosis. *Ann. Neurol.* **52**, 650-653 (2002).
204. Bo,L., Vedeler,C.A., Nyland,H.I., Trapp,B.D., & Mork,S.J. Subpial demyelination in the cerebral cortex of multiple sclerosis patients. *J. Neuropathol. Exp. Neurol.* **62**, 723-732 (2003).
205. Albert,M., Antel,J., Bruck,W., & Stadelmann,C. Extensive cortical remyelination in patients with chronic multiple sclerosis. *Brain Pathol.* **17**, 129-138 (2007).
206. Wegner,C., Esiri,M.M., Chance,S.A., Palace,J., & Matthews,P.M. Neocortical neuronal, synaptic, and glial loss in multiple sclerosis. *Neurology* **67**, 960-967 (2006).
207. Bo,L., Vedeler,C.A., Nyland,H., Trapp,B.D., & Mork,S.J. Intracortical multiple sclerosis lesions are not associated with increased lymphocyte infiltration. *Mult. Scler.* **9**, 323-331 (2003).
208. De,S.N. *et al.* Evidence of early cortical atrophy in MS: relevance to white matter changes and disability. *Neurology* **60**, 1157-1162 (2003).
209. Kutzelnigg,A. *et al.* Widespread demyelination in the cerebellar cortex in multiple sclerosis. *Brain Pathol.* **17**, 38-44 (2007).
210. Bo,L., Geurts,J.J., van,d., V, Polman,C., & Barkhof,F. Lack of correlation between cortical demyelination and white matter pathologic changes in multiple sclerosis. *Arch. Neurol.* **64**, 76-80 (2007).
211. Howell,O.W. *et al.* Meningeal inflammation is widespread and linked to cortical pathology in multiple sclerosis. *Brain* **134**, 2755-2771 (2011).
212. Vercellino,M. *et al.* Grey matter pathology in multiple sclerosis. *J. Neuropathol. Exp. Neurol.* **64**, 1101-1107 (2005).
213. Calabrese,M. & Gallo,P. Magnetic resonance evidence of cortical onset of multiple sclerosis. *Mult. Scler.* **15**, 933-941 (2009).
214. Calabrese,M. *et al.* Detection of cortical inflammatory lesions by double inversion recovery magnetic resonance imaging in patients with multiple sclerosis. *Arch. Neurol.* **64**, 1416-1422 (2007).
215. Calabrese,M. *et al.* Cortical lesions and atrophy associated with cognitive impairment in relapsing-remitting multiple sclerosis. *Arch. Neurol.* **66**, 1144-1150 (2009).
216. Calabrese,M. *et al.* Cortical pathology in multiple sclerosis patients with epilepsy: a 3 year longitudinal study. *J. Neurol. Neurosurg. Psychiatry* **83**, 49-54 (2012).
217. Lucchinetti,C.F. *et al.* Inflammatory cortical demyelination in early multiple sclerosis. *N. Engl. J. Med.* **365**, 2188-2197 (2011).
218. Popescu,B.F., Bunyan,R.F., Parisi,J.E., Ransohoff,R.M., & Lucchinetti,C.F. A case of multiple sclerosis presenting with inflammatory cortical demyelination. *Neurology* **76**, 1705-1710 (2011).
219. Albert,M., Antel,J., Bruck,W., & Stadelmann,C. Extensive cortical remyelination in patients with chronic multiple sclerosis. *Brain Pathol.* **17**, 129-138 (2007).
220. Merkler,D., Ernsting,T., Kerschensteiner,M., Bruck,W., & Stadelmann,C. A new focal EAE model of cortical demyelination: multiple sclerosis-like lesions with rapid resolution of inflammation and extensive remyelination. *Brain* **129**, 1972-1983 (2006).
221. Lopez-Diego,R.S. & Weiner,H.L. Novel therapeutic strategies for multiple sclerosis--a multifaceted adversary. *Nat. Rev. Drug Discov.* **7**, 909-925 (2008).

222. Kieseier,B.C. The mechanism of action of interferon-beta in relapsing multiple sclerosis. *CNS. Drugs* **25**, 491-502 (2011).
223. Interferon beta-1b is effective in relapsing-remitting multiple sclerosis. I. Clinical results of a multicenter, randomized, double-blind, placebo-controlled trial. The IFNB Multiple Sclerosis Study Group. *Neurology* **43**, 655-661 (1993).
224. Jacobs,L.D. *et al.* Intramuscular interferon beta-1a for disease progression in relapsing multiple sclerosis. The Multiple Sclerosis Collaborative Research Group (MSCRG). *Ann. Neurol.* **39**, 285-294 (1996).
225. Randomised double-blind placebo-controlled study of interferon beta-1a in relapsing/remitting multiple sclerosis. PRISMS (Prevention of Relapses and Disability by Interferon beta-1a Subcutaneously in Multiple Sclerosis) Study Group. *Lancet* **352**, 1498-1504 (1998).
226. Polman,C.H. *et al.* Recommendations for clinical use of data on neutralising antibodies to interferon-beta therapy in multiple sclerosis. *Lancet Neurol.* **9**, 740-750 (2010).
227. Weber,M.S. *et al.* Type II monocytes modulate T cell-mediated central nervous system autoimmune disease. *Nat. Med.* **13**, 935-943 (2007).
228. Aharoni,R., Teitelbaum,D., Sela,M., & Arnon,R. Copolymer 1 induces T cells of the T helper type 2 that crossreact with myelin basic protein and suppress experimental autoimmune encephalomyelitis. *Proc. Natl. Acad. Sci. U. S. A* **94**, 10821-10826 (1997).
229. Lalive,P.H. *et al.* Glatiramer acetate in the treatment of multiple sclerosis: emerging concepts regarding its mechanism of action. *CNS. Drugs* **25**, 401-414 (2011).
230. Castro-Borrero,W. *et al.* Current and emerging therapies in multiple sclerosis: a systematic review. *Ther. Adv. Neurol. Disord.* **5**, 205-220 (2012).
231. Filippi,M. *et al.* Glatiramer acetate reduces the proportion of new MS lesions evolving into "black holes". *Neurology* **57**, 731-733 (2001).
232. Johnson,K.P. *et al.* Extended use of glatiramer acetate (Copaxone) is well tolerated and maintains its clinical effect on multiple sclerosis relapse rate and degree of disability. Copolymer 1 Multiple Sclerosis Study Group. *Neurology* **50**, 701-708 (1998).
233. Ford,C. *et al.* Continuous long-term immunomodulatory therapy in relapsing multiple sclerosis: results from the 15-year analysis of the US prospective open-label study of glatiramer acetate. *Mult. Scler.* **16**, 342-350 (2010).
234. Martinelli,V., Radaelli,M., Straffi,L., Rodegher,M., & Comi,G. Mitoxantrone: benefits and risks in multiple sclerosis patients. *Neurol. Sci.* **30 Suppl 2**, S167-S170 (2009).
235. Goodin,D.S., Arnason,B.G., Coyle,P.K., Frohman,E.M., & Paty,D.W. The use of mitoxantrone (Novantrone) for the treatment of multiple sclerosis: report of the Therapeutics and Technology Assessment Subcommittee of the American Academy of Neurology. *Neurology* **61**, 1332-1338 (2003).
236. Hartung,H.P. *et al.* Mitoxantrone in progressive multiple sclerosis: a placebo-controlled, double-blind, randomised, multicentre trial. *Lancet* **360**, 2018-2025 (2002).
237. Marriott,J.J., Miyasaki,J.M., Gronseth,G., & O'Connor,P.W. Evidence Report: The efficacy and safety of mitoxantrone (Novantrone) in the treatment of multiple sclerosis: Report of the Therapeutics and Technology Assessment Subcommittee of the American Academy of Neurology. *Neurology* **74**, 1463-1470 (2010).
238. Polman,C.H. *et al.* A randomized, placebo-controlled trial of natalizumab for relapsing multiple sclerosis. *N. Engl. J. Med.* **354**, 899-910 (2006).
239. Berger,J.R. Progressive multifocal leukoencephalopathy and newer biological agents. *Drug Saf* **33**, 969-983 (2010).
240. Miron,V.E. *et al.* FTY720 modulates human oligodendrocyte progenitor process extension and survival. *Ann. Neurol.* **63**, 61-71 (2008).

241. Coelho,R.P., Payne,S.G., Bittman,R., Spiegel,S., & Sato-Bigbee,C. The immunomodulator FTY720 has a direct cytoprotective effect in oligodendrocyte progenitors. *J. Pharmacol. Exp. Ther.* **323**, 626-635 (2007).
242. Chun,J. & Hartung,H.P. Mechanism of action of oral fingolimod (FTY720) in multiple sclerosis. *Clin. Neuropharmacol.* **33**, 91-101 (2010).
243. Kappos,L. *et al.* A placebo-controlled trial of oral fingolimod in relapsing multiple sclerosis. *N. Engl. J. Med.* **362**, 387-401 (2010).
244. Cohen,J.A. *et al.* Oral fingolimod or intramuscular interferon for relapsing multiple sclerosis. *N. Engl. J. Med.* **362**, 402-415 (2010).
245. Frohman,E.M. *et al.* A randomized, blinded, parallel-group, pilot trial of mycophenolate mofetil (CellCept) compared with interferon beta-1a (Avonex) in patients with relapsing-remitting multiple sclerosis. *Ther. Adv. Neurol. Disord.* **3**, 15-28 (2010).
246. Casetta,I., Iuliano,G., & Filippini,G. Azathioprine for multiple sclerosis. *J. Neurol. Neurosurg. Psychiatry* **80**, 131-132 (2009).
247. La,M.L., Mascoli,N., & Milanese,C. Azathioprine. Safety profile in multiple sclerosis patients. *Neurol. Sci.* **28**, 299-303 (2007).
248. Gray,O., McDonnell,G.V., & Forbes,R.B. Methotrexate for multiple sclerosis. *Cochrane. Database. Syst. Rev.*CD003208 (2004).
249. Bar-Or,A. *et al.* Rituximab in relapsing-remitting multiple sclerosis: a 72-week, open-label, phase I trial. *Ann. Neurol.* **63**, 395-400 (2008).
250. Hawker,K. *et al.* Rituximab in patients with primary progressive multiple sclerosis: results of a randomized double-blind placebo-controlled multicenter trial. *Ann. Neurol.* **66**, 460-471 (2009).
251. Achiron,A. *et al.* Intravenous immunoglobulin treatment following the first demyelinating event suggestive of multiple sclerosis: a randomized, double-blind, placebo-controlled trial. *Arch. Neurol.* **61**, 1515-1520 (2004).
252. Lewanska,M., Siger-Zajdel,M., & Selmaj,K. No difference in efficacy of two different doses of intravenous immunoglobulins in MS: clinical and MRI assessment. *Eur. J. Neurol.* **9**, 565-572 (2002).
253. Elovaara,I. *et al.* EFNS guidelines for the use of intravenous immunoglobulin in treatment of neurological diseases: EFNS task force on the use of intravenous immunoglobulin in treatment of neurological diseases. *Eur. J. Neurol.* **15**, 893-908 (2008).
254. Zivadinov,R. *et al.* Effects of IV methylprednisolone on brain atrophy in relapsing-remitting MS. *Neurology* **57**, 1239-1247 (2001).
255. Sorensen,P.S. *et al.* NORdic trial of oral Methylprednisolone as add-on therapy to Interferon beta-1a for treatment of relapsing-remitting Multiple Sclerosis (NORMIMS study): a randomised, placebo-controlled trial. *Lancet Neurol.* **8**, 519-529 (2009).
256. Comi,G. *et al.* Placebo-controlled trial of oral laquinimod for multiple sclerosis. *N. Engl. J. Med.* **366**, 1000-1009 (2012).
257. O'Connor,P. *et al.* Randomized trial of oral teriflunomide for relapsing multiple sclerosis. *N. Engl. J. Med.* **365**, 1293-1303 (2011).
258. Kappos,L. *et al.* Efficacy and safety of oral fumarate in patients with relapsing-remitting multiple sclerosis: a multicentre, randomised, double-blind, placebo-controlled phase IIb study. *Lancet* **372**, 1463-1472 (2008).
259. Coles,A.J. *et al.* Alemtuzumab vs. interferon beta-1a in early multiple sclerosis. *N. Engl. J. Med.* **359**, 1786-1801 (2008).
260. Wynn,D. *et al.* Daclizumab in active relapsing multiple sclerosis (CHOICE study): a phase 2, randomised, double-blind, placebo-controlled, add-on trial with interferon beta. *Lancet Neurol.* **9**, 381-390 (2010).
261. Kappos,L. *et al.* Ocrelizumab in relapsing-remitting multiple sclerosis: a phase 2, randomised, placebo-controlled, multicentre trial. *Lancet* **378**, 1779-1787 (2011).
262. Bruck,W. & Wegner,C. Insight into the mechanism of laquinimod action. *J. Neurol. Sci.* **306**, 173-179 (2011).

263. Murray,P.J. & Wynn,T.A. Protective and pathogenic functions of macrophage subsets. *Nat. Rev. Immunol.* **11**, 723-737 (2011).
264. Podolsky,S.H. & Tauber,A.I. Darwinism and antibody diversity: a historical perspective. *Res. Immunol.* **147**, 199-202 (1996).
265. Gordon,S. & Taylor,P.R. Monocyte and macrophage heterogeneity. *Nat. Rev. Immunol.* **5**, 953-964 (2005).
266. Geissmann,F. *et al.* Development of monocytes, macrophages, and dendritic cells. *Science* **327**, 656-661 (2010).
267. Auffray,C. *et al.* Monitoring of blood vessels and tissues by a population of monocytes with patrolling behavior. *Science* **317**, 666-670 (2007).
268. Geissmann,F., Jung,S., & Littman,D.R. Blood monocytes consist of two principal subsets with distinct migratory properties. *Immunity.* **19**, 71-82 (2003).
269. Nahrendorf,M. *et al.* The healing myocardium sequentially mobilizes two monocyte subsets with divergent and complementary functions. *J. Exp. Med.* **204**, 3037-3047 (2007).
270. Leuschner,F. *et al.* Therapeutic siRNA silencing in inflammatory monocytes in mice. *Nat. Biotechnol.* **29**, 1005-1010 (2011).
271. Swirski,F.K. *et al.* Identification of splenic reservoir monocytes and their deployment to inflammatory sites. *Science* **325**, 612-616 (2009).
272. Ingersoll,M.A. *et al.* Comparison of gene expression profiles between human and mouse monocyte subsets. *Blood* **115**, e10-e19 (2010).
273. Tacke,F. & Randolph,G.J. Migratory fate and differentiation of blood monocyte subsets. *Immunobiology* **211**, 609-618 (2006).
274. Priller,J. *et al.* Targeting gene-modified hematopoietic cells to the central nervous system: use of green fluorescent protein uncovers microglial engraftment. *Nat. Med.* **7**, 1356-1361 (2001).
275. Hess,D.C. *et al.* Hematopoietic origin of microglial and perivascular cells in brain. *Exp. Neurol.* **186**, 134-144 (2004).
276. Hickey,W.F. & Kimura,H. Perivascular microglial cells of the CNS are bone marrow-derived and present antigen in vivo. *Science* **239**, 290-292 (1988).
277. Ajami,B., Bennett,J.L., Krieger,C., Tetzlaff,W., & Rossi,F.M. Local self-renewal can sustain CNS microglia maintenance and function throughout adult life. *Nat. Neurosci.* **10**, 1538-1543 (2007).
278. Saijo,K. & Glass,C.K. Microglial cell origin and phenotypes in health and disease. *Nat. Rev. Immunol.* **11**, 775-787 (2011).
279. Ginhoux,F. *et al.* Fate mapping analysis reveals that adult microglia derive from primitive macrophages. *Science* **330**, 841-845 (2010).
280. Ajami,B., Bennett,J.L., Krieger,C., McNagny,K.M., & Rossi,F.M. Infiltrating monocytes trigger EAE progression, but do not contribute to the resident microglia pool. *Nat. Neurosci.* **14**, 1142-1149 (2011).
281. Boyle,W.J., Simonet,W.S., & Lacey,D.L. Osteoclast differentiation and activation. *Nature* **423**, 337-342 (2003).
282. Gordon,S.B. & Read,R.C. Macrophage defences against respiratory tract infections. *Br. Med. Bull.* **61**, 45-61 (2002).
283. Surh,C.D. & Sprent,J. T-cell apoptosis detected in situ during positive and negative selection in the thymus. *Nature* **372**, 100-103 (1994).
284. Protzer,U., Maini,M.K., & Knolle,P.A. Living in the liver: hepatic infections. *Nat. Rev. Immunol.* **12**, 201-213 (2012).
285. Gordon,S. The macrophage: past, present and future. *Eur. J. Immunol.* **37 Suppl 1**, S9-17 (2007).
286. Martinez,F.O., Sica,A., Mantovani,A., & Locati,M. Macrophage activation and polarization. *Front Biosci.* **13**, 453-461 (2008).
287. Mantovani,A., Sica,A., & Locati,M. Macrophage polarization comes of age. *Immunity.* **23**, 344-346 (2005).
288. Mosser,D.M. & Edwards,J.P. Exploring the full spectrum of macrophage activation. *Nat. Rev. Immunol.* **8**, 958-969 (2008).

289. Gleissner,C.A., Shaked,I., Little,K.M., & Ley,K. CXC chemokine ligand 4 induces a unique transcriptome in monocyte-derived macrophages. *J. Immunol.* **184**, 4810-4818 (2010).
290. Kadl,A. *et al.* Identification of a novel macrophage phenotype that develops in response to atherogenic phospholipids via Nrf2. *Circ. Res.* **107**, 737-746 (2010).
291. Duluc,D. *et al.* Tumor-associated leukemia inhibitory factor and IL-6 skew monocyte differentiation into tumor-associated macrophage-like cells. *Blood* **110**, 4319-4330 (2007).
292. Wang,Q. *et al.* Fra-1 protooncogene regulates IL-6 expression in macrophages and promotes the generation of M2d macrophages. *Cell Res.* **20**, 701-712 (2010).
293. Martinez,F.O., Helming,L., & Gordon,S. Alternative activation of macrophages: an immunologic functional perspective. *Annu. Rev. Immunol.* **27**, 451-483 (2009).
294. Stout,R.D. *et al.* Macrophages sequentially change their functional phenotype in response to changes in microenvironmental influences. *J. Immunol.* **175**, 342-349 (2005).
295. Stout,R.D. & Suttles,J. Functional plasticity of macrophages: reversible adaptation to changing microenvironments. *J. Leukoc. Biol.* **76**, 509-513 (2004).
296. Sica,A. & Bronte,V. Altered macrophage differentiation and immune dysfunction in tumor development. *J. Clin. Invest* **117**, 1155-1166 (2007).
297. Biswas,S.K. & Mantovani,A. Macrophage plasticity and interaction with lymphocyte subsets: cancer as a paradigm. *Nat. Immunol.* **11**, 889-896 (2010).
298. Chawla,A., Nguyen,K.D., & Goh,Y.P. Macrophage-mediated inflammation in metabolic disease. *Nat. Rev. Immunol.* **11**, 738-749 (2011).
299. Li,A.C. & Glass,C.K. The macrophage foam cell as a target for therapeutic intervention. *Nat. Med.* **8**, 1235-1242 (2002).
300. Woollard,K.J. & Geissmann,F. Monocytes in atherosclerosis: subsets and functions. *Nat. Rev. Cardiol.* **7**, 77-86 (2010).
301. Kamada,N. *et al.* Unique CD14 intestinal macrophages contribute to the pathogenesis of Crohn disease via IL-23/IFN-gamma axis. *J. Clin. Invest* **118**, 2269-2280 (2008).
302. Kamada,N. *et al.* Human CD14+ macrophages in intestinal lamina propria exhibit potent antigen-presenting ability. *J. Immunol.* **183**, 1724-1731 (2009).
303. Kinne,R.W., Brauer,R., Stuhlmuller,B., Palombo-Kinne,E., & Burmester,G.R. Macrophages in rheumatoid arthritis. *Arthritis Res.* **2**, 189-202 (2000).
304. Kennedy,A., Fearon,U., Veale,D.J., & Godson,C. Macrophages in synovial inflammation. *Front Immunol.* **2**, 52 (2011).
305. Hendriks,J.J., Teunissen,C.E., de Vries,H.E., & Dijkstra,C.D. Macrophages and neurodegeneration. *Brain Res. Brain Res. Rev.* **48**, 185-195 (2005).
306. Imler,T.J., Jr. & Petro,T.M. Decreased severity of experimental autoimmune encephalomyelitis during resveratrol administration is associated with increased IL-17+IL-10+ T cells, CD4(-) IFN-gamma+ cells, and decreased macrophage IL-6 expression. *Int. Immunopharmacol.* **9**, 134-143 (2009).
307. Mikita,J. *et al.* Altered M1/M2 activation patterns of monocytes in severe relapsing experimental rat model of multiple sclerosis. Amelioration of clinical status by M2 activated monocyte administration. *Mult. Scler.* **17**, 2-15 (2011).
308. Guiducci,C., Vicari,A.P., Sangaletti,S., Trinchieri,G., & Colombo,M.P. Redirecting in vivo elicited tumor infiltrating macrophages and dendritic cells towards tumor rejection. *Cancer Res.* **65**, 3437-3446 (2005).
309. Titos,E. *et al.* Resolvin D1 and its precursor docosahexaenoic acid promote resolution of adipose tissue inflammation by eliciting macrophage polarization toward an M2-like phenotype. *J. Immunol.* **187**, 5408-5418 (2011).

310. Pinderski,L.J. *et al.* Overexpression of interleukin-10 by activated T lymphocytes inhibits atherosclerosis in LDL receptor-deficient Mice by altering lymphocyte and macrophage phenotypes. *Circ. Res.* **90**, 1064-1071 (2002).
311. Hunter,M.M. *et al.* In vitro-derived alternatively activated macrophages reduce colonic inflammation in mice. *Gastroenterology* **138**, 1395-1405 (2010).
312. Anderson,P. *et al.* Adipose-derived mesenchymal stromal cells induce immunomodulatory macrophages which protect from experimental colitis and sepsis. *Gut*(2012).
313. Hofkens,W., Storm,G., van den Berg,W., & van Lent,P. Inhibition of M1 Macrophage Activation in Favour of M2 Differentiation by Liposomal Targeting of Glucocorticoids to the Synovial Lining During Experimental Arthritis. *Annals of the Rheumatic Diseases* **70**, A40 (2011).
314. Prineas,J.W. & Raine,C.S. Electron microscopy and immunoperoxidase studies of early multiple sclerosis lesions. *Neurology* **26**, 29-32 (1976).
315. Prineas,J.W. *et al.* Multiple sclerosis. Oligodendrocyte proliferation and differentiation in fresh lesions. *Lab Invest* **61**, 489-503 (1989).
316. Prineas,J.W. & Graham,J.S. Multiple sclerosis: capping of surface immunoglobulin G on macrophages engaged in myelin breakdown. *Ann. Neurol.* **10**, 149-158 (1981).
317. Guillemin,G.J. & Brew,B.J. Microglia, macrophages, perivascular macrophages, and pericytes: a review of function and identification. *J. Leukoc. Biol.* **75**, 388-397 (2004).
318. Heppner,F.L. *et al.* Experimental autoimmune encephalomyelitis repressed by microglial paralysis. *Nat. Med.* **11**, 146-152 (2005).
319. Ponomarev,E.D., Shriver,L.P., Maresz,K., & Dittel,B.N. Microglial cell activation and proliferation precedes the onset of CNS autoimmunity. *J. Neurosci. Res.* **81**, 374-389 (2005).
320. Bauer,J. *et al.* The role of macrophages, perivascular cells, and microglial cells in the pathogenesis of experimental autoimmune encephalomyelitis. *Glia* **15**, 437-446 (1995).
321. Brosnan,C.F., Bornstein,M.B., & Bloom,B.R. The effects of macrophage depletion on the clinical and pathologic expression of experimental allergic encephalomyelitis. *J. Immunol.* **126**, 614-620 (1981).
322. Huitinga,I., van Rooijen,N., de Groot,C.J., Uitdehaag,B.M., & Dijkstra,C.D. Suppression of experimental allergic encephalomyelitis in Lewis rats after elimination of macrophages. *J. Exp. Med.* **172**, 1025-1033 (1990).
323. Huitinga,I. *et al.* Macrophages in T cell line-mediated, demyelinating, and chronic relapsing experimental autoimmune encephalomyelitis in Lewis rats. *Clin. Exp. Immunol.* **100**, 344-351 (1995).
324. Mildner,A. *et al.* CCR2+Ly-6Chi monocytes are crucial for the effector phase of autoimmunity in the central nervous system. *Brain* **132**, 2487-2500 (2009).
325. Fife,B.T., Huffnagle,G.B., Kuziel,W.A., & Karpus,W.J. CC chemokine receptor 2 is critical for induction of experimental autoimmune encephalomyelitis. *J. Exp. Med.* **192**, 899-905 (2000).
326. Izikson,L., Klein,R.S., Charo,I.F., Weiner,H.L., & Luster,A.D. Resistance to experimental autoimmune encephalomyelitis in mice lacking the CC chemokine receptor (CCR)2. *J. Exp. Med.* **192**, 1075-1080 (2000).
327. King,I.L., Dickendesher,T.L., & Segal,B.M. Circulating Ly-6C+ myeloid precursors migrate to the CNS and play a pathogenic role during autoimmune demyelinating disease. *Blood* **113**, 3190-3197 (2009).
328. Mrass,P. & Weninger,W. Immune cell migration as a means to control immune privilege: lessons from the CNS and tumors. *Immunol. Rev.* **213**, 195-212 (2006).
329. Larochelle,C., Alvarez,J.I., & Prat,A. How do immune cells overcome the blood-brain barrier in multiple sclerosis? *FEBS Lett.* **585**, 3770-3780 (2011).
330. Engelhardt,B. Immune cell entry into the central nervous system: involvement of adhesion molecules and chemokines. *J. Neurol. Sci.* **274**, 23-26 (2008).

331. Ubogu,E.E., Cossoy,M.B., & Ransohoff,R.M. The expression and function of chemokines involved in CNS inflammation. *Trends Pharmacol. Sci.* **27**, 48-55 (2006).
332. Charo,I.F. & Ransohoff,R.M. The many roles of chemokines and chemokine receptors in inflammation. *N. Engl. J. Med.* **354**, 610-621 (2006).
333. Cartier,L., Hartley,O., Dubois-Dauphin,M., & Krause,K.H. Chemokine receptors in the central nervous system: role in brain inflammation and neurodegenerative diseases. *Brain Res. Brain Res. Rev.* **48**, 16-42 (2005).
334. Szczucinski,A. & Losy,J. Chemokines and chemokine receptors in multiple sclerosis. Potential targets for new therapies. *Acta Neurol. Scand.* **115**, 137-146 (2007).
335. Huang,D.R., Wang,J., Kivisakk,P., Rollins,B.J., & Ransohoff,R.M. Absence of monocyte chemoattractant protein 1 in mice leads to decreased local macrophage recruitment and antigen-specific T helper cell type 1 immune response in experimental autoimmune encephalomyelitis. *J. Exp. Med.* **193**, 713-726 (2001).
336. Bartosik-Psujek,H. & Stelmasiak,Z. The levels of chemokines CXCL8, CCL2 and CCL5 in multiple sclerosis patients are linked to the activity of the disease. *Eur. J. Neurol.* **12**, 49-54 (2005).
337. McManus,C. *et al.* MCP-1, MCP-2 and MCP-3 expression in multiple sclerosis lesions: an immunohistochemical and in situ hybridization study. *J. Neuroimmunol.* **86**, 20-29 (1998).
338. Prinz,M. & Mildner,A. Microglia in the CNS: immigrants from another world. *Glia* **59**, 177-187 (2011).
339. Hanisch,U.K. & Kettenmann,H. Microglia: active sensor and versatile effector cells in the normal and pathologic brain. *Nat. Neurosci.* **10**, 1387-1394 (2007).
340. Olah,M., Biber,K., Vinet,J., & Boddeke,H.W. Microglia phenotype diversity. *CNS. Neurol. Disord. Drug Targets.* **10**, 108-118 (2011).
341. Stence,N., Waite,M., & Dailey,M.E. Dynamics of microglial activation: a confocal time-lapse analysis in hippocampal slices. *Glia* **33**, 256-266 (2001).
342. Kettenmann,H., Hanisch,U.K., Noda,M., & Verkhratsky,A. Physiology of microglia. *Physiol Rev.* **91**, 461-553 (2011).
343. Biber,K. *et al.* Functional expression of CXCR3 in cultured mouse and human astrocytes and microglia. *Neuroscience* **112**, 487-497 (2002).
344. Dijkstra,I.M., Hulshof,S., van,d., V, Boddeke,H.W., & Biber,K. Cutting edge: activity of human adult microglia in response to CC chemokine ligand 21. *J. Immunol.* **172**, 2744-2747 (2004).
345. Rappert,A. *et al.* CXCR3-dependent microglial recruitment is essential for dendrite loss after brain lesion. *J. Neurosci.* **24**, 8500-8509 (2004).
346. Sorensen,T.L. *et al.* Expression of specific chemokines and chemokine receptors in the central nervous system of multiple sclerosis patients. *J. Clin. Invest* **103**, 807-815 (1999).
347. Glabinski,A.R., Tani,M., Strieter,R.M., Tuohy,V.K., & Ransohoff,R.M. Synchronous synthesis of alpha- and beta-chemokines by cells of diverse lineage in the central nervous system of mice with relapses of chronic experimental autoimmune encephalomyelitis. *Am. J. Pathol.* **150**, 617-630 (1997).
348. Godiska,R., Chantry,D., Dietsch,G.N., & Gray,P.W. Chemokine expression in murine experimental allergic encephalomyelitis. *J. Neuroimmunol.* **58**, 167-176 (1995).
349. Liu,L., Callahan,M.K., Huang,D., & Ransohoff,R.M. Chemokine receptor CXCR3: an unexpected enigma. *Curr. Top. Dev. Biol.* **68**, 149-181 (2005).
350. Sorensen,T.L. *et al.* Multiple sclerosis: a study of CXCL10 and CXCR3 co-localization in the inflamed central nervous system. *J. Neuroimmunol.* **127**, 59-68 (2002).

351. Rappert,A. *et al.* Secondary lymphoid tissue chemokine (CCL21) activates CXCR3 to trigger a Cl- current and chemotaxis in murine microglia. *J. Immunol.* **168**, 3221-3226 (2002).
352. Sospedra,M. & Martin,R. Immunology of multiple sclerosis. *Annu. Rev. Immunol.* **23**, 683-747 (2005).
353. Ulvestad,E. *et al.* HLA class II molecules (HLA-DR, -DP, -DQ) on cells in the human CNS studied in situ and in vitro. *Immunology* **82**, 535-541 (1994).
354. Lassmann,H., Zimprich,F., Vass,K., & Hickey,W.F. Microglial cells are a component of the perivascular glia limitans. *J. Neurosci. Res.* **28**, 236-243 (1991).
355. Graeber,M.B., Streit,W.J., Buringer,D., Sparks,D.L., & Kreutzberg,G.W. Ultrastructural location of major histocompatibility complex (MHC) class II positive perivascular cells in histologically normal human brain. *J. Neuropathol. Exp. Neurol.* **51**, 303-311 (1992).
356. Fabriek,B.O. *et al.* CD163-positive perivascular macrophages in the human CNS express molecules for antigen recognition and presentation. *Glia* **51**, 297-305 (2005).
357. Ford,A.L., Goodsall,A.L., Hickey,W.F., & Sedgwick,J.D. Normal adult ramified microglia separated from other central nervous system macrophages by flow cytometric sorting. Phenotypic differences defined and direct ex vivo antigen presentation to myelin basic protein-reactive CD4+ T cells compared. *J. Immunol.* **154**, 4309-4321 (1995).
358. Williams,K., Ulvestad,E., & Antel,J. Immune regulatory and effector properties of human adult microglia studies in vitro and in situ. *Adv. Neuroimmunol.* **4**, 273-281 (1994).
359. Aloisi,F. Immune function of microglia. *Glia* **36**, 165-179 (2001).
360. Gehrmann,J., Banati,R.B., & Kreutzberg,G.W. Microglia in the immune surveillance of the brain: human microglia constitutively express HLA-DR molecules. *J. Neuroimmunol.* **48**, 189-198 (1993).
361. Rezaie,P. & Male,D. Colonisation of the developing human brain and spinal cord by microglia: a review. *Microsc. Res. Tech.* **45**, 359-382 (1999).
362. Kreutzberg,G.W. Microglia: a sensor for pathological events in the CNS. *Trends Neurosci.* **19**, 312-318 (1996).
363. Perry,V.H. A revised view of the central nervous system microenvironment and major histocompatibility complex class II antigen presentation. *J. Neuroimmunol.* **90**, 113-121 (1998).
364. Cash,E., Zhang,Y., & Rott,O. Microglia present myelin antigens to T cells after phagocytosis of oligodendrocytes. *Cell Immunol.* **147**, 129-138 (1993).
365. Boven,L.A. *et al.* Myelin-laden macrophages are anti-inflammatory, consistent with foam cells in multiple sclerosis. *Brain* **129**, 517-526 (2006).
366. de Vos,A.F. *et al.* Transfer of central nervous system autoantigens and presentation in secondary lymphoid organs. *J. Immunol.* **169**, 5415-5423 (2002).
367. Fabriek,B.O. *et al.* In vivo detection of myelin proteins in cervical lymph nodes of MS patients using ultrasound-guided fine-needle aspiration cytology. *J. Neuroimmunol.* **161**, 190-194 (2005).
368. van Zwam,M. *et al.* Brain antigens in functionally distinct antigen-presenting cell populations in cervical lymph nodes in MS and EAE. *J. Mol. Med.* **87**, 273-286 (2009).
369. Karman,J., Ling,C., Sandor,M., & Fabry,Z. Initiation of immune responses in brain is promoted by local dendritic cells. *J. Immunol.* **173**, 2353-2361 (2004).
370. van Zwam,M. *et al.* Myelin ingestion by macrophages promotes their motility and capacity to recruit myeloid cells. *J. Neuroimmunol.* **225**, 112-117 (2010).
371. Forster,R., Davalos-Misslitz,A.C., & Rot,A. CCR7 and its ligands: balancing immunity and tolerance. *Nat. Rev. Immunol.* **8**, 362-371 (2008).

372. Williams,K., Ulvestad,E., Waage,A., Antel,J.P., & McLaurin,J. Activation of adult human derived microglia by myelin phagocytosis in vitro. *J. Neurosci. Res.* **38**, 433-443 (1994).
373. Mosley,K. & Cuzner,M.L. Receptor-mediated phagocytosis of myelin by macrophages and microglia: effect of opsonization and receptor blocking agents. *Neurochem. Res.* **21**, 481-487 (1996).
374. van der Laan,L.J. *et al.* Macrophage phagocytosis of myelin in vitro determined by flow cytometry: phagocytosis is mediated by CR3 and induces production of tumor necrosis factor-alpha and nitric oxide. *J. Neuroimmunol.* **70**, 145-152 (1996).
375. Liu,Y. *et al.* Suppression of microglial inflammatory activity by myelin phagocytosis: role of p47-PHOX-mediated generation of reactive oxygen species. *J. Neurosci.* **26**, 12904-12913 (2006).
376. van Rossum,D., Hilbert,S., Strassenburg,S., Hanisch,U.K., & Bruck,W. Myelin-phagocytosing macrophages in isolated sciatic and optic nerves reveal a unique reactive phenotype. *Glia* **56**, 271-283 (2008).
377. Sun,X. *et al.* Myelin activates FAK/Akt/NF-kappaB pathways and provokes CR3-dependent inflammatory response in murine system. *PLoS. One.* **5**, e9380 (2010).
378. Zhang,Z. *et al.* Parenchymal accumulation of CD163(+) macrophages/microglia in multiple sclerosis brains. *J. Neuroimmunol.* **237**, 73-79 (2011).
379. Durafourt,B.A. *et al.* Comparison of polarization properties of human adult microglia and blood-derived macrophages. *Glia* **60**, 717-727 (2012).
380. Hendriks,J.J. *et al.* Flavonoids inhibit myelin phagocytosis by macrophages; a structure-activity relationship study. *Biochem. Pharmacol.* **65**, 877-885 (2003).
381. Hendriks,J.J. *et al.* Leukemia inhibitory factor modulates production of inflammatory mediators and myelin phagocytosis by macrophages. *J. Neuroimmunol.* **204**, 52-57 (2008).
382. Vougioukas,V.I., Siebert,H., Heinecke,K., & Bruck,W. Effects of the immunomodulator linomide on macrophage migration and myelin phagocytic activity in peripheral nerve trauma: an experimental study. *J. Neurotrauma* **18**, 555-562 (2001).
383. Cohen,G. *et al.* Non-PKC DAG/phorbol-ester receptor(s) inhibit complement receptor-3 and nPKC inhibit scavenger receptor-AI/II-mediated myelin phagocytosis but cPKC, PI3k, and PLCgamma activate myelin phagocytosis by both. *Glia* **53**, 538-550 (2006).
384. Vereyken,E.J. *et al.* Classically and alternatively activated bone marrow derived macrophages differ in cytoskeletal functions and migration towards specific CNS cell types. *J. Neuroinflammation.* **8**, 58 (2011).
385. Bruck,W., Bruck,Y., & Friede,R.L. TNF-alpha suppresses CR3-mediated myelin removal by macrophages. *J. Neuroimmunol.* **38**, 9-17 (1992).
386. Gitik,M., Liraz-Zaltsman,S., Oldenborg,P.A., Reichert,F., & Rotshenker,S. Myelin down-regulates myelin phagocytosis by microglia and macrophages through interactions between CD47 on myelin and SIRPalpha (signal regulatory protein-alpha) on phagocytes. *J. Neuroinflammation.* **8**, 24 (2011).
387. van der Goes,A. *et al.* Reactive oxygen species are required for the phagocytosis of myelin by macrophages. *J. Neuroimmunol.* **92**, 67-75 (1998).
388. Mosley,K. & Cuzner,M.L. Receptor-mediated phagocytosis of myelin by macrophages and microglia: effect of opsonization and receptor blocking agents. *Neurochem. Res.* **21**, 481-487 (1996).
389. Reichert,F. & Rotshenker,S. Complement-receptor-3 and scavenger-receptor-AI/II mediated myelin phagocytosis in microglia and macrophages. *Neurobiol. Dis.* **12**, 65-72 (2003).
390. da Costa,C.C., van der Laan,L.J., Dijkstra,C.D., & Bruck,W. The role of the mouse macrophage scavenger receptor in myelin phagocytosis. *Eur. J. Neurosci.* **9**, 2650-2657 (1997).

391. Rotshenker,S. Microglia and macrophage activation and the regulation of complement-receptor-3 (CR3/MAC-1)-mediated myelin phagocytosis in injury and disease. *J. Mol. Neurosci.* **21**, 65-72 (2003).
392. van der Goes,A., Kortekaas,M., Hoekstra,K., Dijkstra,C.D., & Amor,S. The role of anti-myelin (auto)-antibodies in the phagocytosis of myelin by macrophages. *J. Neuroimmunol.* **101**, 61-67 (1999).
393. Ulvestad,E. *et al.* Reactive microglia in multiple sclerosis lesions have an increased expression of receptors for the Fc part of IgG. *J. Neurol. Sci.* **121**, 125-131 (1994).
394. Lalive,P.H. Autoantibodies in inflammatory demyelinating diseases of the central nervous system. *Swiss. Med. Wkly.* **138**, 692-707 (2008).
395. Breij,E.C. *et al.* Homogeneity of active demyelinating lesions in established multiple sclerosis. *Ann. Neurol.* **63**, 16-25 (2008).
396. Barnett,M.H. & Prineas,J.W. Relapsing and remitting multiple sclerosis: pathology of the newly forming lesion. *Ann. Neurol.* **55**, 458-468 (2004).
397. Lucchinetti,C. *et al.* Heterogeneity of multiple sclerosis lesions: implications for the pathogenesis of demyelination. *Ann. Neurol.* **47**, 707-717 (2000).
398. Linington,C., Bradl,M., Lassmann,H., Brunner,C., & Vass,K. Augmentation of demyelination in rat acute allergic encephalomyelitis by circulating mouse monoclonal antibodies directed against a myelin/oligodendrocyte glycoprotein. *Am. J. Pathol.* **130**, 443-454 (1988).
399. Stefferl,A. *et al.* Myelin oligodendrocyte glycoprotein induces experimental autoimmune encephalomyelitis in the "resistant" Brown Norway rat: disease susceptibility is determined by MHC and MHC-linked effects on the B cell response. *J. Immunol.* **163**, 40-49 (1999).
400. Kerlero de,R.N., Honegger,P., Lassmann,H., & Matthieu,J.M. Demyelination induced in aggregating brain cell cultures by a monoclonal antibody against myelin/oligodendrocyte glycoprotein. *J. Neurochem.* **55**, 583-587 (1990).
401. Schluesener,H.J., Sobel,R.A., Linington,C., & Weiner,H.L. A monoclonal antibody against a myelin oligodendrocyte glycoprotein induces relapses and demyelination in central nervous system autoimmune disease. *J. Immunol.* **139**, 4016-4021 (1987).
402. Piddlesden,S.J., Lassmann,H., Zimprich,F., Morgan,B.P., & Linington,C. The demyelinating potential of antibodies to myelin oligodendrocyte glycoprotein is related to their ability to fix complement. *Am. J. Pathol.* **143**, 555-564 (1993).
403. Piddlesden,S.J. *et al.* Soluble recombinant complement receptor 1 inhibits inflammation and demyelination in antibody-mediated demyelinating experimental allergic encephalomyelitis. *J. Immunol.* **152**, 5477-5484 (1994).
404. Levy-Barazany,H. & Frenkel,D. Expression of Scavenger receptor A on antigen presenting cells is important for CD4+ T-cells proliferation in EAE mouse model. *J. Neuroinflammation.* **9**, 120 (2012).
405. Gaultier,A. *et al.* Low-density lipoprotein receptor-related protein 1 is an essential receptor for myelin phagocytosis. *J. Cell Sci.* **122**, 1155-1162 (2009).
406. Hendriks,J.J., Teunissen,C.E., de Vries,H.E., & Dijkstra,C.D. Macrophages and neurodegeneration. *Brain Res. Brain Res. Rev.* **48**, 185-195 (2005).
407. Raivich,G. & Banati,R. Brain microglia and blood-derived macrophages: molecular profiles and functional roles in multiple sclerosis and animal models of autoimmune demyelinating disease. *Brain Res. Brain Res. Rev.* **46**, 261-281 (2004).
408. Bongarzone,E.R., Pasquini,J.M., & Soto,E.F. Oxidative damage to proteins and lipids of CNS myelin produced by in vitro generated reactive oxygen species. *J. Neurosci. Res.* **41**, 213-221 (1995).
409. Mitrovic,B., Ignarro,L.J., Montestruque,S., Smoll,A., & Merrill,J.E. Nitric oxide as a potential pathological mechanism in demyelination: its differential effects on primary glial cells in vitro. *Neuroscience* **61**, 575-585 (1994).

410. Touil,T., Deloire-Grassin,M.S., Vital,C., Petry,K.G., & Brochet,B. In vivo damage of CNS myelin and axons induced by peroxynitrite. *Neuroreport* **12**, 3637-3644 (2001).
411. Smith,K.J., Kapoor,R., & Felts,P.A. Demyelination: the role of reactive oxygen and nitrogen species. *Brain Pathol.* **9**, 69-92 (1999).
412. Konat,G.W. & Wiggins,R.C. Effect of reactive oxygen species on myelin membrane proteins. *J. Neurochem.* **45**, 1113-1118 (1985).
413. Merrill,J.E., Ignarro,L.J., Sherman,M.P., Melinek,J., & Lane,T.E. Microglial cell cytotoxicity of oligodendrocytes is mediated through nitric oxide. *J. Immunol.* **151**, 2132-2141 (1993).
414. Farias,A.S. *et al.* Nitric oxide and TNFalpha effects in experimental autoimmune encephalomyelitis demyelination. *Neuroimmunomodulation.* **14**, 32-38 (2007).
415. Fischer, M.T., Sharma,R., Lim,J.L., Haider,L., & Lassmann,H. NADPH oxidase expression in active multiple sclerosis lesions in relation to oxidative tissue damage and mitochondrial injury. *Brain* **135**, 886-899 (2012).
416. Koeberle,P.D. & Ball,A.K. Nitric oxide synthase inhibition delays axonal degeneration and promotes the survival of axotomized retinal ganglion cells. *Exp. Neurol.* **158**, 366-381 (1999).
417. MacMicking,J.D., Willenborg,D.O., Weidemann,M.J., Rockett,K.A., & Cowden,W.B. Elevated secretion of reactive nitrogen and oxygen intermediates by inflammatory leukocytes in hyperacute experimental autoimmune encephalomyelitis: enhancement by the soluble products of encephalitogenic T cells. *J. Exp. Med.* **176**, 303-307 (1992).
418. Vladimirova,O. *et al.* Oxidative damage to DNA in plaques of MS brains. *Mult. Scler.* **4**, 413-418 (1998).
419. Vladimirova,O., Lu,F.M., Shawver,L., & Kalman,B. The activation of protein kinase C induces higher production of reactive oxygen species by mononuclear cells in patients with multiple sclerosis than in controls. *Inflamm. Res.* **48**, 412-416 (1999).
420. Ferretti,G. *et al.* Intracellular oxidative activity and respiratory burst of leukocytes isolated from multiple sclerosis patients. *Neurochem. Int.* **48**, 87-92 (2006).
421. Hendriks,J.J. *et al.* Flavonoids influence monocytic GTPase activity and are protective in experimental allergic encephalitis. *J. Exp. Med.* **200**, 1667-1672 (2004).
422. de Groot,C.J., Ruuls,S.R., Theeuwes,J.W., Dijkstra,C.D., & van,d., V Immunocytochemical characterization of the expression of inducible and constitutive isoforms of nitric oxide synthase in demyelinating multiple sclerosis lesions. *J. Neuropathol. Exp. Neurol.* **56**, 10-20 (1997).
423. Bagasra,O. *et al.* Activation of the inducible form of nitric oxide synthase in the brains of patients with multiple sclerosis. *Proc. Natl. Acad. Sci. U. S. A* **92**, 12041-12045 (1995).
424. Liu,J.S., Zhao,M.L., Brosnan,C.F., & Lee,S.C. Expression of inducible nitric oxide synthase and nitrotyrosine in multiple sclerosis lesions. *Am. J. Pathol.* **158**, 2057-2066 (2001).
425. Oleszak,E.L. *et al.* Inducible nitric oxide synthase and nitrotyrosine are found in monocytes/macrophages and/or astrocytes in acute, but not in chronic, multiple sclerosis. *Clin. Diagn. Lab Immunol.* **5**, 438-445 (1998).
426. Hooper,D.C. *et al.* Local nitric oxide production in viral and autoimmune diseases of the central nervous system. *Proc. Natl. Acad. Sci. U. S. A* **92**, 5312-5316 (1995).
427. Okuda,Y. *et al.* Expression of the inducible isoform of nitric oxide synthase in the central nervous system of mice correlates with the severity of actively induced experimental allergic encephalomyelitis. *J. Neuroimmunol.* **62**, 103-112 (1995).

428. Kahl,K.G. *et al.* Protective role of the cytokine-inducible isoform of nitric oxide synthase induction and nitrosative stress in experimental autoimmune encephalomyelitis of the DA rat. *J. Neurosci. Res.* **73**, 198-205 (2003).
429. Okuda,Y., Sakoda,S., Fujimura,H., & Yanagihara,T. Aminoguanidine, a selective inhibitor of the inducible nitric oxide synthase, has different effects on experimental allergic encephalomyelitis in the induction and progression phase. *J. Neuroimmunol.* **81**, 201-210 (1998).
430. Ruuls,S.R., Van Der,L.S., Sontrop,K., Huitinga,I., & Dijkstra,C.D. Aggravation of experimental allergic encephalomyelitis (EAE) by administration of nitric oxide (NO) synthase inhibitors. *Clin. Exp. Immunol.* **103**, 467-474 (1996).
431. Xu,L.Y., Yang,J.S., Link,H., & Xiao,B.G. SIN-1, a nitric oxide donor, ameliorates experimental allergic encephalomyelitis in Lewis rats in the incipient phase: the importance of the time window. *J. Immunol.* **166**, 5810-5816 (2001).
432. van der Veen,R.C., Dietlin,T.A., Dixon,G.J., & Gilmore,W. Macrophage-derived nitric oxide inhibits the proliferation of activated T helper cells and is induced during antigenic stimulation of resting T cells. *Cell Immunol.* **199**, 43-49 (2000).
433. Mahidhara,R.S. *et al.* Nitric oxide-mediated inhibition of caspase-dependent T lymphocyte proliferation. *J. Leukoc. Biol.* **74**, 403-411 (2003).
434. Hurst,R.D. & Fritz,I.B. Nitric oxide-induced perturbations in a cell culture model of the blood-brain barrier. *J. Cell Physiol* **167**, 89-94 (1996).
435. Mayhan,W.G. Nitric oxide donor-induced increase in permeability of the blood-brain barrier. *Brain Res.* **866**, 101-108 (2000).
436. Kean,R.B., Spitsin,S.V., Mikheeva,T., Scott,G.S., & Hooper,D.C. The peroxynitrite scavenger uric acid prevents inflammatory cell invasion into the central nervous system in experimental allergic encephalomyelitis through maintenance of blood-central nervous system barrier integrity. *J. Immunol.* **165**, 6511-6518 (2000).
437. Brian,J.E., Jr., Faraci,F.M., & Heistad,D.D. Recent insights into the regulation of cerebral circulation. *Clin. Exp. Pharmacol. Physiol* **23**, 449-457 (1996).
438. Smith,K.J. & Lassmann,H. The role of nitric oxide in multiple sclerosis. *Lancet Neurol.* **1**, 232-241 (2002).
439. Spuler,S. *et al.* Multiple sclerosis: prospective analysis of TNF-alpha and 55 kDa TNF receptor in CSF and serum in correlation with clinical and MRI activity. *J. Neuroimmunol.* **66**, 57-64 (1996).
440. Maimone,D., Gregory,S., Arnason,B.G., & Reder,A.T. Cytokine levels in the cerebrospinal fluid and serum of patients with multiple sclerosis. *J. Neuroimmunol.* **32**, 67-74 (1991).
441. Sharief,M.K. & Hentges,R. Association between tumor necrosis factor-alpha and disease progression in patients with multiple sclerosis. *N. Engl. J. Med.* **325**, 467-472 (1991).
442. Hofman,F.M., Hinton,D.R., Johnson,K., & Merrill,J.E. Tumor necrosis factor identified in multiple sclerosis brain. *J. Exp. Med.* **170**, 607-612 (1989).
443. Selmaj,K., Raine,C.S., Cannella,B., & Brosnan,C.F. Identification of lymphotoxin and tumor necrosis factor in multiple sclerosis lesions. *J. Clin. Invest* **87**, 949-954 (1991).
444. Renno,T., Krakowski,M., Piccirillo,C., Lin,J.Y., & Owens,T. TNF-alpha expression by resident microglia and infiltrating leukocytes in the central nervous system of mice with experimental allergic encephalomyelitis. Regulation by Th1 cytokines. *J. Immunol.* **154**, 944-953 (1995).
445. Bitsch,A. *et al.* Identification of macrophage populations expressing tumor necrosis factor-alpha mRNA in acute multiple sclerosis. *Acta Neuropathol.* **95**, 373-377 (1998).
446. Akassoglou,K. *et al.* Oligodendrocyte apoptosis and primary demyelination induced by local TNF/p55TNF receptor signaling in the central nervous system of transgenic mice: models for multiple sclerosis with primary oligodendrogliopathy. *Am. J. Pathol.* **153**, 801-813 (1998).

447. Probert,L., Akassoglou,K., Pasparakis,M., Kontogeorgos,G., & Kollias,G. Spontaneous inflammatory demyelinating disease in transgenic mice showing central nervous system-specific expression of tumor necrosis factor alpha. *Proc. Natl. Acad. Sci. U. S. A* **92**, 11294-11298 (1995).
448. Baker,D. *et al.* Control of established experimental allergic encephalomyelitis by inhibition of tumor necrosis factor (TNF) activity within the central nervous system using monoclonal antibodies and TNF receptor-immunoglobulin fusion proteins. *Eur. J. Immunol.* **24**, 2040-2048 (1994).
449. Ruddle,N.H. *et al.* An antibody to lymphotoxin and tumor necrosis factor prevents transfer of experimental allergic encephalomyelitis. *J. Exp. Med.* **172**, 1193-1200 (1990).
450. Selmaj,K.W. & Raine,C.S. Tumor necrosis factor mediates myelin and oligodendrocyte damage in vitro. *Ann. Neurol.* **23**, 339-346 (1988).
451. Finsen,B., Antel,J., & Owens,T. TNFalpha: kill or cure for demyelinating disease? *Mol. Psychiatry* **7**, 820-821 (2002).
452. van Oosten,B.W. *et al.* Increased MRI activity and immune activation in two multiple sclerosis patients treated with the monoclonal anti-tumor necrosis factor antibody cA2. *Neurology* **47**, 1531-1534 (1996).
453. Bauer,J., Berkenbosch,F., Van Dam,A.M., & Dijkstra,C.D. Demonstration of interleukin-1 beta in Lewis rat brain during experimental allergic encephalomyelitis by immunocytochemistry at the light and ultrastructural level. *J. Neuroimmunol.* **48**, 13-21 (1993).
454. Li,J. *et al.* Differential expression and regulation of IL-23 and IL-12 subunits and receptors in adult mouse microglia. *J. Neurol. Sci.* **215**, 95-103 (2003).
455. Li,Y. *et al.* Increased IL-23p19 expression in multiple sclerosis lesions and its induction in microglia. *Brain* **130**, 490-501 (2007).
456. Merrill,J.E. & Scolding,N.J. Mechanisms of damage to myelin and oligodendrocytes and their relevance to disease. *Neuropathol. Appl. Neurobiol.* **25**, 435-458 (1999).
457. Brogi,A., Strazza,M., Melli,M., & Costantino-Ceccarini,E. Induction of intracellular ceramide by interleukin-1 beta in oligodendrocytes. *J. Cell Biochem.* **66**, 532-541 (1997).
458. Ferrari,C.C. *et al.* Reversible demyelination, blood-brain barrier breakdown, and pronounced neutrophil recruitment induced by chronic IL-1 expression in the brain. *Am. J. Pathol.* **165**, 1827-1837 (2004).
459. Jacobs,C.A. *et al.* Experimental autoimmune encephalomyelitis is exacerbated by IL-1 alpha and suppressed by soluble IL-1 receptor. *J. Immunol.* **146**, 2983-2989 (1991).
460. Aggarwal,S., Ghilardi,N., Xie,M.H., de Sauvage,F.J., & Gurney,A.L. Interleukin-23 promotes a distinct CD4 T cell activation state characterized by the production of interleukin-17. *J. Biol. Chem.* **278**, 1910-1914 (2003).
461. Platt,S.R. The role of glutamate in central nervous system health and disease--a review. *Vet. J.* **173**, 278-286 (2007).
462. Matute,C., Sanchez-Gomez,M.V., Martinez-Millan,L., & Miledi,R. Glutamate receptor-mediated toxicity in optic nerve oligodendrocytes. *Proc. Natl. Acad. Sci. U. S. A* **94**, 8830-8835 (1997).
463. McDonald,J.W., Althomsons,S.P., Hyrc,K.L., Choi,D.W., & Goldberg,M.P. Oligodendrocytes from forebrain are highly vulnerable to AMPA/kainate receptor-mediated excitotoxicity. *Nat. Med.* **4**, 291-297 (1998).
464. Stover,J.F. *et al.* Neurotransmitters in cerebrospinal fluid reflect pathological activity. *Eur. J. Clin. Invest* **27**, 1038-1043 (1997).
465. Srinivasan,R., Sailasuta,N., Hurd,R., Nelson,S., & Pelletier,D. Evidence of elevated glutamate in multiple sclerosis using magnetic resonance spectroscopy at 3 T. *Brain* **128**, 1016-1025 (2005).
466. Pampliega,O. *et al.* Association of an EAAT2 polymorphism with higher glutamate concentration in relapsing multiple sclerosis. *J. Neuroimmunol.* **195**, 194-198 (2008).

467. Pitt,D., Werner,P., & Raine,C.S. Glutamate excitotoxicity in a model of multiple sclerosis. *Nat. Med.* **6**, 67-70 (2000).
468. Piani,D., Frei,K., Do,K.Q., Cuenod,M., & Fontana,A. Murine brain macrophages induced NMDA receptor mediated neurotoxicity in vitro by secreting glutamate. *Neurosci. Lett.* **133**, 159-162 (1991).
469. Werner,P., Pitt,D., & Raine,C.S. Multiple sclerosis: altered glutamate homeostasis in lesions correlates with oligodendrocyte and axonal damage. *Ann. Neurol.* **50**, 169-180 (2001).
470. Smith,T., Groom,A., Zhu,B., & Turski,L. Autoimmune encephalomyelitis ameliorated by AMPA antagonists. *Nat. Med.* **6**, 62-66 (2000).
471. Groom,A.J., Smith,T., & Turski,L. Multiple sclerosis and glutamate. *Ann. N. Y. Acad. Sci.* **993**, 229-275 (2003).
472. Kuhlmann,T., Lingfeld,G., Bitsch,A., Schuchardt,J., & Bruck,W. Acute axonal damage in multiple sclerosis is most extensive in early disease stages and decreases over time. *Brain* **125**, 2202-2212 (2002).
473. Herrero-Herranz,E., Pardo,L.A., Gold,R., & Linker,R.A. Pattern of axonal injury in murine myelin oligodendrocyte glycoprotein induced experimental autoimmune encephalomyelitis: implications for multiple sclerosis. *Neurobiol. Dis.* **30**, 162-173 (2008).
474. Tallantyre,E.C. *et al.* Clinico-pathological evidence that axonal loss underlies disability in progressive multiple sclerosis. *Mult. Scler.* **16**, 406-411 (2010).
475. Papadopoulos,D., Pham-Dinh,D., & Reynolds,R. Axon loss is responsible for chronic neurological deficit following inflammatory demyelination in the rat. *Exp. Neurol.* **197**, 373-385 (2006).
476. Wujek,J.R. *et al.* Axon loss in the spinal cord determines permanent neurological disability in an animal model of multiple sclerosis. *J. Neuropathol. Exp. Neurol.* **61**, 23-32 (2002).
477. Huizinga,R. *et al.* Phagocytosis of neuronal debris by microglia is associated with neuronal damage in multiple sclerosis. *Glia* **60**, 422-431 (2012).
478. Piani,D., Frei,K., Do,K.Q., Cuenod,M., & Fontana,A. Murine brain macrophages induced NMDA receptor mediated neurotoxicity in vitro by secreting glutamate. *Neurosci. Lett.* **133**, 159-162 (1991).
479. Piani,D. & Fontana,A. Involvement of the cystine transport system xc- in the macrophage-induced glutamate-dependent cytotoxicity to neurons. *J. Immunol.* **152**, 3578-3585 (1994).
480. Flavin,M.P., Coughlin,K., & Ho,L.T. Soluble macrophage factors trigger apoptosis in cultured hippocampal neurons. *Neuroscience* **80**, 437-448 (1997).
481. Irvine,K.A. & Blakemore,W.F. Remyelination protects axons from demyelination-associated axon degeneration. *Brain* **131**, 1464-1477 (2008).
482. Nguyen,T. *et al.* Axonal protective effects of the myelin-associated glycoprotein. *J. Neurosci.* **29**, 630-637 (2009).
483. Zhang,Y. *et al.* Clonal expansion of IgA-positive plasma cells and axon-reactive antibodies in MS lesions. *J. Neuroimmunol.* **167**, 120-130 (2005).
484. Mathey,E.K. *et al.* Neurofascin as a novel target for autoantibody-mediated axonal injury. *J. Exp. Med.* **204**, 2363-2372 (2007).
485. DeVries,G.H. Cryptic axonal antigens and axonal loss in multiple sclerosis. *Neurochem. Res.* **29**, 1999-2006 (2004).
486. Derfuss,T. *et al.* Contactin-2/TAG-1-directed autoimmunity is identified in multiple sclerosis patients and mediates gray matter pathology in animals. *Proc. Natl. Acad. Sci. U. S. A* **106**, 8302-8307 (2009).
487. Silber,E., Semra,Y.K., Gregson,N.A., & Sharief,M.K. Patients with progressive multiple sclerosis have elevated antibodies to neurofilament subunit. *Neurology* **58**, 1372-1381 (2002).
488. Nikic,I. *et al.* A reversible form of axon damage in experimental autoimmune encephalomyelitis and multiple sclerosis. *Nat. Med.* **17**, 495-499 (2011).
489. Smith,K.J. & Lassmann,H. The role of nitric oxide in multiple sclerosis. *Lancet Neurol.* **1**, 232-241 (2002).

490. Lu,F. *et al.* Oxidative damage to mitochondrial DNA and activity of mitochondrial enzymes in chronic active lesions of multiple sclerosis. *J. Neurol. Sci.* **177**, 95-103 (2000).
491. Kapoor,R., Davies,M., Blaker,P.A., Hall,S.M., & Smith,K.J. Blockers of sodium and calcium entry protect axons from nitric oxide-mediated degeneration. *Ann. Neurol.* **53**, 174-180 (2003).
492. Hendriks,J.J. *et al.* Flavonoids influence monocytic GTPase activity and are protective in experimental allergic encephalitis. *J. Exp. Med.* **200**, 1667-1672 (2004).
493. Marracci,G.H., Jones,R.E., McKeon,G.P., & Bourdette,D.N. Alpha lipoic acid inhibits T cell migration into the spinal cord and suppresses and treats experimental autoimmune encephalomyelitis. *J. Neuroimmunol.* **131**, 104-114 (2002).
494. Liu,Y. *et al.* Bilirubin as a potent antioxidant suppresses experimental autoimmune encephalomyelitis: implications for the role of oxidative stress in the development of multiple sclerosis. *J. Neuroimmunol.* **139**, 27-35 (2003).
495. Rose,J.W. *et al.* Nitric oxide synthase inhibitor, aminoguanidine, reduces inflammation and demyelination produced by Theiler's virus infection. *J. Neuroimmunol.* **81**, 82-89 (1998).
496. Chao,C.C. *et al.* Cytokine-stimulated astrocytes damage human neurons via a nitric oxide mechanism. *Glia* **16**, 276-284 (1996).
497. Chao,C.C., Hu,S., Ehrlich,L., & Peterson,P.K. Interleukin-1 and tumor necrosis factor-alpha synergistically mediate neurotoxicity: involvement of nitric oxide and of N-methyl-D-aspartate receptors. *Brain Behav. Immun.* **9**, 355-365 (1995).
498. Gimsa,U., Peter,S.V., Lehmann,K., Bechmann,I., & Nitsch,R. Axonal damage induced by invading T cells in organotypic central nervous system tissue in vitro: involvement of microglial cells. *Brain Pathol.* **10**, 365-377 (2000).
499. He,B.P., Wen,W., & Strong,M.J. Activated microglia (BV-2) facilitation of TNF-alpha-mediated motor neuron death in vitro. *J. Neuroimmunol.* **128**, 31-38 (2002).
500. Jeohn,G.H., Kong,L.Y., Wilson,B., Hudson,P., & Hong,J.S. Synergistic neurotoxic effects of combined treatments with cytokines in murine primary mixed neuron/glia cultures. *J. Neuroimmunol.* **85**, 1-10 (1998).
501. Tan,J. *et al.* Activation of microglial cells by the CD40 pathway: relevance to multiple sclerosis. *J. Neuroimmunol.* **97**, 77-85 (1999).
502. Ankarcrona,M. *et al.* Glutamate-induced neuronal death: a succession of necrosis or apoptosis depending on mitochondrial function. *Neuron* **15**, 961-973 (1995).
503. Zhang,Y. & Bhavnani,B.R. Glutamate-induced apoptosis in neuronal cells is mediated via caspase-dependent and independent mechanisms involving calpain and caspase-3 proteases as well as apoptosis inducing factor (AIF) and this process is inhibited by equine estrogens. *BMC Neurosci.* **7**, 49 (2006).
504. Sattler,R. & Tymianski,M. Molecular mechanisms of glutamate receptor-mediated excitotoxic neuronal cell death. *Mol. Neurobiol.* **24**, 107-129 (2001).
505. Anthony,D.C. *et al.* Differential matrix metalloproteinase expression in cases of multiple sclerosis and stroke. *Neuropathol. Appl. Neurobiol.* **23**, 406-415 (1997).
506. Lindberg,R.L. *et al.* The expression profile of matrix metalloproteinases (MMPs) and their inhibitors (TIMPs) in lesions and normal appearing white matter of multiple sclerosis. *Brain* **124**, 1743-1753 (2001).
507. Yong,V.W., Power,C., Forsyth,P., & Edwards,D.R. Metalloproteinases in biology and pathology of the nervous system. *Nat. Rev. Neurosci.* **2**, 502-511 (2001).
508. Yong,V.W., Zabad,R.K., Agrawal,S., Goncalves,D.A., & Metz,L.M. Elevation of matrix metalloproteinases (MMPs) in multiple sclerosis and impact of immunomodulators. *J. Neurol. Sci.* **259**, 79-84 (2007).

509. Lu,W., Bhasin,M., & Tsirka,S.E. Involvement of tissue plasminogen activator in onset and effector phases of experimental allergic encephalomyelitis. *J. Neurosci.* **22**, 10781-10789 (2002).

510. Pender,M.P. Demyelination and neurological signs in experimental allergic encephalomyelitis. *J. Neuroimmunol.* **15**, 11-24 (1987).

511. Aboul-Enein,F., Weiser,P., Hoftberger,R., Lassmann,H., & Bradl,M. Transient axonal injury in the absence of demyelination: a correlate of clinical disease in acute experimental autoimmune encephalomyelitis. *Acta Neuropathol.* **111**, 539-547 (2006).

512. Coman,I. *et al.* Nodal, paranodal and juxtaparanodal axonal proteins during demyelination and remyelination in multiple sclerosis. *Brain* **129**, 3186-3195 (2006).

513. Androdias,G. *et al.* Meningeal T cells associate with diffuse axonal loss in multiple sclerosis spinal cords. *Ann. Neurol.* **68**, 465-476 (2010).

514. Schirmer,L., Antel,J.P., Bruck,W., & Stadelmann,C. Axonal loss and neurofilament phosphorylation changes accompany lesion development and clinical progression in multiple sclerosis. *Brain Pathol.* **21**, 428-440 (2011).

515. Li,C. *et al.* Myelination in the absence of myelin-associated glycoprotein. *Nature* **369**, 747-750 (1994).

516. Griffiths,I. *et al.* Axonal swellings and degeneration in mice lacking the major proteolipid of myelin. *Science* **280**, 1610-1613 (1998).

517. Lappe-Siefke,C. *et al.* Disruption of Cnp1 uncouples oligodendroglial functions in axonal support and myelination. *Nat. Genet.* **33**, 366-374 (2003).

518. Schirmer,L., Merkler,D., Konig,F.B., Bruck,W., & Stadelmann,C. Neuroaxonal Regeneration is More Pronounced in Early Multiple Sclerosis than in Traumatic Brain Injury Lesions. *Brain Pathol.*(2012).

519. Kerschensteiner,M. *et al.* Remodeling of axonal connections contributes to recovery in an animal model of multiple sclerosis. *J. Exp. Med.* **200**, 1027-1038 (2004).

520. Goldschmidt,T., Antel,J., Konig,F.B., Bruck,W., & Kuhlmann,T. Remyelination capacity of the MS brain decreases with disease chronicity. *Neurology* **72**, 1914-1921 (2009).

521. Patani,R., Balaratnam,M., Vora,A., & Reynolds,R. Remyelination can be extensive in multiple sclerosis despite a long disease course. *Neuropathol. Appl. Neurobiol.* **33**, 277-287 (2007).

522. Lee,S. *et al.* Distinct macrophage phenotypes contribute to kidney injury and repair. *J. Am. Soc. Nephrol.* **22**, 317-326 (2011).

523. Umemura,N. *et al.* Tumor-infiltrating myeloid-derived suppressor cells are pleiotropic-inflamed monocytes/macrophages that bear M1- and M2-type characteristics. *J. Leukoc. Biol.* **83**, 1136-1144 (2008).

524. Kotter,M.R., Stadelmann,C., & Hartung,H.P. Enhancing remyelination in disease--can we wrap it up? *Brain* **134**, 1882-1900 (2011).

525. Morell,P. *et al.* Gene expression in brain during cuprizone-induced demyelination and remyelination. *Mol. Cell Neurosci.* **12**, 220-227 (1998).

526. Arnett,H.A., Wang,Y., Matsushima,G.K., Suzuki,K., & Ting,J.P. Functional genomic analysis of remyelination reveals importance of inflammation in oligodendrocyte regeneration. *J. Neurosci.* **23**, 9824-9832 (2003).

527. Li,W.W., Setzu,A., Zhao,C., & Franklin,R.J. Minocycline-mediated inhibition of microglia activation impairs oligodendrocyte progenitor cell responses and remyelination in a non-immune model of demyelination. *J. Neuroimmunol.* **158**, 58-66 (2005).

528. Prineas,J.W. & Raine,C.S. Electron microscopy and immunoperoxidase studies of early multiple sclerosis lesions. *Neurology* **26**, 29-32 (1976).

529. Raine,C.S. The Norton Lecture: a review of the oligodendrocyte in the multiple sclerosis lesion. *J. Neuroimmunol.* **77**, 135-152 (1997).

530. Wolswijk,G. Oligodendrocyte precursor cells in the demyelinated multiple sclerosis spinal cord. *Brain* **125**, 338-349 (2002).

531. Miller,R.H. Contact with central nervous system myelin inhibits oligodendrocyte progenitor maturation. *Dev. Biol.* **216**, 359-368 (1999).
532. Kotter,M.R., Li,W.W., Zhao,C., & Franklin,R.J. Myelin impairs CNS remyelination by inhibiting oligodendrocyte precursor cell differentiation. *J. Neurosci.* **26**, 328-332 (2006).
533. Kotter,M.R., Zhao,C., van Rooijen,N., & Franklin,R.J. Macrophage-depletion induced impairment of experimental CNS remyelination is associated with a reduced oligodendrocyte progenitor cell response and altered growth factor expression. *Neurobiol. Dis.* **18**, 166-175 (2005).
534. Takahashi,K., Prinz,M., Stagi,M., Chechneva,O., & Neumann,H. TREM2-transduced myeloid precursors mediate nervous tissue debris clearance and facilitate recovery in an animal model of multiple sclerosis. *PLoS Med.* **4**, e124 (2007).
535. Syed,Y.A. *et al.* Inhibition of oligodendrocyte precursor cell differentiation by myelin-associated proteins. *Neurosurg. Focus.* **24**, E5 (2008).
536. Baer,A.S. *et al.* Myelin-mediated inhibition of oligodendrocyte precursor differentiation can be overcome by pharmacological modulation of Fyn-RhoA and protein kinase C signalling. *Brain* **132**, 465-481 (2009).
537. Schwartz,M. & Kipnis,J. Protective autoimmunity and neuroprotection in inflammatory and noninflammatory neurodegenerative diseases. *J. Neurol. Sci.* **233**, 163-166 (2005).
538. Linker,R.A. *et al.* Leukemia inhibitory factor deficiency modulates the immune response and limits autoimmune demyelination: a new role for neurotrophic cytokines in neuroinflammation. *J. Immunol.* **180**, 2204-2213 (2008).
539. Butzkueven,H., Emery,B., Cipriani,T., Marriott,M.P., & Kilpatrick,T.J. Endogenous leukemia inhibitory factor production limits autoimmune demyelination and oligodendrocyte loss. *Glia* **53**, 696-703 (2006).
540. Vanderlocht,J. *et al.* Leukemia inhibitory factor is produced by myelin-reactive T cells from multiple sclerosis patients and protects against tumor necrosis factor-alpha-induced oligodendrocyte apoptosis. *J. Neurosci. Res.* **83**, 763-774 (2006).
541. Stadelmann,C. *et al.* BDNF and gp145trkB in multiple sclerosis brain lesions: neuroprotective interactions between immune and neuronal cells? *Brain* **125**, 75-85 (2002).
542. Elkabes,S., DiCicco-Bloom,E.M., & Black,I.B. Brain microglia/macrophages express neurotrophins that selectively regulate microglial proliferation and function. *J. Neurosci.* **16**, 2508-2521 (1996).
543. Barouch,R., Appel,E., Kazimirsky,G., & Brodie,C. Macrophages express neurotrophins and neurotrophin receptors. Regulation of nitric oxide production by NT-3. *J. Neuroimmunol.* **112**, 72-77 (2001).
544. Caroleo,M.C., Costa,N., Bracci-Laudiero,L., & Aloe,L. Human monocyte/macrophages activate by exposure to LPS overexpress NGF and NGF receptors. *J. Neuroimmunol.* **113**, 193-201 (2001).
545. Kerschensteiner,M. *et al.* Activated human T cells, B cells, and monocytes produce brain-derived neurotrophic factor in vitro and in inflammatory brain lesions: a neuroprotective role of inflammation? *J. Exp. Med.* **189**, 865-870 (1999).
546. Batchelor,P.E. *et al.* Activated macrophages and microglia induce dopaminergic sprouting in the injured striatum and express brain-derived neurotrophic factor and glial cell line-derived neurotrophic factor. *J. Neurosci.* **19**, 1708-1716 (1999).
547. Chari,D.M., Zhao,C., Kotter,M.R., Blakemore,W.F., & Franklin,R.J. Corticosteroids delay remyelination of experimental demyelination in the rodent central nervous system. *J. Neurosci. Res.* **83**, 594-605 (2006).
548. Foote,A.K. & Blakemore,W.F. Inflammation stimulates remyelination in areas of chronic demyelination. *Brain* **128**, 528-539 (2005).
549. Arnett,H.A. *et al.* TNF alpha promotes proliferation of oligodendrocyte progenitors and remyelination. *Nat. Neurosci.* **4**, 1116-1122 (2001).

550. Mason,J.L., Suzuki,K., Chaplin,D.D., & Matsushima,G.K. Interleukin-1beta promotes repair of the CNS. *J. Neurosci.* **21**, 7046-7052 (2001).
551. Ness,J.K. & Wood,T.L. Insulin-like growth factor I, but not neurotrophin-3, sustains Akt activation and provides long-term protection of immature oligodendrocytes from glutamate-mediated apoptosis. *Mol. Cell Neurosci.* **20**, 476-488 (2002).
552. Hsieh,J. *et al.* IGF-I instructs multipotent adult neural progenitor cells to become oligodendrocytes. *J. Cell Biol.* **164**, 111-122 (2004).
553. Butovsky,O. *et al.* Induction and blockage of oligodendrogenesis by differently activated microglia in an animal model of multiple sclerosis. *J. Clin. Invest* **116**, 905-915 (2006).
554. Liu,B.P., Cafferty,W.B., Budel,S.O., & Strittmatter,S.M. Extracellular regulators of axonal growth in the adult central nervous system. *Philos. Trans. R. Soc. Lond B Biol. Sci.* **361**, 1593-1610 (2006).
555. Prinjha,R. *et al.* Inhibitor of neurite outgrowth in humans. *Nature* **403**, 383-384 (2000).
556. GrandPre,T., Nakamura,F., Vartanian,T., & Strittmatter,S.M. Identification of the Nogo inhibitor of axon regeneration as a Reticulon protein. *Nature* **403**, 439-444 (2000).
557. Mukhopadhyay,G., Doherty,P., Walsh,F.S., Crocker,P.R., & Filbin,M.T. A novel role for myelin-associated glycoprotein as an inhibitor of axonal regeneration. *Neuron* **13**, 757-767 (1994).
558. Wang,K.C. *et al.* Oligodendrocyte-myelin glycoprotein is a Nogo receptor ligand that inhibits neurite outgrowth. *Nature* **417**, 941-944 (2002).
559. Ji,B. *et al.* LINGO-1 antagonist promotes functional recovery and axonal sprouting after spinal cord injury. *Mol. Cell Neurosci.* **33**, 311-320 (2006).
560. Mi,S. *et al.* LINGO-1 antagonist promotes spinal cord remyelination and axonal integrity in MOG-induced experimental autoimmune encephalomyelitis. *Nat. Med.* **13**, 1228-1233 (2007).
561. Karnezis,T. *et al.* The neurite outgrowth inhibitor Nogo A is involved in autoimmune-mediated demyelination. *Nat. Neurosci.* **7**, 736-744 (2004).
562. Merkler,D. *et al.* Locomotor recovery in spinal cord-injured rats treated with an antibody neutralizing the myelin-associated neurite growth inhibitor Nogo-A. *J. Neurosci.* **21**, 3665-3673 (2001).
563. Liu,X., Hawkes,E., Ishimaru,T., Tran,T., & Sretavan,D.W. EphB3: an endogenous mediator of adult axonal plasticity and regrowth after CNS injury. *J. Neurosci.* **26**, 3087-3101 (2006).
564. Yin,Y. *et al.* Macrophage-derived factors stimulate optic nerve regeneration. *J. Neurosci.* **23**, 2284-2293 (2003).
565. Lorber,B., Berry,M., & Logan,A. Lens injury stimulates adult mouse retinal ganglion cell axon regeneration via both macrophage- and lens-derived factors. *Eur. J. Neurosci.* **21**, 2029-2034 (2005).
566. Yin,Y. *et al.* Oncomodulin is a macrophage-derived signal for axon regeneration in retinal ganglion cells. *Nat. Neurosci.* **9**, 843-852 (2006).
567. Bolin,L.M., Verity,A.N., Silver,J.E., Shooter,E.M., & Abrams,J.S. Interleukin-6 production by Schwann cells and induction in sciatic nerve injury. *J. Neurochem.* **64**, 850-858 (1995).
568. Toulmond,S., Vige,X., Fage,D., & Benavides,J. Local infusion of interleukin-6 attenuates the neurotoxic effects of NMDA on rat striatal cholinergic neurons. *Neurosci. Lett.* **144**, 49-52 (1992).
569. Gruol,D.L. & Nelson,T.E. Physiological and pathological roles of interleukin-6 in the central nervous system. *Mol. Neurobiol.* **15**, 307-339 (1997).
570. Bruck,W. *et al.* Macrophages in multiple sclerosis. *Immunobiology* **195**, 588-600 (1996).
571. Hultqvist,M. *et al.* Enhanced autoimmunity, arthritis, and encephalomyelitis in mice with a reduced oxidative burst due to a mutation in the Ncf1 gene. *Proc. Natl. Acad. Sci. U. S. A* **101**, 12646-12651 (2004).

572. Komohara,Y. *et al.* AM-3K, an anti-macrophage antibody, recognizes CD163, a molecule associated with an anti-inflammatory macrophage phenotype. *J. Histochem. Cytochem.* **54**, 763-771 (2006).
573. Abraham,N.G. & Drummond,G. CD163-Mediated hemoglobin-heme uptake activates macrophage HO-1, providing an antiinflammatory function. *Circ. Res.* **99**, 911-914 (2006).
574. Spann,N.J. *et al.* Regulated accumulation of desmosterol integrates macrophage lipid metabolism and inflammatory responses. *Cell* **151**, 138-152 (2012).
575. Lawrence,T., Willoughby,D.A., & Gilroy,D.W. Anti-inflammatory lipid mediators and insights into the resolution of inflammation. *Nat. Rev. Immunol.* **2**, 787-795 (2002).
576. Brem-Exner,B.G. *et al.* Macrophages driven to a novel state of activation have anti-inflammatory properties in mice. *J. Immunol.* **180**, 335-349 (2008).
577. Hikawa,N. & Takenaka,T. Myelin-stimulated macrophages release neurotrophic factors for adult dorsal root ganglion neurons in culture. *Cell Mol. Neurobiol.* **16**, 517-528 (1996).
578. Blewett,M.M. Lipid autoreactivity in multiple sclerosis. *Med. Hypotheses* **74**, 433-442 (2010).
579. Hong,C. & Tontonoz,P. Coordination of inflammation and metabolism by PPAR and LXR nuclear receptors. *Curr. Opin. Genet. Dev.* **18**, 461-467 (2008).
580. Bensinger,S.J. & Tontonoz,P. Integration of metabolism and inflammation by lipid-activated nuclear receptors. *Nature* **454**, 470-477 (2008).
581. Valledor,A.F. & Ricote,M. Nuclear receptor signaling in macrophages. *Biochem. Pharmacol.* **67**, 201-212 (2004).
582. Bouhlel,M.A. *et al.* PPARgamma activation primes human monocytes into alternative M2 macrophages with anti-inflammatory properties. *Cell Metab* **6**, 137-143 (2007).
583. Evans,R.M., Barish,G.D., & Wang,Y.X. PPARs and the complex journey to obesity. *Nat. Med.* **10**, 355-361 (2004).
584. Kang,K. *et al.* Adipocyte-derived Th2 cytokines and myeloid PPARdelta regulate macrophage polarization and insulin sensitivity. *Cell Metab* **7**, 485-495 (2008).
585. Lovren,F. *et al.* Adiponectin primes human monocytes into alternative anti-inflammatory M2 macrophages. *Am. J. Physiol Heart Circ. Physiol* **299**, H656-H663 (2010).
586. Odegaard,J.I. *et al.* Macrophage-specific PPARgamma controls alternative activation and improves insulin resistance. *Nature* **447**, 1116-1120 (2007).
587. Odegaard,J.I. *et al.* Alternative M2 activation of Kupffer cells by PPARdelta ameliorates obesity-induced insulin resistance. *Cell Metab* **7**, 496-507 (2008).
588. Joseph,S.B., Castrillo,A., Laffitte,B.A., Mangelsdorf,D.J., & Tontonoz,P. Reciprocal regulation of inflammation and lipid metabolism by liver X receptors. *Nat. Med.* **9**, 213-219 (2003).
589. Castrillo,A., Joseph,S.B., Marathe,C., Mangelsdorf,D.J., & Tontonoz,P. Liver X receptor-dependent repression of matrix metalloproteinase-9 expression in macrophages. *J. Biol. Chem.* **278**, 10443-10449 (2003).
590. Ghisletti,S. *et al.* Parallel SUMOylation-dependent pathways mediate gene- and signal-specific transrepression by LXRs and PPARgamma. *Mol. Cell* **25**, 57-70 (2007).
591. Ghisletti,S. *et al.* Cooperative NCoR/SMRT interactions establish a corepressor-based strategy for integration of inflammatory and anti-inflammatory signaling pathways. *Genes Dev.* **23**, 681-693 (2009).
592. Ogawa,S. *et al.* Molecular determinants of crosstalk between nuclear receptors and toll-like receptors. *Cell* **122**, 707-721 (2005).
593. Mukundan,L. *et al.* PPAR-delta senses and orchestrates clearance of apoptotic cells to promote tolerance. *Nat. Med.* **15**, 1266-1272 (2009).
594. Kodama,T. *et al.* Type I macrophage scavenger receptor contains alpha-helical and collagen-like coiled coils. *Nature* **343**, 531-535 (1990).

595. Nakamura,K. *et al.* Possible role of scavenger receptor SRCL in the clearance of amyloid-beta in Alzheimer's disease. *J. Neurosci. Res.* **84**, 874-890 (2006).
596. Vanderlocht,J., Hellings,N., Hendriks,J.J., & Stinissen,P. The ambivalent nature of T-cell infiltration in the central nervous system of patients with multiple sclerosis. *Crit Rev. Immunol.* **27**, 1-13 (2007).
597. Glim,J.E., Vereyken,E.J., Heijnen,D.A., Vallejo,J.J., & Dijkstra,C.D. The release of cytokines by macrophages is not affected by myelin ingestion. *Glia* **58**, 1928-1936 (2010).
598. Barnett,M.H., Henderson,A.P., & Prineas,J.W. The macrophage in MS: just a scavenger after all? Pathology and pathogenesis of the acute MS lesion. *Mult. Scler.* **12**, 121-132 (2006).
599. Pineau,I. & Lacroix,S. Proinflammatory cytokine synthesis in the injured mouse spinal cord: multiphasic expression pattern and identification of the cell types involved. *J. Comp Neurol.* **500**, 267-285 (2007).
600. Miller,E., Mrowicka,M., Zolynski,K., & Kedziora,J. [Oxidative stress in multiple sclerosis]. *Pol. Merkur Lekarski.* **27**, 499-502 (2009).
601. Gredler,V. *et al.* Impact of human myelin on the maturation and function of human monocyte-derived dendritic cells. *Clin. Immunol.* **134**, 296-304 (2010).
602. Hendriks,J.J. *et al.* Leukemia inhibitory factor modulates production of inflammatory mediators and myelin phagocytosis by macrophages. *J. Neuroimmunol.* **204**, 52-57 (2008).
603. Norton,W.T. & Poduslo,S.E. Myelination in rat brain: changes in myelin composition during brain maturation. *J. Neurochem.* **21**, 759-773 (1973).
604. Vandenbark,A.A., Gill,T., & Offner,H. A myelin basic protein-specific T lymphocyte line that mediates experimental autoimmune encephalomyelitis. *J. Immunol.* **135**, 223-228 (1985).
605. Lescano-De-Souza,A., Jr. & Curi,R. Cholesterol inhibits glutamine metabolism in LLC WRC256 tumour cells but does not affect it in lymphocytes: possible implications for tumour cell proliferation. *Cell Biochem. Funct.* **17**, 223-228 (1999).
606. Nishiyama,A., Cavaglieri,C.R., Curi,R., & Calder,P.C. Arachidonic acid-containing phosphatidylcholine inhibits lymphocyte proliferation and decreases interleukin-2 and interferon-gamma production from concanavalin A-stimulated rat lymphocytes. *Biochim. Biophys. Acta* **1487**, 50-60 (2000).
607. Rodriguez,P.C. *et al.* Arginase I production in the tumor microenvironment by mature myeloid cells inhibits T-cell receptor expression and antigen-specific T-cell responses. *Cancer Res.* **64**, 5839-5849 (2004).
608. Rodriguez,P.C. *et al.* L-arginine consumption by macrophages modulates the expression of CD3 zeta chain in T lymphocytes. *J. Immunol.* **171**, 1232-1239 (2003).
609. Peres,C.M., Otton,R., & Curi,R. Modulation of lymphocyte proliferation by macrophages and macrophages loaded with arachidonic acid. *Cell Biochem. Funct.* **23**, 373-381 (2005).
610. Denham,S. & Rowland,I.J. Inhibition of the reactive proliferation of lymphocytes by activated macrophages: the role of nitric oxide. *Clin. Exp. Immunol.* **87**, 157-162 (1992).
611. Bingisser,R.M., Tilbrook,P.A., Holt,P.G., & Kees,U.R. Macrophage-derived nitric oxide regulates T cell activation via reversible disruption of the Jak3/STAT5 signaling pathway. *J. Immunol.* **160**, 5729-5734 (1998).
612. Munn,D.H. *et al.* Inhibition of T cell proliferation by macrophage tryptophan catabolism. *J. Exp. Med.* **189**, 1363-1372 (1999).
613. Zea,A.H. *et al.* Arginase-producing myeloid suppressor cells in renal cell carcinoma patients: a mechanism of tumor evasion. *Cancer Res.* **65**, 3044-3048 (2005).
614. Ahvazi,B.C., Jacobs,P., & Stevenson,M.M. Role of macrophage-derived nitric oxide in suppression of lymphocyte proliferation during blood-stage malaria. *J. Leukoc. Biol.* **58**, 23-31 (1995).

615. Albina,J.E., Abate,J.A., & Henry,W.L., Jr. Nitric oxide production is required for murine resident peritoneal macrophages to suppress mitogen-stimulated T cell proliferation. Role of IFN-gamma in the induction of the nitric oxide-synthesizing pathway. *J. Immunol.* **147**, 144-148 (1991).
616. Isobe,K. & Nakashima,I. Feedback suppression of staphylococcal enterotoxin-stimulated T-lymphocyte proliferation by macrophages through inductive nitric oxide synthesis. *Infect. Immun.* **60**, 4832-4837 (1992).
617. Shimizu,T., Cai,S., & Tomioka,H. Roles of reactive nitrogen intermediates and transforming growth factor-beta produced by immunosuppressive macrophages in the expression of suppressor activity against T cell proliferation induced by TCR stimulation. *Cytokine* **30**, 7-13 (2005).
618. Hamilton,M.J. *et al.* TLR agonists that induce IFN-beta abrogate resident macrophage suppression of T cells. *J. Immunol.* **185**, 4545-4553 (2010).
619. Ignarro,L.J. Biosynthesis and metabolism of endothelium-derived nitric oxide. *Annu. Rev. Pharmacol. Toxicol.* **30**, 535-560 (1990).
620. Carrasco,Y.R. & Batista,F.D. B cells acquire particulate antigen in a macrophage-rich area at the boundary between the follicle and the subcapsular sinus of the lymph node. *Immunity.* **27**, 160-171 (2007).
621. Junt,T. *et al.* Subcapsular sinus macrophages in lymph nodes clear lymph-borne viruses and present them to antiviral B cells. *Nature* **450**, 110-114 (2007).
622. Phan,T.G., Grigorova,I., Okada,T., & Cyster,J.G. Subcapsular encounter and complement-dependent transport of immune complexes by lymph node B cells. *Nat. Immunol.* **8**, 992-1000 (2007).
623. Phan,T.G., Green,J.A., Gray,E.E., Xu,Y., & Cyster,J.G. Immune complex relay by subcapsular sinus macrophages and noncognate B cells drives antibody affinity maturation. *Nat. Immunol.* **10**, 786-793 (2009).
624. Barral,P. *et al.* CD169(+) macrophages present lipid antigens to mediate early activation of iNKT cells in lymph nodes. *Nat. Immunol.* **11**, 303-312 (2010).
625. O'Brien,N.C., Charlton,B., Cowden,W.B., & Willenborg,D.O. Nitric oxide plays a critical role in the recovery of Lewis rats from experimental autoimmune encephalomyelitis and the maintenance of resistance to reinduction. *J. Immunol.* **163**, 6841-6847 (1999).
626. Kahl,K.G. *et al.* Protective role of the cytokine-inducible isoform of nitric oxide synthase induction and nitrosative stress in experimental autoimmune encephalomyelitis of the DA rat. *J. Neurosci. Res.* **73**, 198-205 (2003).
627. Cowden,W.B., Cullen,F.A., Staykova,M.A., & Willenborg,D.O. Nitric oxide is a potential down-regulating molecule in autoimmune disease: inhibition of nitric oxide production renders PVG rats highly susceptible to EAE. *J. Neuroimmunol.* **88**, 1-8 (1998).
628. Staykova,M.A., Paridaen,J.T., Cowden,W.B., & Willenborg,D.O. Nitric oxide contributes to resistance of the Brown Norway rat to experimental autoimmune encephalomyelitis. *Am. J. Pathol.* **166**, 147-157 (2005).
629. Wang,P.Y., Kitchens,R.L., & Munford,R.S. Phosphatidylinositides bind to plasma membrane CD14 and can prevent monocyte activation by bacterial lipopolysaccharide. *J. Biol. Chem.* **273**, 24309-24313 (1998).
630. Jou,I. *et al.* Gangliosides trigger inflammatory responses via TLR4 in brain glia. *Am. J. Pathol.* **168**, 1619-1630 (2006).
631. Jeon,S.B., Yoon,H.J., Park,S.H., Kim,I.H., & Park,E.J. Sulfatide, a major lipid component of myelin sheath, activates inflammatory responses as an endogenous stimulator in brain-resident immune cells. *J. Immunol.* **181**, 8077-8087 (2008).
632. Lee,C., An,H.J., Kim,J.L., Lee,H., & Paik,S.G. Inhibitory effect of a phosphatidyl ethanolamine derivative on LPS-induced sepsis. *Mol. Cells* **27**, 251-255 (2009).
633. Ghosn,E.E. *et al.* Two physically, functionally, and developmentally distinct peritoneal macrophage subsets. *Proc. Natl. Acad. Sci. U. S. A* **107**, 2568-2573 (2010).

634. Bogie,J.F., Stinissen,P., Hellings,N., & Hendriks,J.J. Myelin-phagocytosing macrophages modulate autoreactive T cell proliferation. *J. Neuroinflammation.* **8**, 85 (2011).
635. Cannella,B. & Raine,C.S. The adhesion molecule and cytokine profile of multiple sclerosis lesions. *Ann. Neurol.* **37**, 424-435 (1995).
636. Alberti,S. *et al.* Hepatic cholesterol metabolism and resistance to dietary cholesterol in LXRbeta-deficient mice. *J. Clin. Invest* **107**, 565-573 (2001).
637. Gentleman,R.C. *et al.* Bioconductor: open software development for computational biology and bioinformatics. *Genome Biol.* **5**, R80 (2004).
638. Gautier,L., Cope,L., Bolstad,B.M., & Irizarry,R.A. affy--analysis of Affymetrix GeneChip data at the probe level. *Bioinformatics.* **20**, 307-315 (2004).
639. Irizarry,R.A. *et al.* Exploration, normalization, and summaries of high density oligonucleotide array probe level data. *Biostatistics.* **4**, 249-264 (2003).
640. Edgar,R., Domrachev,M., & Lash,A.E. Gene Expression Omnibus: NCBI gene expression and hybridization array data repository. *Nucleic Acids Res.* **30**, 207-210 (2002).
641. Huang,d.W., Sherman,B.T., & Lempicki,R.A. Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. *Nat. Protoc.* **4**, 44-57 (2009).
642. Vandesompele,J. *et al.* Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome Biol.* **3**, RESEARCH0034 (2002).
643. Nelissen,K., Smeets,K., Mulder,M., Hendriks,J.J., & Ameloot,M. Selection of reference genes for gene expression studies in rat oligodendrocytes using quantitative real time PCR. *J. Neurosci. Methods* **187**, 78-83 (2010).
644. Papenfuss,T.L. *et al.* Induction of Golli-MBP expression in CNS macrophages during acute LPS-induced CNS inflammation and experimental autoimmune encephalomyelitis (EAE). *ScientificWorldJournal.* **7**, 112-120 (2007).
645. Oram,J.F. & Yokoyama,S. Apolipoprotein-mediated removal of cellular cholesterol and phospholipids. *J. Lipid Res.* **37**, 2473-2491 (1996).
646. Kennedy,M.A. *et al.* ABCG1 has a critical role in mediating cholesterol efflux to HDL and preventing cellular lipid accumulation. *Cell Metab* **1**, 121-131 (2005).
647. Vaughan,A.M. & Oram,J.F. ABCG1 redistributes cell cholesterol to domains removable by high density lipoprotein but not by lipid-depleted apolipoproteins. *J. Biol. Chem.* **280**, 30150-30157 (2005).
648. Smith,J.D. *et al.* ABCA1 mediates concurrent cholesterol and phospholipid efflux to apolipoprotein A-I. *J. Lipid Res.* **45**, 635-644 (2004).
649. Wang,N., Lan,D., Chen,W., Matsuura,F., & Tall,A.R. ATP-binding cassette transporters G1 and G4 mediate cellular cholesterol efflux to high-density lipoproteins. *Proc. Natl. Acad. Sci. U. S. A* **101**, 9774-9779 (2004).
650. Wu,X., Bayle,J.H., Olson,D., & Levine,A.J. The p53-mdm-2 autoregulatory feedback loop. *Genes Dev.* **7**, 1126-1132 (1993).
651. Barak,Y., Juven,T., Haffner,R., & Oren,M. mdm2 expression is induced by wild type p53 activity. *EMBO J.* **12**, 461-468 (1993).
652. Espinosa,J.M. & Emerson,B.M. Transcriptional regulation by p53 through intrinsic DNA/chromatin binding and site-directed cofactor recruitment. *Mol. Cell* **8**, 57-69 (2001).
653. Hofmann,T.G. *et al.* Regulation of p53 activity by its interaction with homeodomain-interacting protein kinase-2. *Nat. Cell Biol.* **4**, 1-10 (2002).
654. D'Orazi,G. *et al.* Homeodomain-interacting protein kinase-2 phosphorylates p53 at Ser 46 and mediates apoptosis. *Nat. Cell Biol.* **4**, 11-19 (2002).
655. Lai,Y. *et al.* Interleukin-8 induces the endothelial cell migration through the activation of phosphoinositide 3-kinase-Rac1/RhoA pathway. *Int. J. Biol. Sci.* **7**, 782-791 (2011).
656. Fox,R. *et al.* PSGL-1 and mTOR regulate translation of ROCK-1 and physiological functions of macrophages. *EMBO J.* **26**, 505-515 (2007).
657. Alfaro,C. *et al.* Carcinoma-derived interleukin-8 disorients dendritic cell migration without impairing T-cell stimulation. *PLoS. One.* **6**, e17922 (2011).

658. Matsumoto,T. *et al.* Pivotal role of interleukin-8 in the acute respiratory distress syndrome and cerebral reperfusion injury. *J. Leukoc. Biol.* **62**, 581-587 (1997).
659. Smythies,L.E. *et al.* Mucosal IL-8 and TGF-beta recruit blood monocytes: evidence for cross-talk between the lamina propria stroma and myeloid cells. *J. Leukoc. Biol.* **80**, 492-499 (2006).
660. Sordi,V. *et al.* Differential effects of immunosuppressive drugs on chemokine receptor CCR7 in human monocyte-derived dendritic cells: selective upregulation by rapamycin. *Transplantation* **82**, 826-834 (2006).
661. Vega,F.M., Fruhwirth,G., Ng,T., & Ridley,A.J. RhoA and RhoC have distinct roles in migration and invasion by acting through different targets. *J. Cell Biol.* **193**, 655-665 (2011).
662. Gitik,M., Reichert,F., & Rotshenker,S. Cytoskeleton plays a dual role of activation and inhibition in myelin and zymosan phagocytosis by microglia. *FASEB J.* **24**, 2211-2221 (2010).
663. Ogden,C.A., Kowalewski,R., Peng,Y., Montenegro,V., & Elkon,K.B. IGM is required for efficient complement mediated phagocytosis of apoptotic cells in vivo. *Autoimmunity* **38**, 259-264 (2005).
664. Ogden,C.A. *et al.* C1q and mannose binding lectin engagement of cell surface calreticulin and CD91 initiates macropinocytosis and uptake of apoptotic cells. *J. Exp. Med.* **194**, 781-795 (2001).
665. Weinstein,S.L. *et al.* Phosphatidylinositol 3-kinase and mTOR mediate lipopolysaccharide-stimulated nitric oxide production in macrophages via interferon-beta. *J. Leukoc. Biol.* **67**, 405-414 (2000).
666. Essayan,D.M. Cyclic nucleotide phosphodiesterase (PDE) inhibitors and immunomodulation. *Biochem. Pharmacol.* **57**, 965-973 (1999).
667. Paterniti,I. *et al.* PDE 7 inhibitors: new potential drugs for the therapy of spinal cord injury. *PLoS. One.* **6**, e15937 (2011).
668. Castano,T. *et al.* Synthesis, structural analysis, and biological evaluation of thioxoquinazoline derivatives as phosphodiesterase 7 inhibitors. *ChemMedChem.* **4**, 866-876 (2009).
669. Guo,R.F., Riedemann,N.C., & Ward,P.A. Role of C5a-C5aR interaction in sepsis. *Shock* **21**, 1-7 (2004).
670. Iynedjian,P.B. Molecular physiology of mammalian glucokinase. *Cell Mol. Life Sci.* **66**, 27-42 (2009).
671. Jeyakumar,M., Butters,T.D., Dwek,R.A., & Platt,F.M. Glycosphingolipid lysosomal storage diseases: therapy and pathogenesis. *Neuropathol. Appl. Neurobiol.* **28**, 343-357 (2002).
672. Jou,I. *et al.* Gangliosides trigger inflammatory responses via TLR4 in brain glia. *Am. J. Pathol.* **168**, 1619-1630 (2006).
673. Park,E.J. *et al.* Dietary ganglioside inhibits acute inflammatory signals in intestinal mucosa and blood induced by systemic inflammation of Escherichia coli lipopolysaccharide. *Shock* **28**, 112-117 (2007).
674. Chawla,A. *et al.* A PPAR gamma-LXR-ABCA1 pathway in macrophages is involved in cholesterol efflux and atherogenesis. *Mol. Cell* **7**, 161-171 (2001).
675. Zhang,Y. *et al.* Liver X receptor agonist TO-901317 upregulates SCD1 expression in renal proximal straight tubule. *Am. J. Physiol Renal Physiol* **290**, F1065-F1073 (2006).
676. Tall,A.R. Cholesterol efflux pathways and other potential mechanisms involved in the athero-protective effect of high density lipoproteins. *J. Intern. Med.* **263**, 256-273 (2008).
677. Tabas,I. Consequences of cellular cholesterol accumulation: basic concepts and physiological implications. *J. Clin. Invest* **110**, 905-911 (2002).
678. Peet,D.J. *et al.* Cholesterol and bile acid metabolism are impaired in mice lacking the nuclear oxysterol receptor LXR alpha. *Cell* **93**, 693-704 (1998).
679. Repa,J.J. & Mangelsdorf,D.J. The liver X receptor gene team: potential new players in atherosclerosis. *Nat. Med.* **8**, 1243-1248 (2002).

680. Bjorkhem,I. & Meaney,S. Brain cholesterol: long secret life behind a barrier. *Arterioscler. Thromb. Vasc. Biol.* **24**, 806-815 (2004).
681. Cui,G. *et al.* Liver X receptor (LXR) mediates negative regulation of mouse and human Th17 differentiation. *J. Clin. Invest* **121**, 658-670 (2011).
682. Xu,J., Wagoner,G., Douglas,J.C., & Drew,P.D. Liver X receptor agonist regulation of Th17 lymphocyte function in autoimmunity. *J. Leukoc. Biol.* **86**, 401-409 (2009).
683. Hindinger,C. *et al.* Liver X receptor activation decreases the severity of experimental autoimmune encephalomyelitis. *J. Neurosci. Res.* **84**, 1225-1234 (2006).
684. Giorelli,M., Livrea,P., Minervini,M.G., & Trojano,M. Immunomodulatory properties of increased levels of liver X receptor beta in peripheral blood mononuclear cells from multiple sclerosis patients. *Exp. Neurol.* **204**, 759-766 (2007).
685. Bogie,J.F. *et al.* Myelin-derived lipids modulate macrophage activity by liver X receptor activation. *PLoS. One.* **7**, e44998 (2012).
686. Fadok,V.A. *et al.* A receptor for phosphatidylserine-specific clearance of apoptotic cells. *Nature* **405**, 85-90 (2000).
687. Zwaal,R.F. & Schroit,A.J. Pathophysiologic implications of membrane phospholipid asymmetry in blood cells. *Blood* **89**, 1121-1132 (1997).
688. Fadok,V.A. *et al.* Macrophages that have ingested apoptotic cells in vitro inhibit proinflammatory cytokine production through autocrine/paracrine mechanisms involving TGF-beta, PGE2, and PAF. *J. Clin. Invest* **101**, 890-898 (1998).
689. Hoffmann,P.R. *et al.* Interaction between phosphatidylserine and the phosphatidylserine receptor inhibits immune responses in vivo. *J. Immunol.* **174**, 1393-1404 (2005).
690. Hoffmann,P.R. *et al.* Phosphatidylserine (PS) induces PS receptor-mediated macropinocytosis and promotes clearance of apoptotic cells. *J. Cell Biol.* **155**, 649-659 (2001).
691. Huynh,M.L., Fadok,V.A., & Henson,P.M. Phosphatidylserine-dependent ingestion of apoptotic cells promotes TGF-beta1 secretion and the resolution of inflammation. *J. Clin. Invest* **109**, 41-50 (2002).
692. Kleinclauss,F. *et al.* Intravenous apoptotic spleen cell infusion induces a TGF-beta-dependent regulatory T-cell expansion. *Cell Death. Differ.* **13**, 41-52 (2006).
693. Burgess,A. *et al.* Loss of human Greatwall results in G2 arrest and multiple mitotic defects due to deregulation of the cyclin B-Cdc2/PP2A balance. *Proc. Natl. Acad. Sci. U. S. A* **107**, 12564-12569 (2010).
694. Coll,T. *et al.* Activation of peroxisome proliferator-activated receptor-{delta} by GW501516 prevents fatty acid-induced nuclear factor-{kappa}B activation and insulin resistance in skeletal muscle cells. *Endocrinology* **151**, 1560-1569 (2010).
695. Degenhardt,T. *et al.* Three members of the human pyruvate dehydrogenase kinase gene family are direct targets of the peroxisome proliferator-activated receptor beta/delta. *J. Mol. Biol.* **372**, 341-355 (2007).
696. Schmuth,M. *et al.* Peroxisome proliferator-activated receptor (PPAR)-beta/delta stimulates differentiation and lipid accumulation in keratinocytes. *J. Invest Dermatol.* **122**, 971-983 (2004).
697. Aramaki,Y., Nitta,F., Matsuno,R., Morimura,Y., & Tsuchiya,S. Inhibitory effects of negatively charged liposomes on nitric oxide production from macrophages stimulated by LPS. *Biochem. Biophys. Res. Commun.* **220**, 1-6 (1996).
698. De,S.R., Ajmone-Cat,M.A., Nicolini,A., & Minghetti,L. Expression of phosphatidylserine receptor and down-regulation of pro-inflammatory molecule production by its natural ligand in rat microglial cultures. *J. Neuropathol. Exp. Neurol.* **61**, 237-244 (2002).

699. Harel-Adar,T. *et al.* Modulation of cardiac macrophages by phosphatidylserine-presenting liposomes improves infarct repair. *Proc. Natl. Acad. Sci. U. S. A* **108**, 1827-1832 (2011).
700. Ma,H.M., Wu,Z., & Nakanishi,H. Phosphatidylserine-containing liposomes suppress inflammatory bone loss by ameliorating the cytokine imbalance provoked by infiltrated macrophages. *Lab Invest* **91**, 921-931 (2011).
701. Otsuka,M., Tsuchiya,S., & Aramaki,Y. Involvement of ERK, a MAP kinase, in the production of TGF-beta by macrophages treated with liposomes composed of phosphatidylserine. *Biochem. Biophys. Res. Commun.* **324**, 1400-1405 (2004).
702. Zhang,J. *et al.* Involvement of COX-1 and up-regulated prostaglandin E synthases in phosphatidylserine liposome-induced prostaglandin E2 production by microglia. *J. Neuroimmunol.* **172**, 112-120 (2006).
703. Ramos,G.C. *et al.* Apoptotic mimicry: phosphatidylserine liposomes reduce inflammation through activation of peroxisome proliferator-activated receptors (PPARs) in vivo. *Br. J. Pharmacol.* **151**, 844-850 (2007).
704. Ho,P.P. *et al.* Identification of naturally occurring fatty acids of the myelin sheath that resolve neuroinflammation. *Sci. Transl. Med.* **4**, 137ra73 (2012).
705. Diab,A. *et al.* Peroxisome proliferator-activated receptor-gamma agonist 15-deoxy-Delta(12,14)-prostaglandin J(2) ameliorates experimental autoimmune encephalomyelitis. *J. Immunol.* **168**, 2508-2515 (2002).
706. Polak,P.E. *et al.* Protective effects of a peroxisome proliferator-activated receptor-beta/delta agonist in experimental autoimmune encephalomyelitis. *J. Neuroimmunol.* **168**, 65-75 (2005).
707. Feinstein,D.L. *et al.* Peroxisome proliferator-activated receptor-gamma agonists prevent experimental autoimmune encephalomyelitis. *Ann. Neurol.* **51**, 694-702 (2002).
708. Gocke,A.R. *et al.* Transcriptional modulation of the immune response by peroxisome proliferator-activated receptor-{alpha} agonists in autoimmune disease. *J. Immunol.* **182**, 4479-4487 (2009).
709. Kanakasabai,S. *et al.* Peroxisome proliferator-activated receptor delta agonists inhibit T helper type 1 (Th1) and Th17 responses in experimental allergic encephalomyelitis. *Immunology* **130**, 572-588 (2010).
710. Lovett-Racke,A.E. *et al.* Peroxisome proliferator-activated receptor alpha agonists as therapy for autoimmune disease. *J. Immunol.* **172**, 5790-5798 (2004).
711. Natarajan,C. & Bright,J.J. Peroxisome proliferator-activated receptor-gamma agonists inhibit experimental allergic encephalomyelitis by blocking IL-12 production, IL-12 signaling and Th1 differentiation. *Genes Immun.* **3**, 59-70 (2002).
712. Niino,M. *et al.* Amelioration of experimental autoimmune encephalomyelitis in C57BL/6 mice by an agonist of peroxisome proliferator-activated receptor-gamma. *J. Neuroimmunol.* **116**, 40-48 (2001).
713. Nakamura,K., Funakoshi,H., Miyamoto,K., Tokunaga,F., & Nakamura,T. Molecular cloning and functional characterization of a human scavenger receptor with C-type lectin (SRCL), a novel member of a scavenger receptor family. *Biochem. Biophys. Res. Commun.* **280**, 1028-1035 (2001).
714. Nakamura,K., Funakoshi,H., Tokunaga,F., & Nakamura,T. Molecular cloning of a mouse scavenger receptor with C-type lectin (SRCL)(1), a novel member of the scavenger receptor family. *Biochim. Biophys. Acta* **1522**, 53-58 (2001).
715. Ohtani,K. *et al.* The membrane-type collectin CL-P1 is a scavenger receptor on vascular endothelial cells. *J. Biol. Chem.* **276**, 44222-44228 (2001).
716. Jang,S. *et al.* Scavenger receptor collectin placenta 1 (CL-P1) predominantly mediates zymosan phagocytosis by human vascular endothelial cells. *J. Biol. Chem.* **284**, 3956-3965 (2009).
717. Coombs,P.J., Graham,S.A., Drickamer,K., & Taylor,M.E. Selective binding of the scavenger receptor C-type lectin to Lewisx trisaccharide and related glycan ligands. *J. Biol. Chem.* **280**, 22993-22999 (2005).

718. Feinberg,H., Taylor,M.E., & Weis,W.I. Scavenger receptor C-type lectin binds to the leukocyte cell surface glycan Lewis(x) by a novel mechanism. *J. Biol. Chem.* **282**, 17250-17258 (2007).
719. Elola,M.T. *et al.* Lewis x antigen mediates adhesion of human breast carcinoma cells to activated endothelium. Possible involvement of the endothelial scavenger receptor C-type lectin. *Breast Cancer Res. Treat.* **101**, 161-174 (2007).
720. Yoshida,T. *et al.* SRCL/CL-P1 recognizes GalNAc and a carcinoma-associated antigen, Tn antigen. *J. Biochem.* **133**, 271-277 (2003).
721. Hendriks,J.J. *et al.* Flavonoids inhibit myelin phagocytosis by macrophages; a structure-activity relationship study. *Biochem. Pharmacol.* **65**, 877-885 (2003).
722. Kirkeby,S. & Thomsen,C.E. Quantitative immunohistochemistry of fluorescence labelled probes using low-cost software. *J. Immunol. Methods* **301**, 102-113 (2005).
723. Lee,S.C., Moore,G.R., Golenwsky,G., & Raine,C.S. Multiple sclerosis: a role for astroglia in active demyelination suggested by class II MHC expression and ultrastructural study. *J. Neuropathol. Exp. Neurol.* **49**, 122-136 (1990).
724. Kuhlmann,T. *et al.* Differential regulation of myelin phagocytosis by macrophages/microglia, involvement of target myelin, Fc receptors and activation by intravenous immunoglobulins. *J. Neurosci. Res.* **67**, 185-190 (2002).
725. Carson,M.J. *et al.* A rose by any other name? The potential consequences of microglial heterogeneity during CNS health and disease. *Neurotherapeutics.* **4**, 571-579 (2007).
726. Polfliet,M.M. *et al.* Meningeal and perivascular macrophages of the central nervous system play a protective role during bacterial meningitis. *J. Immunol.* **167**, 4644-4650 (2001).
727. Williams,K.C. *et al.* Perivascular macrophages are the primary cell type productively infected by simian immunodeficiency virus in the brains of macaques: implications for the neuropathogenesis of AIDS. *J. Exp. Med.* **193**, 905-915 (2001).
728. Tontonoz,P., Nagy,L., Alvarez,J.G., Thomazy,V.A., & Evans,R.M. PPARgamma promotes monocyte/macrophage differentiation and uptake of oxidized LDL. *Cell* **93**, 241-252 (1998).
729. Joseph,S.B. *et al.* LXR-dependent gene expression is important for macrophage survival and the innate immune response. *Cell* **119**, 299-309 (2004).
730. Guo,Y. *et al.* Structural basis for distinct ligand-binding and targeting properties of the receptors DC-SIGN and DC-SIGNR. *Nat. Struct. Mol. Biol.* **11**, 591-598 (2004).
731. Appelmelk,B.J. *et al.* Cutting edge: carbohydrate profiling identifies new pathogens that interact with dendritic cell-specific ICAM-3-grabbing nonintegrin on dendritic cells. *J. Immunol.* **170**, 1635-1639 (2003).
732. Svajger,U., Anderluh,M., Jeras,M., & Obermajer,N. C-type lectin DC-SIGN: an adhesion, signalling and antigen-uptake molecule that guides dendritic cells in immunity. *Cell Signal.* **22**, 1397-1405 (2010).
733. Ehrhardt,C., Kneuer,C., & Bakowsky,U. Selectins-an emerging target for drug delivery. *Adv. Drug Deliv. Rev.* **56**, 527-549 (2004).
734. Nair,A., Frederick,T.J., & Miller,S.D. Astrocytes in multiple sclerosis: a product of their environment. *Cell Mol. Life Sci.* **65**, 2702-2720 (2008).
735. Farina,C., Aloisi,F., & Meinl,E. Astrocytes are active players in cerebral innate immunity. *Trends Immunol.* **28**, 138-145 (2007).
736. Nair,A., Frederick,T.J., & Miller,S.D. Astrocytes in multiple sclerosis: a product of their environment. *Cell Mol. Life Sci.* **65**, 2702-2720 (2008).
737. Huizinga,R., Hintzen,R.Q., Assink,K., van,M.M., & Amor,S. T-cell responses to neurofilament light protein are part of the normal immune repertoire. *Int. Immunol.* **21**, 433-441 (2009).

738. Forooghian,F., Cheung,R.K., Smith,W.C., O'Connor,P., & Dosch,H.M. Enolase and arrestin are novel nonmyelin autoantigens in multiple sclerosis. *J. Clin. Immunol.* **27**, 388-396 (2007).
739. Gautier,E.L. *et al.* Gene-expression profiles and transcriptional regulatory pathways that underlie the identity and diversity of mouse tissue macrophages. *Nat. Immunol.* **13**, 1118-1128 (2012).
740. Korach-Andre,M. *et al.* Separate and overlapping metabolic functions of LXRalpha and LXRbeta in C57Bl/6 female mice. *Am. J. Physiol Endocrinol. Metab* **298**, E167-E178 (2010).
741. Lund,E.G. *et al.* Different roles of liver X receptor alpha and beta in lipid metabolism: effects of an alpha-selective and a dual agonist in mice deficient in each subtype. *Biochem. Pharmacol.* **71**, 453-463 (2006).
742. Lee,J.H. *et al.* Differential SUMOylation of LXRalpha and LXRbeta mediates transrepression of STAT1 inflammatory signaling in IFN-gamma-stimulated brain astrocytes. *Mol. Cell* **35**, 806-817 (2009).
743. Janowski,B.A. *et al.* Structural requirements of ligands for the oxysterol liver X receptors LXRalpha and LXRbeta. *Proc. Natl. Acad. Sci. U. S. A* **96**, 266-271 (1999).
744. Fruhwirth,G.O., Loidl,A., & Hermetter,A. Oxidized phospholipids: from molecular properties to disease. *Biochim. Biophys. Acta* **1772**, 718-736 (2007).
745. Xu,H.E. *et al.* Molecular recognition of fatty acids by peroxisome proliferator-activated receptors. *Mol. Cell* **3**, 397-403 (1999).
746. Maltarollo,V.G. & Honorio,K.M. Ligand- and structure-based drug design strategies and PPARdelta/alpha selectivity. *Chem. Biol. Drug Des* **80**, 533-544 (2012).
747. van,Z.M. *et al.* Myelin ingestion alters macrophage antigen-presenting function in vitro and in vivo. *J. Leukoc. Biol.* **90**, 123-132 (2011).
748. Miyake,Y. *et al.* Critical role of macrophages in the marginal zone in the suppression of immune responses to apoptotic cell-associated antigens. *J. Clin. Invest* **117**, 2268-2278 (2007).
749. Green,D.R., Ferguson,T., Zitvogel,L., & Kroemer,G. Immunogenic and tolerogenic cell death. *Nat. Rev. Immunol.* **9**, 353-363 (2009).
750. Asano,K. *et al.* CD169-positive macrophages dominate antitumor immunity by crosspresenting dead cell-associated antigens. *Immunity.* **34**, 85-95 (2011).
751. Wan,Y.J. & Badr,M.Z. Inhibition of Carrageenan-Induced Cutaneous Inflammation by PPAR Agonists Is Dependent on Hepatocyte-Specific Retinoid X ReceptorAlpha. *PPAR. Res.* **2006**, 96341 (2006).
752. Youssef,J. & Badr,M. Role of Peroxisome Proliferator-Activated Receptors in Inflammation Control. *J. Biomed. Biotechnol.* **2004**, 156-166 (2004).
753. Arsenijevic,D. *et al.* Increased infarct size and lack of hyperphagic response after focal cerebral ischemia in peroxisome proliferator-activated receptor beta-deficient mice. *J. Cereb. Blood Flow Metab* **26**, 433-445 (2006).
754. Quintana,F.J., Yeste,A., Weiner,H.L., & Covacu,R. Lipids and lipid-reactive antibodies as biomarkers for multiple sclerosis. *J. Neuroimmunol.* **248**, 53-57 (2012).
755. Sadatipour,B.T., Greer,J.M., & Pender,M.P. Increased circulating antiganglioside antibodies in primary and secondary progressive multiple sclerosis. *Ann. Neurol.* **44**, 980-983 (1998).
756. Bidot,C.J. *et al.* Clinical and neuroimaging correlates of antiphospholipid antibodies in multiple sclerosis: a preliminary study. *BMC. Neurol.* **7**, 36 (2007).
757. Sung,B., Park,S., Yu,B.P., & Chung,H.Y. Modulation of PPAR in aging, inflammation, and calorie restriction. *J. Gerontol. A Biol. Sci. Med. Sci.* **59**, 997-1006 (2004).
758. Kelly,C., Jefferies,C., & Cryan,S.A. Targeted liposomal drug delivery to monocytes and macrophages. *J. Drug Deliv.* **2011**, 727241 (2011).

759. Torchilin,V.P. Recent advances with liposomes as pharmaceutical carriers. *Nat. Rev. Drug Discov.* **4**, 145-160 (2005).

760. Kaundal,R.K. & Sharma,S.S. Peroxisome proliferator-activated receptor gamma agonists as neuroprotective agents. *Drug News Perspect.* **23**, 241-256 (2010).

761. Bright,J.J., Walline,C.C., Kanakasabai,S., & Chakraborty,S. Targeting PPAR as a therapy to treat multiple sclerosis. *Expert. Opin. Ther. Targets.* **12**, 1565-1575 (2008).

762. Racke,M.K. *et al.* Nuclear receptors and autoimmune disease: the potential of PPAR agonists to treat multiple sclerosis. *J. Nutr.* **136**, 700-703 (2006).

763. Wang,D. *et al.* Crosstalk between peroxisome proliferator-activated receptor delta and VEGF stimulates cancer progression. *Proc. Natl. Acad. Sci. U. S. A* **103**, 19069-19074 (2006).

764. Zhao,C. & Dahlman-Wright,K. Liver X receptor in cholesterol metabolism. *J. Endocrinol.* **204**, 233-240 (2010).

765. Zhu,Y. & Li,Y. Liver X receptors as potential therapeutic targets in atherosclerosis. *Clin. Invest Med.* **32**, E383-E394 (2009).

766. Bordoni,A., Di,N.M., Danesi,F., & Biagi,P.L. Polyunsaturated fatty acids: From diet to binding to ppars and other nuclear receptors. *Genes Nutr.* **1**, 95-106 (2006).

767. Riccio,P., Rossano,R., & Liuzzi,G.M. May diet and dietary supplements improve the wellness of multiple sclerosis patients? A molecular approach. *Autoimmune. Dis.* **2010**, 249842 (2011).

768. SWANK,R.L. Multiple sclerosis; a correlation of its incidence with dietary fat. *Am. J. Med. Sci.* **220**, 421-430 (1950).

769. SWANK,R.L. & Goodwin,J. Review of MS patient survival on a Swank low saturated fat diet. *Nutrition* **19**, 161-162 (2003).

Curriculum vitae

Jeroen Bogie werd geboren op 16 juli 1983 te Geleen. In 2001 behaalde hij zijn diploma Voortgezet Wetenschappelijk Onderwijs (VWO) aan het Graafhuyn College te Geleen. Vervolgens startte hij zijn opleiding Biomedische Wetenschappen aan de Vrije Universiteit Amsterdam, waar hij in 2006 zijn diploma bachelor in de Biomedische Wetenschappen behaalde. Aansluitend behaalde hij zijn diploma master in de Biomedische Wetenschappen in 2008. Zijn eindwerken, getiteld " De invloed van myeline-fagocyterende macrofagen op autoreactieve T cel reactiviteit", "Dendritische cel-gebaseerde immunotherapie targeting Mucine-1" en "Regulatoire T cel ontwikkeling: thymus *versus* periferie" werden uitgevoerd aan, respectievelijk, het Biomedisch Onderzoeksinsituut (BIOMED) van de Universiteit Hasselt (o.l.v Prof dr. Jerome Hendriks en Prof dr. Niels Hellings), de afdeling Interne Geneeskunde van Universiteit Maastricht (o.l.v. Prof. Dr Wilfred Germeraad en Prof. dr. Gerard Bos) en de afdeling Reumatologie van Universiteit Leiden (o.l.v Prof. dr Rene Toes). In september 2008 startte hij zijn doctoraat aan BIOMED in het kader van een BOF-beurs. In januari 2009 behaalde hij een IWT-beurs (score A - hoogste score), getiteld "Macrofagen als mediatoren van protectieve autoimmuniteit in multiple sclerose". Tijdens de hieropvolgende jaren deed hij actief mee in het onderwijs van de universiteit Hasselt en volgde hij de cursussen proefdierkunde (module I & II), biosafety, good scientific conduct and lab book taking, transcriptomics data analyse, presentatietechnieken, Oppino entrepeneurship (module I & II) en parametrische/niet-parametrische statistische methoden voor levens wetenschappen (module I & II). Bovendien heeft hij een FWO doctoraatsbeurs geschreven en actief deelgenomen aan het schrijven van een FWO krediet aan navorser. Ten slotte, hij ontving een reisbeurs om de ISNI 2009 (Istanbul) bij te wonen en hij won de posterprijs op Euron PhD days in 2010.

Bibliography

Publications resulting from this work

Myelin-phagocytosing macrophages modulate T cell proliferation
Jeroen FJ Bogie, Piet Stinissen, Niels Hellings and Jerome JA Hendriks
J. Neuroinflammation 2011 jul 25; 8: 85 (IF: 5.7)

Myelin-derived lipids modulate macrophage activity by liver X receptor activation
Jeroen FJ Bogie, Silke Timmermans, Vân Anh Huynh-Thu, Alexandre Irrthum, Hubert JM Smeets, Jan-Åke Gustafsson, Knut R Steffensen, Monique Mulder, Piet Stinissen, Niels Hellings and Jerome JA Hendriks
PLoS One 2012*; 7(9): e44998 (IF: 4.1)*

Phosphatidylserine present in myelin alters the inflammatory phenotype of myelin-phagocytosing macrophages by activating PPARs
Jeroen FJ Bogie, Winde Jorissen, Jo Mailleux, Philip G Nijland, Noam Zelcer, Tim Vanmierlo, Jack van Horssen, Piet Stinissen, Niels Hellings and Jerome JA Hendriks
Submitted

Characterization of collectin sub-family 12 expression on myelin-phagocytosing macrophages and in active MS lesion
Jeroen FJ Bogie, Tim Vanmierlo, Jack van Horssen, Piet Stinissen, Niels Hellings and Jerome JA Hendriks
In preparation

Macrophages and microglia in multiple sclerosis: A blessing in disguise
Jeroen FJ Bogie, Piet Stinissen, Niels Hellings and Jerome Hendriks
In preparation

Publications in collaboration with other team members

A high fat diet exacerbates neuroinflammation by activation of the brain RAS
Silke Timmermans, **Jeroen FJ Bogie**, Tim Vanmierlo, Piet Stinissen, Niels Hellings and Jerome JA Hendriks
In preparation

LXRβ activation ameliorates EAE by inhibiting immune cell infiltraton
Silke Timmermans, **Jeroen FJ Bogie**, Tim Vanmierlo, Piet Stinissen, Niels Hellings and Jerome JA Hendriks
In preparation

Oncostatin M elicits a type 2 immune response in the brain that prevents autoimmune encephalomyelitis
Helena Slaets, Kris Janssens, **Jeroen FJ Bogie**, Helga deVries, Veerle Baekelandt Chris Van Den Haute, Piet stinissen, Jerome JA Hendriks and Niels Hellings
Submitted

Immunological and developmental features of Wharton's jelly-derived mesenchymal-like cells
Raf Donders, **Jeroen FJ Bogie**, Stelios Ravanidis, Herbert Smeets, Wilfried Gyselaers, Ivo Lambrichts, Piet Stinissen and Niels Hellings
In preparation

Published abstracts

Myelin-phagocytosing macrophages show immunomodulatory properties
Jeroen FJ Bogie, Silke Timmermans, Piet Stinissen, Niels Hellings, Jerome JA Hendriks
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Oral presentations

Damage and Healing in Multiple Sclerosis
Jeroen FJ Bogie, Piet Stinissen, Niels Hellings and Jerome JA Hendriks
Immunology of Autoimmunity meeting, *14th November* 2009, Leuven, Belgium

Myelin-phagocytosing macrophages show immunomodulatory properties
Jeroen FJ Bogie, Piet Stinissen, Niels Hellings and Jerome JA Hendriks
Ms research days, 13th-14th November 2009, Groningen, The Netherlands

Myelin-phagocytosing macrophages show immunomodulatory properties
Jeroen FJ Bogie, Piet Stinissen, Niels Hellings and Jerome JA Hendriks
FWO-WOG MS symposium, 13th December 2011, Brussel, Belgium

Phosphatidylserine present in myelin alters the inflammatory phenotype of myelin-phagocytosing macrophages by activating PPARs
Jeroen FJ Bogie, Winde Jorissen, Jo Mailleux, Philip G Nijland, Noam Zelcer, Tim Vanmierlo, Jack van Horssen, Piet Stinissen, Niels Hellings and Jerome JA Hendriks
PhD student symposium Hasselt University & VUMC Amsterdam, *23th August 2012, Hasselt, Belgium*

Macrophages as mediators of protective autoimmunity in multiple sclerosis
Jeroen FJ Bogie, Piet Stinissen, Niels Hellings and Jerome JA Hendriks
Alma in Silico meeting, 24th October 2012, Liege, Belgium

Phosphatidylserine present in myelin alters the inflammatory phenotype of myelin-phagocytosing macrophages by activating PPARs
Jeroen FJ Bogie, Winde Jorissen, Jo Mailleux, Philip G Nijland, Noam Zelcer, Tim Vanmierlo, Jack van Horssen, Piet Stinissen, Niels Hellings and Jerome JA Hendriks
Ms research days, 29th-30th November 2012, Nijmegen, The Netherlands

Poster presentations

Myelin-phagocytosing macrophages show immunomodulatory properties
Jeroen FJ Bogie, Piet Stinissen, Niels Hellings and Jerome JA Hendriks

- *MS research days, 12th-13th November 2008, Oegstgeest, The Netherlands*
- *Immunology of Autoimmunity meeting, 14th November 2009, Leuven, Belgium*
- *Annual Meeting of the Belgian Immunological Society (BIS), 16th November 2009, Ghent, Belgium*
- *Biomedica, 17th-18th March 2010, Aachen, Germany.*
- *Euron PhD days, 8th October 2010, Hasselt Belgium*
- *BSCDB meeting, 22thOctober 2010, Hasselt, Belgium*
- *10th, International Congress of Neuroimmunology (ISNI), 26th-30th October, Sitges 2010, Spain*
- *MS research days 14th-15th November 2010, Alphen aan den Rijn, The Netherlands*
- *FWO-WOG MS symposium, 13th December 2011, Brussel, Belgium*

The role of liver X receptors in central nervous system inflammation
Silke Timmermans, **Jeroen FJ Bogie**, Niels Hellings and Jerome JA Hendriks

- *Euron PhD days, 8th October 2010, Hasselt Belgium*
- *BSCDB meeting, 22thOctober 2010, Hasselt, Belgium*
- *Benelux Nuclear receptor meeting, 9th November 2010, Ghent, Belgium*
- *MS research days 14th-15th November 2010, Alphen aan den Rijn, The Netherlands*

Myelin-derived lipids modulate macrophage activity by liver X receptor activation
Silke Timmermans, **Jeroen FJ Bogie**, Monique Mulder, Niels Hellings and Jerome JA Hendriks

- *25th Annual Meeting of the European Macrophage and Dendritic Cell Society (EMDS),22th -24th September 2011, Brussels, Belgium*
- *Annual Meeting of the Belgian Immunological Society (BIS), 18th November 2011, Hasselt, Belgium*

- *FWO-WOG MS symposium, 13th December 2011, Brussel, Belgium*
- *European Lipoprotein Club, 10th -13th September 2012, Tutzing, Germany*
- *Nuclear Receptor Research Network Meeting, 15th November 2012, Leuven Belgium*
- *Ms research days, 29th-30th November 2012, Nijmegen, The Netherlands*

Phosphatidylserine present in myelin alters the inflammatory phenotype of myelin-phagocytosing macrophages by activating PPARs
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- *Annual Meeting of the Belgian Immunological Society (BIS), 18th November 2011, Hasselt, Belgium*
- *25th Annual Meeting of the European Macrophage and Dendritic Cell Society (EMDS),22th -24th September 2011, Brussels, Belgium*
- *11th, International Congress of Neuroimmunology (ISNI), 4th-8th November 2012, Boston, Spain*
- *Nuclear Receptor Research Network Meeting, 15th November 2012, Leuven Belgium*
- *Ms research days, 29th-30th November 2012, Nijmegen, The Netherlands*

Poster presentation award

Myelin-phagocytosing macrophages show immunomodulatory properties
Jeroen FJ Bogie, Piet Stinissen, Niels Hellings and Jerome JA Hendriks
Euron PhD days, 8th October 2010, Hasselt Belgium