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GENEESKUNDE

master in de biomedische wetenschappen: klinische moleculaire wetenschappen

2010
2011

Masterproef

A role for myelin-derived lipids in the induction of an anti-inflammatory phenotype in macrophages

Promotor :
Prof. dr. Jerome HENDRIKS

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Masterproef voorgedragen tot het bekomen van de graad van master in de biomedische wetenschappen , afstudeerrichting klinische moleculaire wetenschappen

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ABBREVIATIONS

ANOVA = Analysis **o**f **Va**riance

APC = **A**ntigen **P**resenting **C**ell

ARG1 = **Arg**inase 1

BBB = **B**lood **B**rain **B**arrier

BCA = **Bic**inchoninic **A**cid

BSA = **B**ovine **S**erum **A**lbumin

CFA = Complete **F**reund's **A**djuvant

CNS = **C**entral **N**ervous **S**ystem

CSF = Cerebro**s**pinal **F**luid

DAG = **Dia**cyl**g**lycerol

DC = Dendritic **C**ell

DHR = **Dih**ydro**r**hodamine

DMSO = **Dim**ethyl**s**ulf**o**xide

EAE = **E**xperimental **A**utoimmune **E**ncephalomyelitis

ECM = **E**xtra**c**ellular **M**atrix

EDTA = **E**thylene **D**iamine **T**etraacetic **A**cid

FACS = **F**luorescence **A**ctivated **C**ell **S**orting

FCS = **F**etal **C**alf **S**erum

FIZZ1 = **F**ound In **I**nflammatory **Z**one **1**

GA = **G**anglioside

HLA-DR = **H**uman **L**eukocyte **A**ntigen **DR**

IFN-γ = **I**nter**f**ero**n γ**

IL- = **I**nter**l**eukin

IL-1ra = **I**nter**l**eukin **1 r**eceptor **a**ntagonist

iNOS = **i**nducable **N**itric **O**xide **S**ynthase

L-arg = L-arginine

LDL = **L**ow **D**ensity **L**ipoprotein

L-orn = L-ornithine

LPS = **L**ipo**p**oly**s**accharide

LTB4 = **L**euko**T**riene **B4**

MAG = **M**yelin **A**ssociated **G**lycoprotein

MBP = **M**yelin **B**asic **P**rotein

MHC = **M**ajor **H**istocompatibility **C**omplex

MMM = Marginal **M**etallophilic **M**acrophage

MOG = **M**yelin **O**ligodendrocyte **G**lycoprotein

MPS = Mononuclear **P**hagocytic **S**ystem

MRI = **M**agnetic **R**esonance **I**maging

MS = **M**ultiple **S**clerosis

MZ = Marginal **Z**one

MZM = Marginal **Z**one **M**acrophage

NADPH = Nicotinamide **A**denine **D**inucleotide **P**hosphate

NFκB = Nuclear **F**actor **κ B**

NK cell = **N**atural **K**iller cell

NO = **N**itric **O**xide

$O2\cdot^-$ = Superoxide anion

OCT = Optimal **C**utting **T**emperature

OncoM = Oncostatin **M**

ORO = **O**il **R**ed **O**

P/S = **P**enicillin/ **S**treptomycin

PBMC = Peripheral **B**lood **M**ononuclear **C**ells

PBS = **P**hosphate **B**uffered **S**aline

PC = **P**hosphatidyl**c**holine

PCL = Phosphatidyl**c**holine **l**iposomes

PE = **P**hosphatidyl**e**thanolamine

PFA = **P**ara**f**orm**a**ldehyde

PGE_2 = **P**rosta**g**landin **E2**

PHOX = Phagocyte **Ox**idase

PI = **P**hospho**i**nositides

PLP = Proteo**l**ipo**p**rotein

PMA = **P**horbol **M**yristate **A**cetate

PP-MS = **P**rimary **P**rogressive **M**ultiple **S**clerosis

PS = **P**hosphatidyl**s**erine

PSL = Phosphatidyl**s**erine **l**iposomes

PtdIns = **P**hospha**t**idyl**in**o**s**itol

RA = Rheumatoid **A**rtritis

ROS = **R**adical **O**xygen **S**pecies

RR-MS = **R**elapse **R**emitting **M**ultiple **S**clerosis

RT PCR = Real **T**ime **P**olymerase **C**hain **R**eaction

SBB = **S**udan **B**lack **B**

SEM = **S**tandard **E**rror of **M**ean

SPHK1 = **Sp**hingosine **K**inase **1**

SP-MS = **S**econdary **P**rogressive **M**ultiple **S**clerosis

SU = **Su**lfatide

TCR = T cell **R**eceptor

TGF-β1 = Transforming **G**rowth **F**actor **β1**

TNFα = **T**umor **N**ecrosis **F**actor **α**

ABSTRACT

Multiple Sclerosis (MS) is an autoimmune disease, characterized by chronic inflammation and demyelination in the central nervous system (CNS). Current therapies for MS partially reduce new lesion development and prevent clinical disease activity to a certain degree, but none can halt the progression or cure the disease. Macrophages play a central role in the disease process of MS by phagocytosing myelin and releasing inflammatory and toxic mediators in the CNS. Although classically regarded to be detrimental, recent evidence has revealed the presence of a more "M2-like", anti-inflammatory phenotype of macrophages in MS, especially following myelin phagocytosis. These M2 or alternatively activated macrophages, generally associated with T_H2 responses, might play a neuroprotective role during MS pathogenesis. The myelin component inducing the anti-inflammatory phenotype, however, is not yet known. Interestingly, recent studies have indicated a potential role for lipids in the induction of an "M2-like" phenotype in macrophages. We hypothesize that myelin-derived lipids may be responsible for the "M2-like" phenotype in macrophages. In this study, we compared the effect of five selected myelin-derived lipids (e.g. sulfatide (SU), gangliosides (GA), phosphatidylserine (PS), phosphatidylethanolamine (PE) and phosphatidylinositides (PI)) on the phenotype of macrophages. To this end, we performed phagocytosis assays, real time PCR, FACS analysis, griess reagent assays, and immunohistochemistry. Additionally, we validated *in vitro* data in an *in vivo* chronic EAE model. Here, we report that phosphatidylserine induces an anti-inflammatory phenotype in macrophages through the suppression of nitric oxide production, the upregulation of the expression of the genes ARG1, TGF-β1 and IL-11 and the simultaneous suppression of the genes iNOS, CXCL10 and TNFα *in vitro*. Moreover, specific delivery of PS loaded liposomes to macrophages in an *in vivo* chronic EAE rat model resulted in significantly lower EAE scores and weight loss compared to control animals. These results suggest that phosphatidylserine loaded liposomes are likely to induce a more "M2-like" phenotype in macrophages, and that these M2 macrophages may have a therapeutic effect in inflammatory diseases such as EAE and MS.

Keywords: *Multiple sclerosis, M2 macrophages, anti-inflammatory, myelin, phosphatidylserine*

SAMENVATTING

Multiple Sclerose (MS) is een autoimmuunziekte die gekarakteriseerd wordt door chronische inflammatie en demyelinatie in het centrale zenuwstelsel (CZS). Huidige therapieën voor MS verminderen de ontwikkeling van nieuwe lesies gedeeltelijk, en verhinderen de klinische ziekte-activiteit tot een bepaalde graad, maar geen enkele therapie verhindert de progressie van de ziekte of geneest ze. Macrofagen spelen een centrale rol in het ziekteproces van MS door het opnemen van myeline en het vrijzetten van inflammatoire en toxische moleculen in het CZS. Tot voorkort werd er verondersteld dat macrofagen een pro-inflammatoir (M1) fenotype aannemen in MS. Recent bewijs, echter, heeft de aanwezigheid van een meer anti-inflammatoir (M2) fenotype van macrofagen in MS aangetoond, vooral na de opname van myeline. Deze M2 of alternatief geactiveerde macrofagen, die geassocieerd zijn met T_H2 responsen, zouden een neuroprotectieve rol kunnen hebben in het ziekteproces van MS. De myeline component die dit anti-inflammatoire fenotype induceert, is echter nog niet gekend. Recente studies hebben een mogelijke rol voor lipiden voorgesteld in de inductie van een anti-inflammatoir fenotype in macrofagen. Wij verwachtten dat lipiden afgeleid uit myeline verantwoordelijk zouden kunnen zijn voor het M2 fenotype dat gezien wordt in macrofagen na opname van myeline. In deze studie vergeleken we het effect van vijf geselecteerde lipiden, aanwezig in myeline (e.g. sulfatide (SU), gangliosides (GA), phosphatidylserine (PS), phosphatidylethanolamine (PE) en phosphatidylinositides (PI)), op het fenotype van macrofagen. Hiervoor gebruikten we fagocytose assays, real time PCR, FACS analyses, griess reagent assays en immunohistochemie. Verder werden de *in vitro* data gevalideerd in een *in vivo* chronisch EAE rat model. In deze studie rapporteren we dat phosphatidylserine een anti-inflammatoir fenotype induceert in macrofagen door de onderdrukking van stikstofmonoxide, de inductie van de expressie van de genen ARG1, TGF-β1 en IL-11 en de gelijktijdige suppressie van de genes iNOS, CXCL10 en TNFα *in vitro*. Verder lijdt de specifieke aflevering van PS beladen liposomen aan macrofagen in een *in vivo* chronisch EAE ratten model tot significant verlaagde EAE scores en significant verminderd gewichtsverlies in vergelijking met de controle groep. Deze resultaten suggesteren dat phosphatidylserine beladen liposomen waarschijnlijk een M2 fenotype induceren in macrofagen, en dat deze M2 macrofagen een therapeutisch effect zouden kunnen uitoefenen in inflammatoire aandoeningen zoals EAE en MS.

Sleutelwoorden: *Multiple sclerose, M2 macrofagen, anti-inflammatoir, myeline, phosphatidylserine*

1. GENERAL INTRODUCTION

1.1. Multiple sclerosis

Multiple sclerosis (MS) is an autoimmune disease, characterized by chronic inflammation and demyelination in the central nervous system (CNS). MS is the most common neurological disorder in the world, affecting 2.5 million people worldwide (1). Most patients are young adults, with onset of the disease between 18 and 35 years. Women are affected about two times more frequently than men.

Clinically, MS is characterized by unpredictable periods of symptoms and periods of remission with no disease activity. According to the prevalence of these two phases, two different forms of MS can be distinguished. The first form, relapse remitting MS (RR-MS), is the most frequent form (80-90%). This form is characterized by periods of relapses followed by episodes of remissions. After 5 to 15 years most RR-MS patients develop secondary progressive MS (SP-MS) which is characterized by an increased number of relapses. The second form, primary progressive MS (PP-MS), is only present in a minority (10-20%) of the patients. This form is characterized by a gradual increase in neurodegeneration and reduced inflammation (2).

Current MS treatment consists of the administration of anti-inflammatory (glucocorticosteroids), immunosuppressive (chemotherapy) or immunomodulatory (beta Interferon, Natalizumab) drugs. These drugs are capable of delaying the progression in the early phase of the disease, and are most effective in RR-MS. However, these therapies are non-specific and are frequently accompanied by a number of complications. At present there is no effective treatment for MS.

1.2. The pathogenesis of multiple sclerosis

The onset of MS is characterized by the degradation of the blood brain barrier (BBB). Inflammatory cells such as macrophages, plasma cells, CD4+ T-cells and CD8+ T-cells can then infiltrate the brain (Fig. 1), where they are involved in the formation of multiple lesions by inducing an autoimmune response against myelin components. The disruption of the BBB typically lasts for about a month and then resolves (3-5). The most affected areas in the CNS are the brain stem, the optic nerves, the ventricle-surrounding-tissue and the spinal cord (2). Magnetic resonance imaging (MRI) (6) and histological evaluation (7) in morphological studies can visualize these plaques. The complete pathway underlying the disease mechanism is not yet fully understood, both genes and environmental factors are involved (8-11).

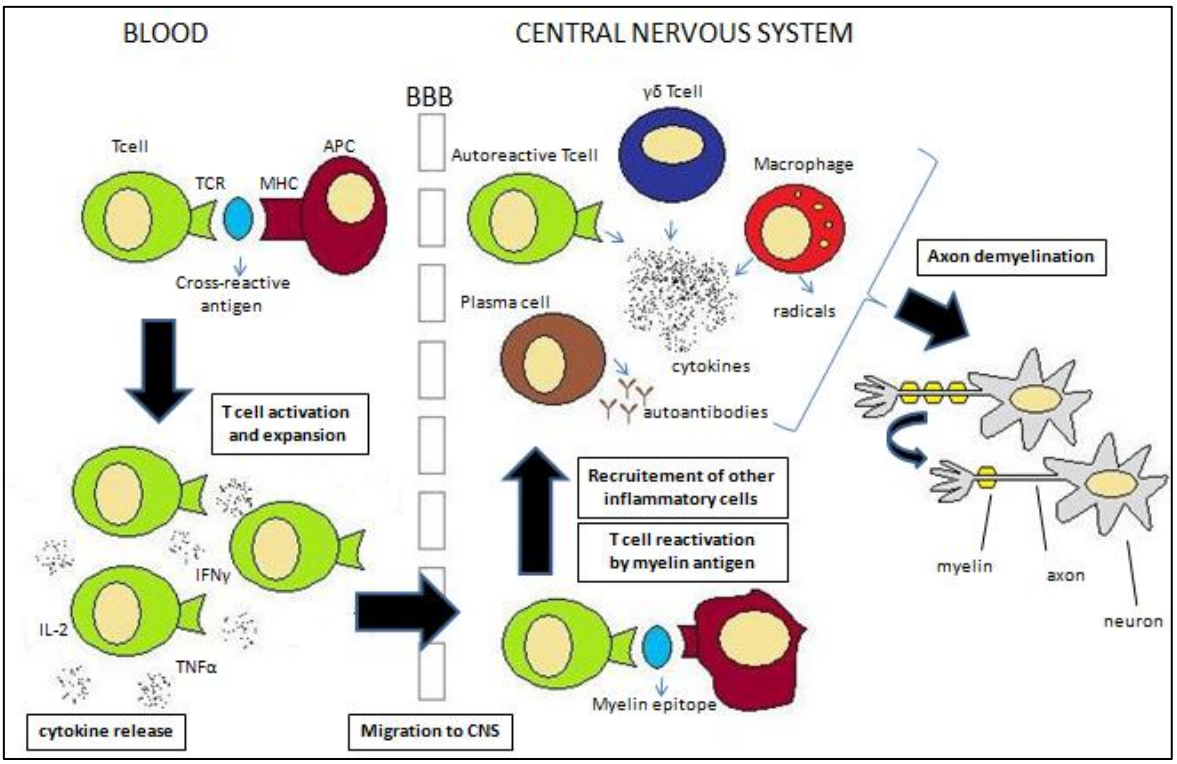

Fig. 1 The pathogenesis of multiple sclerosis. T cells become activated in the periphery by cross-reactive antigens, causing them to expand and release inflammatory cytokines. Damage occurs to the blood brain barrier, allowing inflammatory cells to cross the barrier. After being reactivated by myelin epitopes in the central nervous system, these cells secrete cytokines, autoantibodies, and chemokines. The latter result in the recruitment of even more inflammatory cells. Axonal demyelination takes place, mainly by activated macrophages in the CNS.

Autoreactive T-cells that can directly damage axons are present in inflammatory MS lesions (12). Therefore it is generally accepted that autoreactive T-cells are involved in the formation of these lesions. Other evidence comes from the animal model of MS, experimental autoimmune encephalomyelitis (EAE), that shares many clinical and histological features with MS. EAE can be induced in a variety of animals (e.g. rats, mice, guinea pigs, rabbits, macaques, rhesus monkeys and marmosets) by adoptive transfer of myelin reactive T-cells or by generating T-cell mediated immunoreactivity to CNS antigens (13). Several studies indicate that these autoreactive T-cells show specificity for myelin-derived antigens (e.g. myelin basic protein (MBP), proteolipoprotein (PLP), myelin oligodendrocyte glycoprotein (MOG)) (14, 15). However, the initial trigger in MS, which activates the autoreactive T-cells in the periphery, remain to be elucidated. Possible mechanisms are superantigens or molecular mimicry. In molecular mimicry, antigens from viruses or microbes are able to cross-activate T-cells specific for myelin epitopes (5) (Fig. 1). More specifically, myelin-specific T_H1 and T_H17-cells are regarded to be activated in the periphery. Activated T helper cells cross the BBB and initiate an immune response against myelin in the brain.

1.3. A dual role for macrophages in multiple sclerosis

Besides T lymphocytes, macrophages play a central role in the disease process of MS, since depletion of macrophages inhibits the manifestation of acute (16) or chronic (17) EAE in Lewis rats. Macrophages are known to phagocytose myelin and to release toxic mediators in the CNS of EAE animals and MS patients, hereby playing a role in demyelination and axon degeneration (18).

In literature, different phenotypes of macrophages have been described, of which M1 and M2 are thought to be extremes (19, 20) (Fig. 2). M1, or classically activated macrophages, become activated in the presence of mediators (e.g. IFN-γ, TNFα) secreted by T_H1 lymphocytes and NK cells, or LPS. These M1 macrophages secrete pro-inflammatory, T_H1/T_H17 associated cytokines (e.g. ROS, TNFα, IL-1β, IL-12, IL-23, CXCL10) and nitric oxygen (NO), that enable them to eliminate harmful pathogens. For a long time, it was thought that macrophages could solely adapt a pro-inflammatory phenotype in MS. Recent experiments, however, have shown the presence of a more 'M2-like', anti-inflammatory phenotype of macrophages in MS, especially following myelin phagocytosis (19, 21). Moreover, these macrophages acquire distinct phenotypes depending on the micro-location in the lesion. These M2 or alternatively activated (e.g. by IL-4, IL-13, IL-10 or PGE_2) macrophages, are associated with T_H2 responses and are likely to be anti-inflammatory, neuroprotective and immunosuppressive (21, 22). Generally, M2 macrophages secrete anti-inflammatory products (e.g. Il-10, TGFβ, IL-4, CCL18, CCL22, IL-1ra, PGE_2) and express ARG1, YM1, FIZZ1 and SPHK1. Importantly, macrophage polarization is not permanent, which indicates that they can switch their effector functions depending on divergent cues in the local environment (23).

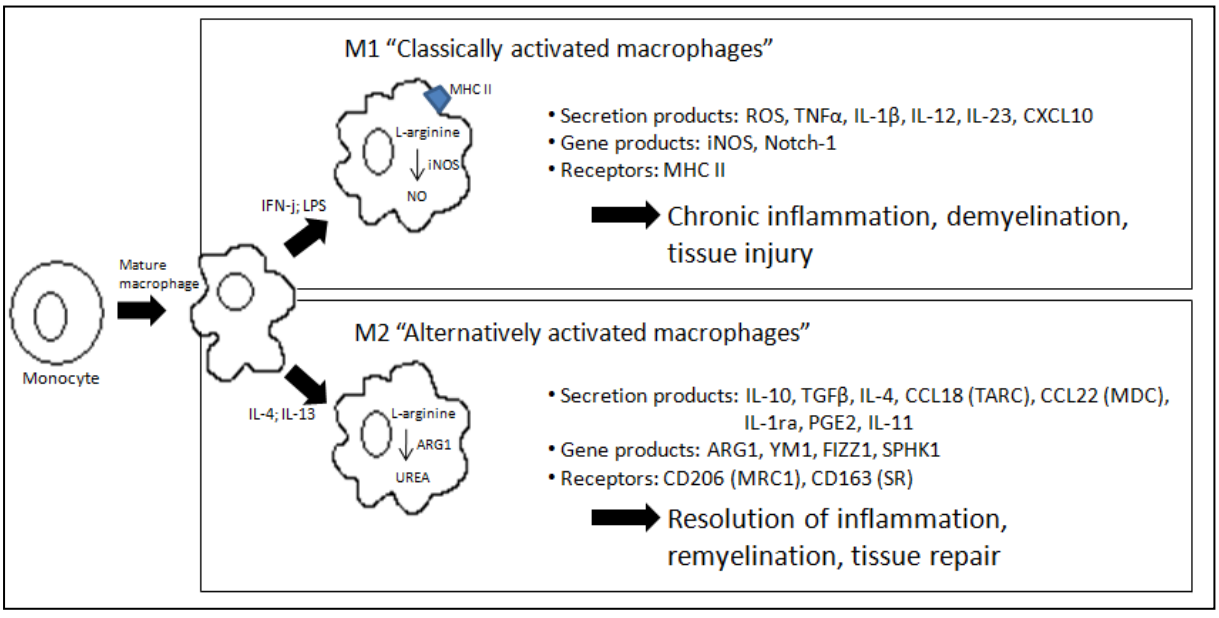

Fig. 2 Classically vs. alternatively activated macrophages. Classically activated or M1 macrophages show a T_H1-like phenotype. They secrete pro-inflammatory molecules (e.g. ROS, TNFα, IL-1β, CXCL10), and upregulate iNOS and Notch-1 gene expression. These M1 macrophages induce an inflammatory environment, destruct extracellular matrix (ECM), and promote apoptosis, leading to tissue injury and chronic inflammation. Alternatively or M2 macrophages, in contrast, display a

T_H2-like phenotype, secrete anti-inflammatory molecules (e.g. IL-10, TGFβ, IL-4, CCL18, IL-1ra, PGE_2 and IL-11) and express the genes ARG1, YM1, FIZZ1 and SPHK1. Although both phenotypes are important components of both the innate and adaptive immune systems, the classically activated macrophages tend to elicit chronic inflammation and tissue injury whereas the alternatively activated macrophages tend to resolve inflammation by suppressing the immune response and allowing tissue repair.

1.4. Anti-inflammatory capacities of myelin-laden macrophages

Myelin is a highly ordered membrane structure that is essential for the conduction of nerve impulses in the CNS. It consists for ~20% of proteins (e.g. MBP, MAG, MOG and PLP) and for ~80% of lipids. Cholesterol and the glycolipids 'galactocerebrosides' are the most abundant lipid components of human myelin (24). Other myelin lipid components are phosphoinositides (PI), phosphatidylethanolamine (PE), phosphatidylcholine (PC), phosphatidylserine (PS), plasmalogen, sulfatide (SU), and ganglioside (GA). To date, even though lipids are abundantly present in myelin, much attention has focused on the role of myelin-derived proteins in MS. Importantly, the composition of myelin-derived lipid components in the brain differs between patients suffering from MS (26). As mentioned, recent studies have suggested that myelin-derived lipids may induce an anti-inflammatory, "M2-like" phenotype in macrophages (21, 27).

Myelin-laden macrophages are ubiquitously present in the CNS of MS patients and in animals with EAE. Activated macrophages acquire a foamy appearance through the ingestion of large amounts of myelin-derived lipids. Foamy macrophages in MS lesions contain vesicles with neutral lipids, express high levels of HLA-DR, CD11b, CD163 and secrete anti-inflammatory molecules (e.g. IL-1ra, CCL18, IL-10, IL-4 and TGF-β). Moreover, these macrophages lack the expression of M1associated pro-inflammatory cytokines such as TNFα, IL-12p40 and IL-1β (21). Even though the exact function of foamy macrophages in MS lesions remains to be elucidated, these cells already show some characteristics to modulate inflammation.

A disease that might provide further relevant insight into the function of foam cells, is atherosclerosis. Atherosclerotic plaques contain a characteristic inflammatory infiltrate, including monocytes, numerous monocyte-derived macrophages, modified lipid-laden macrophages (foam cells) and T lymphocytes. Foam cell formation in atherosclerosis is thought to be induced by the ingestion of oxidized lipids (e.g. low density lipoprotein (LDL)) and cholesterol (26). Macrophages that have just arrived at an atherosclerotic plaque tend to express effector molecules that are pro-inflammatory, cytotoxic and chemotactic. Furthermore, these macrophages secrete enzymes that can degrade the extracellular matrix (ECM), leading to destabilization of plaques and an increased risk of rupture. When the macrophages have acquired a certain amount of lipids, however, they show the potential to drive tissue remodeling and ultimately vascular repair (27). The resulting lipid-laden cells have been shown to be anti-inflammatory (28-30). Correspondingly, the uptake of LDL inhibits TNF-induced TNF expression of foam cells and stimulates them to produce IL-10 (29).

1.5. Myelin-derived lipids with anti-inflammatory features

Hashioka et al. (31) have demonstrated that myelin-derived phospholipids (e.g. PS and PC) can inhibit both superoxide anion ($O2\cdot^-$) and nitric oxide (NO) production of microglia, stimulated with lipopolysaccharide (LPS)/phorbol myristate acetate (PMA). In addition, preliminary results at Biomed have shown that sulfatide and PS are potential candidates to induce an anti-inflammatory, "M2-like" phenotype in peritoneal rat macrophages. Whereas sulfatide incorporation lowers macrophage TNFα and IL-6 production, the engulfment of PS induces IL-10 production. Altogether, these results suggest a role for myelin-derived lipids in the induction of an anti-inflammatory phenotype in macrophages.

1.5.1. Sulfatides

Sulfatide, or sulfated galactocerebroside, is a glycolipid found at high concentrations exclusively in the brain, kidney and spleen (32, 33). In the brain, sulfatide is not solely a structural component of myelin, it also has an important function in several biological processes, including axon-myelin interactions, neuronal plasticity, regulation of cell growth, cell adhesion and protein trafficking (34, 35). In the CNS, about 20% of all galactolipids - a group of glycolipids - occurs in the form of SU (3'-sulfogalactosyl ceramide) in which the 3'-OH moiety on galactose is sulfated, and the carbohydrate moiety is attached to ceramide in a β-linkage (Fig. 3).

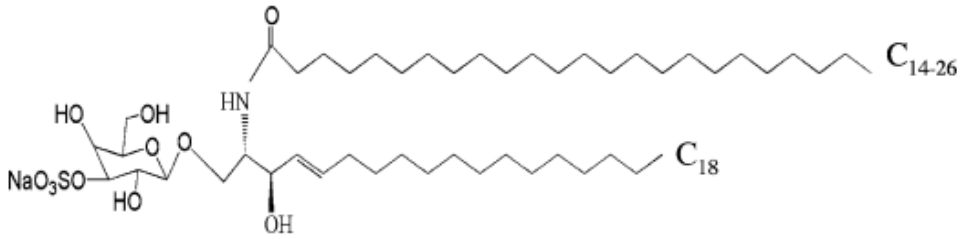

Fig. 3 Chemical structure of myelin-derived sulfatide. (Figure reproduced from Halder, R.C. et al., 2007 (36)).

Previous studies have shown that the administration of sulfatide, emulsified with complete Freund's adjuvant, in mice does not protect them from EAE (37). Adjuvant-free injection (i.p.) of sulfatide at the time of myelin-protein immunization, however, completely protects mice from EAE (38). This protection is mediated trough CD1d, presumably expressed on macrophages/ microglia, as mice deficient in CD1d are not protected. Furthermore, preliminary data of Maricic et al. (36) have shown that intraperitoneal injection of sulfatide after disease onset is able to reverse ongoing disease, indicating that sulfatide can modify ongoing EAE in SJL/J mice. Collectively, these studies indicate that sulfatide might play an important role in MS and perhaps in the specific phenotype of macrophages following myelin internalization.

1.5.2. Gangliosides

Gangliosides are sialic acid-containing glycosphingolipids that are components of the mammalian cell membrane. Neuronal cell membranes are particularly rich in gangliosides. Besides being constituents

of membranes, gangliosides also play an important role in biological processes such as cell adhesion, signal transduction, neuronal sprouting and growth of injured peripheral nerves (39, 40). Several *in vitro* studies suggest anti-inflammatory effects of gangliosides. Incubation of human epithelial cells with gangliosides results in the inhibition of prostaglandin E2 (PGE_2) production by 75% (compared with controls) in response to TNFα (40). Purified bovine gangliosides GM1 and GD1a are able to suppress nuclear factor kappa B (NFκB) binding activity in T cells and reduce expression of the cytokines IL-2 and IFN-γ. These gangliosides can also suppress T cell function, induce T cell apoptosis (41) and impair the antigen-presenting function of human dendritic cells (42). Furthermore, *in vivo* studies have shown that the natural ganglioside mixture (GM1 + GD1a + GD1b + GT1b) from bovine brain has a strong anti-inflammatory activity in rodents (43). Moreover, dietary gangliosides, fed to rats with acute gut inflammation, can lower pro-inflammatory cytokines (IL-1β, TNFα and leukotriene B4 (LTB4)) in the inflamed tissue and plasma (44). Nonetheless, several studies have shown that exposure of brain microglia and astrocytes to gangliosides, both *in vivo* and *in vitro*, can induce the production of various inflammatory mediators, such as cytokines and inducible nitric oxide synthase (iNOS) (45).

1.5.3. Phospholipids

Phosphatidylserine (PS), a phospholipid, has known functions in all organs and is highly concentrated in the brain, where it is essential for effective neurotransmission and synaptic communication. Moreover, PS plays an important role in preserving the function of the membrane surrounding the mitochondria. Inadequacies of function of the mitochondrial membrane compromise energy production, and thus threaten the viability of the neuron. Importantly, in MS patients, a reduction of PS in the serum was found, compared to healthy subjects (46), and deficiencies in cellular communication are ubiquitously present in brain lesions from MS patients. Adequate amounts of PS are thus required not only to preserve, but also to enhance the ability of nerves to transmit information. Furthermore, PS is a marker for apoptosis, being redistributed from the inner leaflet to the outer leaflet of the plasma membrane early in the process of apoptosis. Importantly, PS already has several applications in neurodegenerative diseases including Parkinson and Alzheimer's disease (47, 48).

Besides PS there are 3 other types of phosphatides, which all play an important role in brain function and structure: phosphatidylcholine (PC), phosphatidylethanolamine (PE), and phosphatidylinositol (PtdIns). Phosphatidylethanolamine (PE) constitutes the backbone of cell membranes and contributes to the fluidity and the structural environment of cells. In regard to the brain and nervous tissue, PE comprises more than 10% of total human CNS myelin (24), and plays an important role in myelin structure and nerve endings in the brain. Phoshoinositides (PI) are phosphorylated forms of PtdIns. Three of the five hydroxyl residues on the inositol ring can be reversibely phosphorylated, individually or in combination, to generate seven species of PI (Fig. 4). Phosphorilation and dephosphorilation by lipid kinases and phosphatases respectively regulate their biosynthesis. The resulting PtIns mono- /di-

or tri-sulfates were previously thought to have merely a structural role. However, through interactions mediated by their headgroups, PI play a fundamental role in controlling membrane–cytosol interfaces. Moreover, they regulate membrane trafficking, the cytoskeleton, signal transduction at the cell surface (49), neurite extension and neuronal development (50). Given PI's essential roles in (brain) cell metabolism and development, it is not surprising that the misregulation of PI's and PI binding proteins has been implicated in a growing number of diseases and developmental disorders (e.g. Lowe syndrome, Charcot-Marie-Tooth).

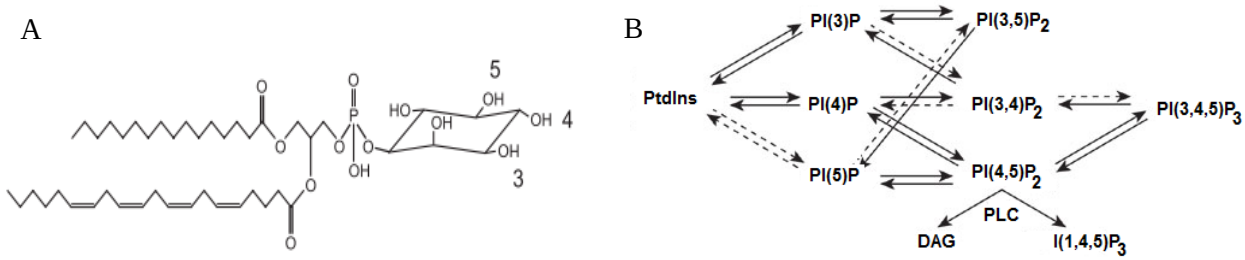

Fig. 4 (A) Chemical structure of PtdIns. (B) Metabolic pathways interconverting the different PI. Positions 3, 4, and 5 on the inositol ring can be phosphorylated to produce seven different PI species: PI(3)P, PI(4)P, PI(5)P, PI(3,5)P_2, PI(3,4)P_2, PI(4,5)P_2 and PI(3,4,5)P_3. Abbreviations: DAG = diacylglycerol, PLC = phospholipase C, I(1,4,5)P_3 = inositol-3,4,5-trisphosphate. (Figure adapted from Di Paolo, G.& De Camilli, P., 2006 (51); Skwarek, L.C. & Boulianne, G.L., 2009 (50)).

AIMS

Myelin phagocytosis by infiltrated macrophages and activated microglia in the CNS of MS patients is not just a hallmark of multiple sclerosis, but also a key determinant of lesion development, disease progression, and most likely also disease resolution. Recent evidence, has revealed the presence of a more "M2-like", anti-inflammatory phenotype of macrophages in MS, especially following myelin phagocytosis. The myelin component inducing the anti-inflammatory phenotype, however, is not yet known. We hypothesized that myelin-derived lipids may be responsible for the anti-inflammatory phenotype in macrophages following myelin phagocytosis. In this study, we will compare the effect of myelin-derived lipid components (i.e. PI, PE, PS, SU and GA) on the phenotype of macrophages. Macrophage uptake of myelin-derived lipids will be assessed using phagocytosis assays. The macrophage phenotype will be further elucidated by making use of griess reagent assays, Real Time PCR, immunohistochenistry and FACS. Additionally, data of the lipid with the greatest capacity to induce an 'M2-like' phenotype in macrophages, will be validated *in vivo* in a chronic EAE rat model.

New insight into possible anti-inflammatory inducing mechanisms of myelin-derived lipids in macrophages can lead to new applications to control lesion development in MS. Since none of the MS therapies can halt the progression or cure the disease, the identification of anti-inflammatory components can be used for the development of new commercial therapeutics, with the goal of inducing repair mechanisms in MS.

2. MATERIALS & METHODS

2.1. Myelin isolation from rat brains

Myelin was isolated from Wistar rat brains using a modification of the method of Norton and Poduslo (52). Briefly, brains were removed from rats, frozen in -80°C and stored in liquid nitrogen. One day in advance of myelin isolation, brains were transferred to -20°C. For myelin isolation, brain tissue was homogenized and dissolved in ice-cold 0.32M sucrose (Merck, Overijse, Belgium). 0,85M sucrose (Merck) was brought into ultracentrifuge tubes and overlaid with the homogenate. Tubes were centrifuged at 75 000g for 30 min. at 4°C. Myelin was removed from the 0.32 M/ 0.85 M-sucrose interface and subjected to hypo-osmotic shock by resuspension in ice cold MilliQ, followed by centrifugation (75 000g, 30 min., 4°C). The shocked membrane was recovered through centrifugation at 12 000g for 10 min at 4°C. The discontinuous sucrose gradient centrifugation was repeated. Purified myelin was resuspended in phosphate buffered saline (PBS) (Lonza, Verviers, Belgium) and stored at -20 °C. The myelin protein concentration was measured using a bicinchoninic acid (BCA) protein assay kit (Thermo Fisher Scientific, Erembodegem, Belgium). In short, a sample of the isolated myelin, was compared to a bovine serum albumin (BSA) standard curve. The standard was prepared by diluting a stock of 2 mg/mL BSA. 25 µl of each standard and sample was added to 200µL BCA reagent and incubated for 30 min at 37 °C. Absorbance was measured at 550 nm using a microplate reader (Biorad, Nazareth, Belgium). Isolated myelin contained a neglectable amount of endotoxin ($<1.8x10^{-3}$ pg/µg myelin).

2.2. DiI labeling of myelin

The myelin was isolated using density-gradient centrifugation. Myelin (6,7mg protein/mL) was fluorescently labeled as described by van der Laan et al. (53). In short, the lipophilic fluorescent dye 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate (DiI) (Sigma-Aldrich, Bornem, Belgium) was added to the myelin at a concentration of 40µM, followed by a 30 min. incubation period in humified atmosphere at 37°C. Excess of DiI was removed with several PBS washing steps. DiI-labeled myelin was stored in cryovials at -20°C.

2.3. Cell culture

Alveolar macrophage-derived NR8383 macrophages were cultured in RPMI supplemented with 0,5% penicillin/streptomycin (P/S) (all from Invitrogen, Merelbeke, Belgium) and 10% fetal calf serum (FCS) (Hyclone, Erembodegem, Belgium). Cell cultures were incubated in humidified atmosphere at 37°C and 5% CO_2, and passaged twice a week to ensure a continuous vigorous growth. Cells were counted using a Fuchs-Rosenthal counting chamber. Cell viability was assessed using the Trypan blue (Sigma-Aldrich) exclusion method, and cells were replated in 6-, 24- or 96 well plates (Greiner Bio One, Wemmel, Belgium) at a density of 1.10^6 cells/mL culture medium.

2.4. Preparation of liposomes

Liposomes were composed of phosphatidylcholine (Sigma-Aldrich) only (PCL), or of a combination of PC:PS at a molar ratio of 7:3 respectively (PSL). Briefly, the lipids PC or PC:PS, present in a solvent of chloroform or chloroform/methanol (95:5) respectively, were dried under nitrogen. Dried lipid films were resolved in PBS and vigorously vortexed. The liposomes were formed using a sonicating bath with ice, for 15 min. Liposomes were again vortexed, stored at 4°C, and immediately used. In some cases, DiI-labeled liposomes were used. To this end, liposomes were made followed by an incubation step with DiI (2.5µL DiI/mL liposomes; DiI conc. 10mg/mL) for 10 min. (dark). DiI-labeled liposomes were centrifuged at 16000 xG for 10 min., followed by several washing steps with PBS. DiI- labeled liposomes were vortexed, stored at 4°C and immediately used.

2.5. Phagocytosis assay

2.5.1. Myelin / liposome phagocytosis – Flow cytometry

Macrophages were cultured in 24 well plates (500 000 cells/well) in culture medium. DiI -myelin, DiI-PSL or DiI-PCL were added respectively to the cultures at a concentration of 100µg/ mL, for 24 hours. After the incubation period, cells were detached by vigorously pipetting, centrifuged for 3 min. at 2000rpm, resuspended in fluorescence activated cell sorting (FACS) buffer and transferred to FACS tubes. DiI fluorescence (i.e. relative myelin phagocytosis) was measured with a FACS Calibur flow cytometer (BD biosciences, Erembodegem, Belgium). Macrophage cultures without DiI-myelin supplementation were used as negative controls.

2.5.2. Myelin and myelin-derived lipid incorporation – Histochemical staining

Macrophages were cultured in 24 well plates (500 000 cells/well) on an inserted glass plate, in culture medium. Myelin or one of the myelin-derived lipids, e.g. phosphatidylserine (PS), phosphatidylethanolamine (PE), phosphoinositides (PI), sulfatide (SU) (All from Sigma-Aldrich) or gangliosides (GA) (Sercolab, Merksem, Belgium) were added respectively to the cultures for 24 hours at a concentration of 100µg/mL. Cultures were fixed with 4% paraformaldehyde (PFA) (Sigma-Aldrich) and stained with Oil red O (ORO)/ hematoxylin or Sudan Black B (SBB) (all from Sigma-Aldrich). In short, after fixation the cultures were washed three times with MilliQ, and stained for 30 min. with the respective dye. Next, ORO cultures were washed for 3 seconds with 60% isopropanol (Sigma-Aldrich), and SBB cultures were washed three times for 15 seconds, with 70% EtOH (Sigma-Aldrich). Cultures were then washed three times with MilliQ. ORO cultures were counterstained for 5-8 min. with hematoxylin, followed by another three washing steps with MillliQ. The glass plates were mounted in fluorescent mounting medium (Dako, Heverlee, Belgium). Myelin or lipid phagocytosis were observed using an Eclipse 80i microscope (Nikon). Unstained cells and macrophage cultures stained without the supplementation of myelin or lipids were used as negative controls.

2.6. Radical oxygen species production

Macrophages were cultured in 24 well plates (500 000 cells/well) in culture medium. Myelin was added to the respective cultures at a concentration of 100µg/mL. After 24 hours of incubation, medium was removed and phorbol myristate acetate (PMA) (Sigma-Aldrich) was added to the cultures at a concentration of 100ng/mL, and incubated for 15 min. in humidified atmosphere, 37°C, 5% CO_2. Medium was removed and cultures were incubated with 10µM dihydrorhodamine (DHR) (Sigma-Aldrich) for 15 min. in humified atmosphere, 37°C and 5% CO_2. Finally, cells were detached by vigorously pipetting and were centrifuged for 3 min. at 2000rpm. The supernatant was discarded, cells were resuspended in FACSbuffer and transferred to FACS tubes. Radical oxygen species (ROS) production was measured with a FACS Calibur flow cytometer. Controls included cells without supplemented myelin though stimulated with PMA, and cells with or without supplemented myelin without PMA stimulation.

2.7. Cell viability

Macrophages were cultured in 96 well plates (100 000 cells/well) in culture medium. Cultures were treated with myelin or one of the respective lipids (e.g PS, PE, PI, GA or SU) at a concentration of 100µg/mL. After a 24h incubation period, cells were further cultured for another 24h with an LPS stimulus (Calbiochem, USA) (100ng/mL) or no stimulus. Medium was removed and macrophages were incubated with 0,5mg/mL 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Sigma-Aldrich) for 4h, in humidified atmosphere at 37°C and 5% CO_2. Cells were centrifuged, the medium was removed and a mixture of dimethylsulfoxide (DMSO) (VWR)/0,1M glycine (Sigma-Aldrich) was added to the macrophages. Absorption was measured at 550nm with a microplate reader (Biorad). As a control, macrophage cultures without supplementary myelin, lipids or LPS stimulus were used.

2.8. Nitric Oxide production

Macrophages were cultured in 96 well plates (100 000 cells/well), in macrophage culture medium. Cultures were treated with myelin, one of the respective myelin-derived lipids (e.g PS, PE, PI, GA or SU), PSL or PCL at a concentration of 100µg/mL for 24h. After the incubation period, cells were further cultured for 24h with an LPS stimulus (100ng/mL) or no stimulus. NO production was measured with a Griess reagent kit (Promega, Heusden-Zolder, Belgium), following manufacturer's instructions. Briefly, 50µL of macrophage supernatant was incubated with an equal volume of sulphanilamide solution for 10min. at room temperature (dark). Next, samples were incubated with 50 µL N-1-napthylethylenediaminedihydrochloride (NED) under the same conditions. Nitrite concentrations in macrophage culture media were calculated using a nitrite standard curve. As a control, macrophage cultures without supplementary myelin, lipids or LPS stimulus were used.

2.9. Reverse transcription – Real time polymerase chain reaction

Total RNA was isolated from NR8383 macrophages using a High Pure RNA isolation kit (Roche Diagnostics, Vilvoorde, Belgium) according to manufacturer instructions. In short, macrophages were exposed to a lysis buffer and transferred to a High Pure Filter Tube placed in a collection tube. A DNase step was performed to remove genomic DNA. Following several washing steps, RNA was eluted from the filter tube. Next, total RNA concentration and RNA purity were verified with a Nanodrop Spectrophotometer ND-1000 (Isogen life science, St-Pieters-Leeuw, Belgium). If necessary, the RNA was stored at -20°C / -80°C for later use. RNA was reverse transcribed using a cDNA synthesis kit (Promega). Briefly, a cDNA-synthesis mix (25mM $MgCl_2$, 10x RTase buffer, 10mM dNTP mixture, 20-40 U/µL RNasin, 20 U/µL AMV RTase, 0.5 µg/µL Oligo dT primer) was added to the RNA sample, followed by a polymerase chain reaction (60' 42°C, 5' 95°C, 5' 4°C, ∞ 12°C) in a DNA Thermal Cycler (Biorad). Quantitative PCR was performed in an optical 96-well plate with an ABI PRISM 7500 fast sequence detection system (Applied Biosystems, Carlsbad, California) and universal cycling conditions (10' 95°C, (15''95°C)x40 and 60'' 60°C). Each 10µl reaction contained 5µl SYBR Green Master Mix (Applied Biosystems), 0.3µl gene-specific forward and reverse primers (all from Eurogentec, Seraing, Belgium) (10µM), 1.9µl nuclease-free water and 2.5µl pre-diluted cDNA. Amplification by the 7500 Fast Real-Time PCR System (Applied biosystems, Halle, Belgium) was followed by an analysis of the melting curve to check PCR product specificity. The PCR products were also loaded on 2% agarose gels to confirm the specificity of amplification and the absence of primer dimer formation. Data were analyzed using the $^{\Delta\Delta C}$t method (54). Expression levels were normalized using the most stable housekeeping genes, determined with geNorm (55) and NormFinder (56). The used primer sequences for real time PCR are listed in Table 1 (*see:* Addendum).

2.10. Rats

Dark agouti (DA) rats (Harlan) were used as an animal model for MS. For anesthesia, rats were held in a transparent chamber with 3% isofluorane gas. The animal was removed from the chamber at the onset of drowsiness. Its mouth and nose was then placed within an anesthesia mask, which was connected to a circuit designed to deliver and evacuate the gas through one tube. Rats continued spontaneous breathing and the anesthesia depth level was maintained unchanged throughout the entire experiment. Rats were also placed on a warn blanket to keep their body temperature at 37.5 °C ± 0.5 °C throughout the entire experiment. All rats were housed under specific pathogen–free conditions in an animal facility. All animal procedures were approved by the Hasselt University ethics committee for animals.

2.11. *In vivo* pharmacokinetics of liposomes

Healthy DA rats were intravenously injected in the tail vein with 5mg/kg (in 400µL) DiI-labeled PCL or PSL liposome suspension. As a control, an untreated rat was used. 18h after injection, animals were

sacrificed, and blood, liver, spleen, lungs, brains, spinal cord, thymus, and lymph nodes were collected.

Half of the organ tissue was homogenized in a 70µm cellstrainer (BD biosciences) in PBS. On cells of the spleen and the blood, a ficoll density gradient centrifugation was performed to obtain peripheral blood mononuclear cells (PBMC's). Cells of the different organs respectively were incubated with CD11b antibodies (Biolegend, Antwerpen, Belgium) and analyzed with a FACS Calibur flow cytometer. Cells of the untreated rat or cells receiving no CD11b staining were used as a negative control.

The remaining half of the organ tissue was used for immunohistochemistry. Tissue was embedded in optimal cutting temperature (OCT) compound Tissue-Tek (Sakura Finetek, Berchem, Belgium) and frozen in liquid nitrogen for cryosectioning and immunohistochemistry. The spleens of rats were cryosectioned (5µm) using a cryostat (Leica, Groot-Bijgaarden, Belgium), and stained for the presence of liposome-loaded macrophages. Part of the cryosectioned tissue was stained with ORO/ hematoxylin. Briefly, freshly cut sections were stained with ORO for 10 min., washed for 30 seconds in tab water, counterstained for 5 min. with hematoxylin, and rinsed for 5 min. with running tab water. Tissue was mounted in aqueous mounting medium (Dako, Heverlee, Belgium) and coverslipped. Other tissue sections were stained with macrophage marker mouse anti-rat CD68 (AbD Serotec, Düsseldorf, Germany) or mouse anti-rat CD169 (AbD Serotec) and a secondary detection antibody goat anti-mouse Alexa Fluor 488 (Invitrogen). In short, sections were fixed in ice cold aceton for 10 min. immediately after cutting. Slides were dried for 30 min., shortly rinsed with 1xPBS/ Tween (0,05%) (Merck) and blocked for 20 min. with 10% goat serum (Millipore, Brussels, Belgium). Spleen sections were then incubated overnight with a primary antibody diluted in 1xPBS. Following 3 rinses with 1xPBS/ Tween, sections were incubated for at least 2h with the secondary antibody diluted in 1xPBS/ 10% goat serum. Slides were then washed 3 times in 1xPBS/ Tween, followed by a dipwash in 70% EtOH, 5 dipwashes in 1xPBS and a 5 min. wash in MilliQ. Sections were mounted in fluorescent mounting medium (Dako) and coverslipped. Spleen sections of the untreated animal were used as a negative control. Tissue stained with secondary antibody though without prior staining with primary antibody was used to verify the absence of aspecific secondary antibody binding. Micrographs were made using an Eclipse 80i microscope (Nikon).

2.12. The effect of PS liposomes on chronic EAE

Dark agouti rats were immunized at day 0 with myelin oligodendrocyte glycoprotein (MOG) emulsified in complete Freund's adjuvant (Sigma-Aldrich) as a 1:1 mixture, or with a combination of MOG-CFA and PS liposomes (5mg/kg). 200µL of the emulsified MOG–CFA or MOG-CFA-PSL was injected subcutaneously near the base of the tail of each rat, at 2 contra-lateral sites (100 µl per area to minimize backflow). From day 6 on, rats were injected intravenously in the tail vein once a day with

PSL in PBS (5mg/kg in 400µL) or with PBS (control group). Weight and neurological scores were closely monitored (clinical EAE scoring system 0-5) and compared to controls.

2.13. Statistical analysis

Statistical analysis was performed with GraphPad Prism 4 software. Values given are means ± standard error of mean (SEM). To determine statistical differences between 2 groups, a t- test was used. Statistical differences between 3 or more groups, were determined with a one-way analysis of variance (ANOVA), using the non parametric Kruskal-Wallis test followed by the Dunns post hoc test, or using the parametric Dunnetts's or Tukey's multiple comparison tests. Differences were considered to be statistically significant at $p<0,05$. *$p<0.05$; **$p<0,01$; ***$p<0,001$.

3. RESULTS

3.1. Macrophages take up myelin and myelin-derived lipids *in vitro*

Myelin-loaded macrophages are derived from CNS resident microglia and infiltrating monocytes (57) that have phagocytosed large amounts of myelin debris. After the phagocytosis of myelin components, macrophages adopt an anti-inflammatory "M2-like" phenotype. The myelin component inducing the anti-inflammatory phenotype, however, is yet to be elucidated. In this study, we compared the effect of five selected myelin-derived lipid components (e.g. sulfatide (SU), gangliosides (GA), phosphatidylserine (PS), phosphatidylethanolamine (PE) and phosphatidylinositides (PI)) on the phenotype of macrophages.

To determine whether NR8383 macrophages phagocytose myelin and the myelin-derived lipids, different incorporation assays were performed. First, myelin phagocytosis was assessed using FACS. To this end, macrophages were cultured for 24h with supplemented DiI-labeled myelin. FACS analysis demonstrated that myelin was significantly taken up by the macrophages (Fig. 5A).

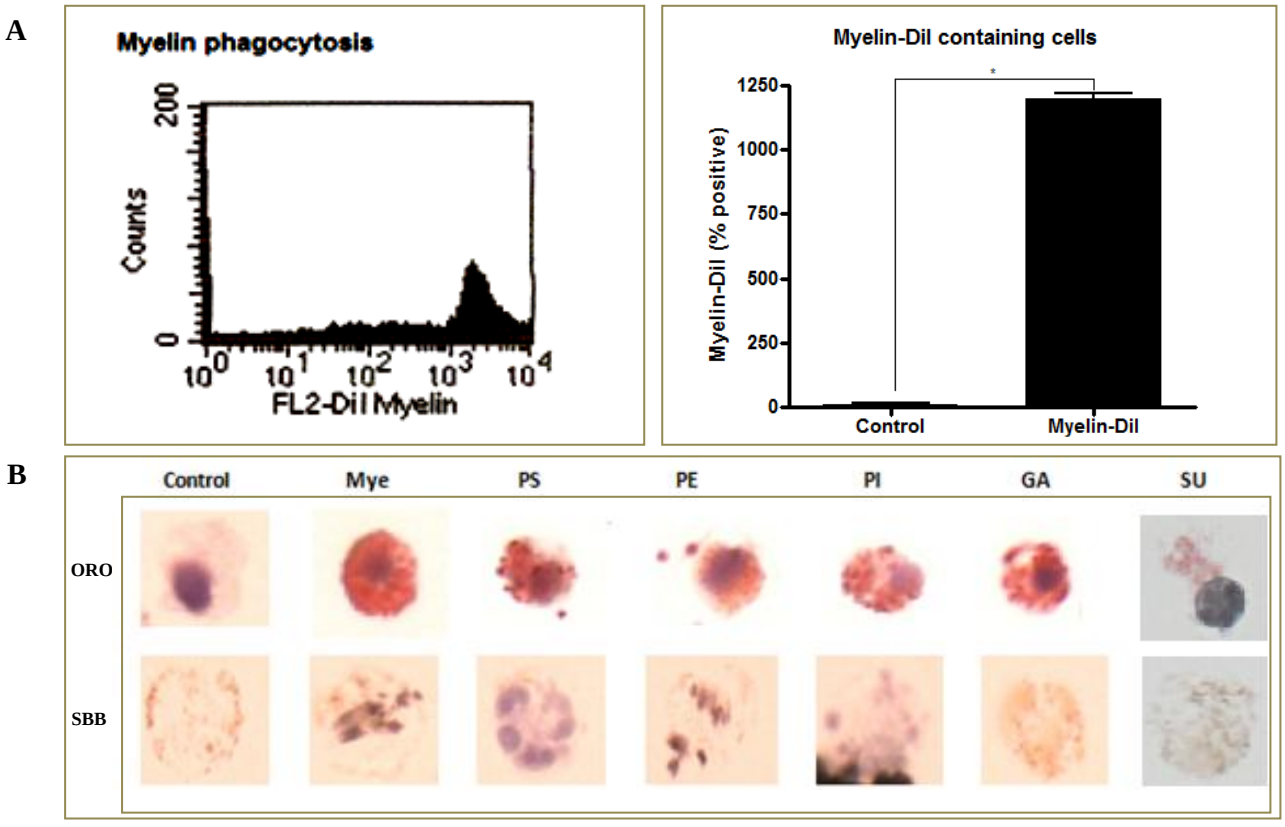

Fig. 5 (A) Macrophages readily phagocytose myelin. Macrophages were incubated with DiI-labeled myelin (100µg/mL) for 24h. The number of myelin-DiI positive macrophages was assessed by flow cytometry. A representative example shown in the left panel depicts fluorescence of myelin-DiI-laden macrophages. The graph in the right panel shows that macrophages had significantly taken up DiI-labeled myelin, compared to macrophages without supplemented myelin (control). Data are presented as mean ± SEM of one experiment with triplicate measurements per experiment. *p<0.05 (T-test). (B)

Macrophages incorporate myelin-derived lipids (e.g. PS, PE, PI, GA or SU). Macrophages were incubated with the myelin or one of the respective lipids (100µg/mL) for 24h, followed by fixation and staining with ORO/ hematoxylin (upper panel) or SBB (lower panel). As observed by light microscopy, macrophages had taken up myelin and the myelin-derived lipids. Whereas ORO stained neutral lipids red, SBB stained phospholipids grey/purple. Results are representative of 2 independent experiments with duplicate measurements per experiment.

To determine internalization of the divergent lipids, macrophages were incubated with the respective lipids for 24h, followed by fixation and staining with ORO/ hematoxylin or SBB. Analysis of cell staining by light microscopy showed that macrophages had incorporated myelin and the myelin-derived lipids (Fig. 5B). ORO stained all the lipids red, since Oil-soluble dyes stain lipids by being more soluble in the lipids than in their solvents. SBB stained the phospholipids (e.g. PS, PE and PI) grey/purple, because the dye is slightly basic and combines with acidic groups. These results demonstrate that macrophages readily phagocytose the divergent lipids used in this study.

3.2. Myelin phagocytosis induces the production of radical oxygen species *in vitro*

Radical oxygen species (ROS) are reactive molecules that are produced by the reduction of molecular oxygen and include species such as superoxide anion ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2) and the hydroxyl radical ($OH^{\bullet}$). Phagocytes produce ROS during the respiratory burst as a defense mechanism against pathogens. The intracellular sources that contribute to the generation of ROS in monocytes are diverse, including cyclooxygenases, lipoxygenases, mitochondrial respiration and nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (58). However, ROS are involved in more cell regulating functions, including cell growth, differentiation, proliferation, apoptosis and gene expression (59). Moreover, ROS are of possible importance in mediating and regulating inflammation (25, 60).

In both MS and EAE, myelin phagocytosis has been shown to induce the generation of reactive oxygen species (ROS) (18, 25) by macrophages. To verify whether our macrophages could reproduce these findings, we studied the effect of myelin incorporation on PMA-induced ROS production in macrophages. Our findings (Fig. 6) showed that the process of myelin phagocytosis (Ma Mye / - PMA) did not induce the production of ROS by itself, compared to the control cells (Ma / - PMA). PMA stimulation, however, significantly induced ROS production in macrophages (Ma /+ PMA). Moreover, pre-incubation with myelin appeared to induce an even stronger ROS production in macrophages after PMA stimulation (Ma Mye / + PMA).

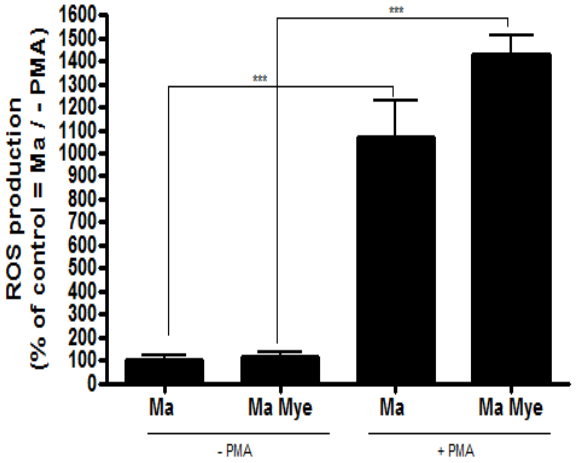

Fig. 6 Myelin phagocytosis induces the production of reactive oxygen species in PMA stimulated macrophages. Macrophages were incubated with myelin (100µg/ mL) for 24h, followed by PMA stimulation and analysis of ROS production by FACS. Myelin phagocytosis did not induce ROS production as such (Ma Mye / - PMA). Stimulation with PMA, however, induced the production of reactive oxygen species by macrophages. Macrophages that had been incubated with myelin produced somewhat higher amounts of ROS as compared to control macrophages (Ma / + PMA). Data are presented as mean ± SEM of one experiment with triplicate measurements per experiment. ***p<0.001 (One way ANOVA, Dunnett's Multiple Comparison Test).

3.3. PS incorporation suppresses NO production in macrophages *in vitro*

Nitric oxide (NO) is a free radical that is synthesized by a family of enzymes, referred to as the nitric oxide synthases (NOSs). L-Arginine (L-arg) is metabolized to NO by inducible nitric oxide synthase (iNOS) or to urea and L-ornithine (L-orn) by arginase (ARG1). A raised NO production is a typical hallmark of the M1 phenotype of macrophages. For this reason, we assessed macrophage NO production after pre-incubating them for 24h, 48h (data not shown) or 72h (data not shown) with myelin (100µg/mL), or one of the respective myelin-derived lipids (100µg/mL), followed by a 24h LPS (100ng/mL) stimulus, or no stimulus. As we observed the strongest effects after a 24h incubation period with myelin or the lipids, we continued to use this incubation period in following experiments.

NO production patterns of macrophages with LPS stimulation showed that gangliosides (GA) and sulfatides (SU) induced a somewhat similar pattern of NO production as myelin did, which was, a slight decline in NO production as compared to the control macrophages (Ma - / LPS) (Fig. 7). Phosphatidylserine (PS), however, seemed to act as a major inhibitor of NO production in LPS activated macrophages.

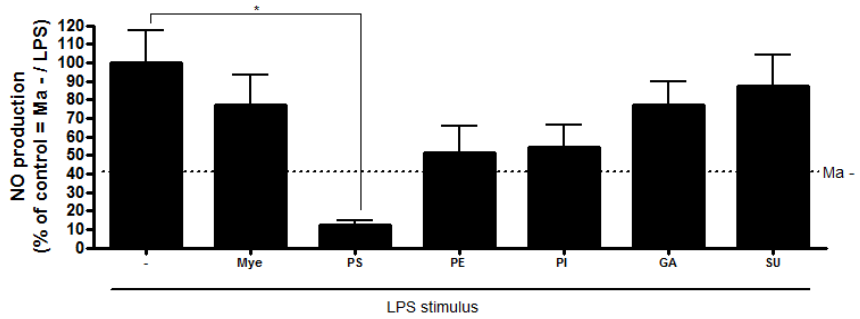

Fig. 7 PS incorporation suppresses the secretion of NO by LPS activated macrophages. Macrophages were incubated with myelin (100µg/mL) or one of the respective lipids (e.g. PS, PE, PI, GA or SU) (100µg/mL) for 24h, followed by a 24h

stimulus with LPS (100ng/mL) or no stimulus (Ma -). Nitric oxide levels in culture media were analyzed using a test based on the Gries reaction. The phagocytic engulfment of the myelin-derived lipids gangliosides (GA) and sulfatides (SU) induced somewhat similar NO production patterns in macrophages as seen after myelin phagocytosis. PS incorporation, however, significantly suppressed NO production in macrophages. Data are presented as mean ± SEM of two experiments with triplicate measurements per experiment. *p<0.05 (One way ANOVA, Kruskal-Wallis test and Dunn's post hoc analysis).

Analysis of cell viability with a Thiazolyl Blue Tetrazolium Bromide (MTT) reduction assay indicated that there were no significant differences in viability of the cells (data not shown). Since only moderate effects on NO production were seen after phagocytosis of PE, PI, GA and SU, the subsequent experiments were performed with myelin and phosphatidylserine. Of note, subsequent experiments revealed that the inhibitory effect of phosphatidylserine on macrophage-produced NO was seen not only in macrophage cultures stimulated with LPS , but also in cultures that received no stimulus (Fig. 8).

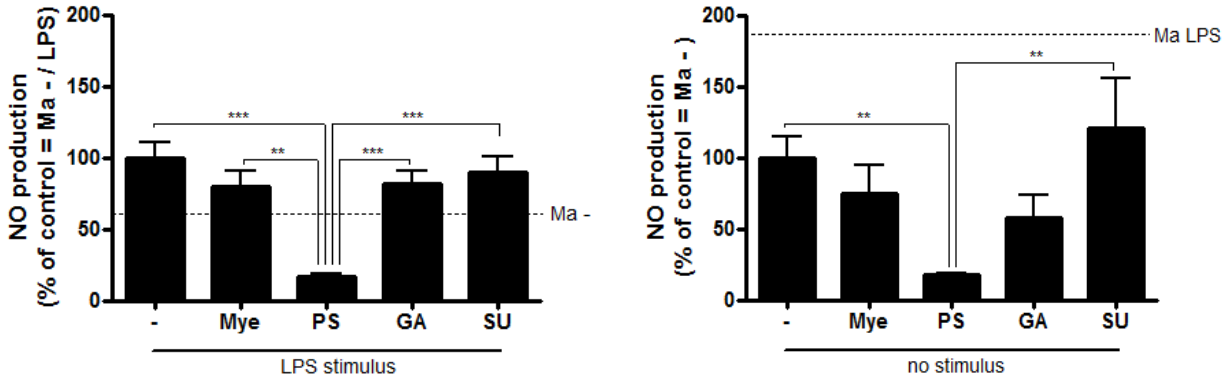

Fig. 8 Uptake of PS suppresses NO secretion by macrophages. Macrophages were cultured for 24h with myelin (100µg/mL) or one of the respective myelin-derived lipids (e.g. PS, GA or SU) (100µg/mL), followed by a 24h LPS (100ng/mL) stimulus (left graph) or no stimulus (right graph). Nitric oxide levels in culture media were analyzed using a test based on the Gries reaction. PS significantly suppressed macrophage NO production in macrophage cultures. Data are presented as mean ± SEM of four experiments with quadruplicate measurements per experiment. **p<0.01, ***p<0.001 (One way ANOVA, Kruskal-Wallis test and Dunn's post hoc analysis).

3.4. The incorporation of PS induces the expression of genes associated with an M2 phenotype and suppresses the expression of genes associated with an M1 phenotype

To further elucidate the phenotype of myelin- and phosphatidylserine-phagocytosing macrophages, gene expression of several pro- and anti-inflammatory genes was assessed by quantitative Real Time PCR. Three genes associated with an M1 phenotype (i.e. iNOS, TNFα and CXCL10) and three genes associated with an M2 phenotype (i.e. ARG1, IL-11, TGF-β1) were chosen. Additionally, gene expression of Oncostatin M was analyzed. Gene expression was measured after a 24h incubation

period with myelin or one of the selected lipids followed by a 24h period with an LPS stimulus. As a control, cells without supplementary lipids, though stimulated with LPS were used.

Here, we observed a significant downregulation of the pro-inflammatory genes iNOS, TNFα and CXCL10 after the incorporation of PS by LPS stimulated macrophages (Fig. 9). Phagocytosis of myelin significantly suppressed the expression of both iNOS and TNFα, but upregulated the expression of CXCL10. Furthermore, unlike myelin, the incorporation of PS in macrophages increased the expression of the genes ARG1, IL-11 and TGF-β1, which are all associated with an M2 phenotype in macrophages. An upregulation of the gene oncostatin M (OncoM) was seen after PS phagocytosis in LPS stimulated macrophages. Myelin uptake, however, suppressed the expression of OncoM.

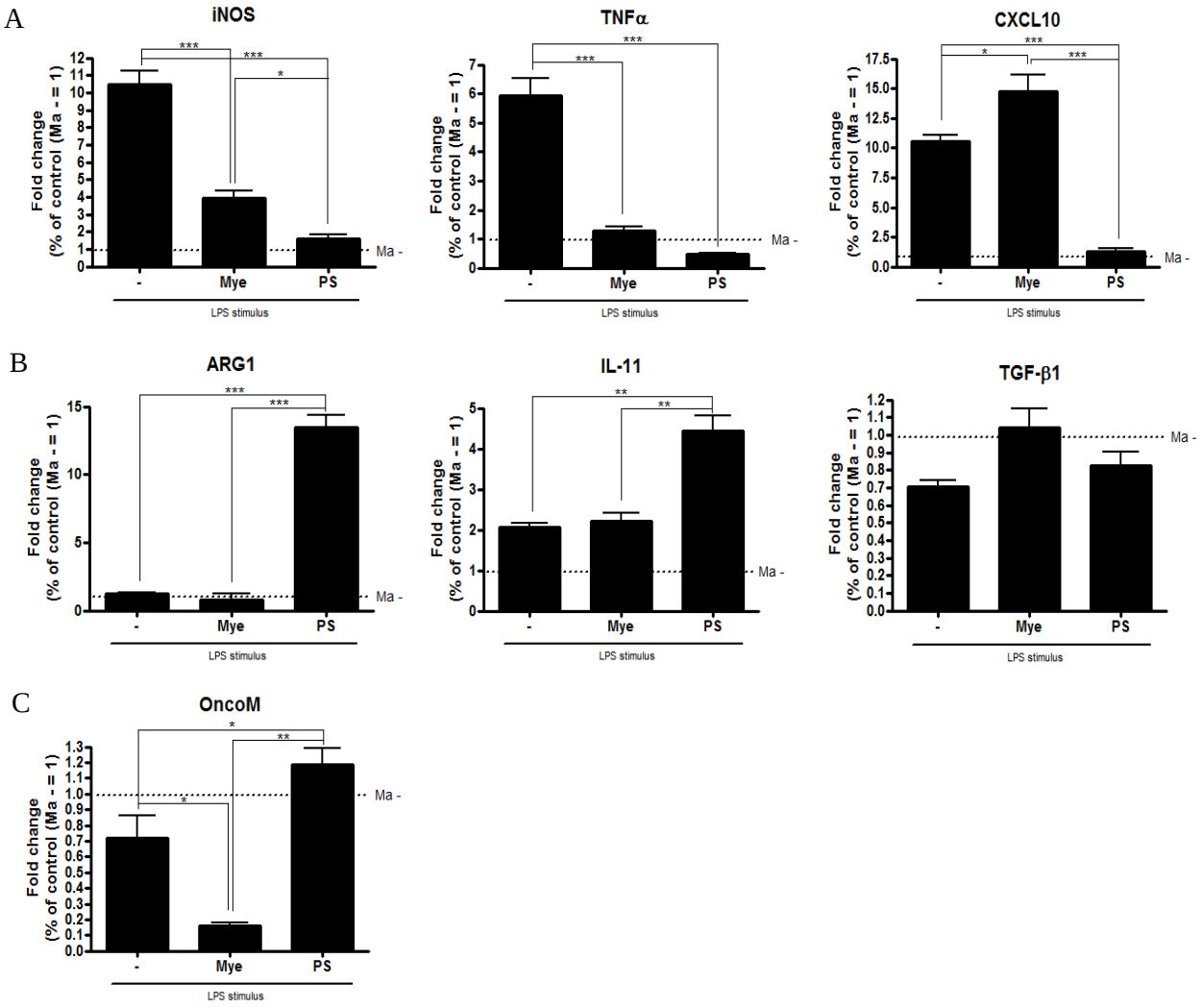

Fig. 9 The effect of PS and myelin incorporation on gene expression in LPS stimulated macrophages. Macrophages were incubated with myelin or PS (100µg/mL) for 24h, followed by a 24h stimulus with LPS (100ng/mL) or no stimulus (Ma -). mRNA was analyzed with real time quantitative polymerase chain reaction (RT PCR). (A) PS incorporation significantly suppressed the expression of the pro-inflammatory genes iNOS, TNFα and CXCL10 in LPS stimulated NR8383 macrophages, compared to macrophages without lipid incubation though stimulated with LPS (- / LPS). Myelin phagocytosis, however, significantly suppressed the expression of iNOS and TNFα, but upregulated the expression of

CXCL10. (B) The uptake of PS or myelin by LPS stimulated macrophages significantly upregulated the expression of the anti-inflammatory genes ARG1 and IL-11 but induced only a slight raise in the expression of TGF-β1, as compared to macrophages without lipid supplementation, though stimulated with LPS. (C) The uptake of PS by LPS stimulated macrophages significantly upregulated the expression of the gene OncoM as compared to macrophages without lipid incubation though stimulated with LPS. Myelin uptake, however, suppressed OncoM expression. Data were calculated as a percentage of unstimulated cells (Ma - = 1). Data are presented as mean ± SEM of one experiment, with triplicate measurements per experiment, and representative of at least three independent experiments with similar results. *p<0.05, **p<0.01, ***p<0.001 (One way ANOVA, Tukey's Multiple Comparison Test).

3.5. PS loaded liposomes are taken up by macrophages *in vitro*

Liposomes are frequently used as particulate carriers, that are taken up naturally by cells of the mononuclear phagocytic system (MPS), especially by macrophages. In view of the possible use of PS in an *in vivo* chronic EAE rat model, loading our PS lipids into liposomes could therefore be an efficient way of targeting our macrophages. PS liposomes (PSL) were made up out of phosphatidylcholine (PC) and phosphatidylserine in a molar ratio of 7:3.

To verify whether our macrophages could take up PS-loaded liposmes (PSL), we constructed PSL, labeled them with DiI and incubated them for 24h with macrophages followed by a FACS analysis. As a control, DiI-labeled PC liposomes (PCL) (100µg/mL) were used. Fig. 10 shows that macrophages readily incorporate DiI-labeled liposomes.

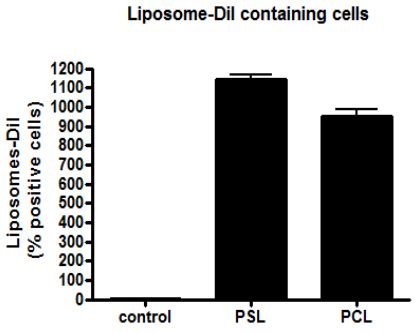

Fig. 10 Macrophages take up DiI-labeled liposomes *in vitro*. Macrophages were incubated with DiI labeled PSL or PCL (100µg/mL) for 24h, followed by FACS analysis. The liposomes were taken up by the macrophages, as shown by DiI fluorescence. Data are presented as mean ± SEM of one experiment, with duplicate measurements. (One way ANOVA, Kruskal-Wallis test and Dunn's post hoc analysis).

3.6. The uptake of PS liposomes suppresses LPS-induced NO production *in vitro*

To assess whether the phagocytosis of PS liposomes could suppress NO production to a similar extent as seen after PS incorporation, we incubated macrophages with PSL (100µg/mL) for 24h and then stimulated them for another 24h with LPS. As a control, DiI-labeled PCL (100µg/mL) were used. As shown in Fig. 11, uptake of PSL, but not PCL by macrophages significantly reduced NO production.

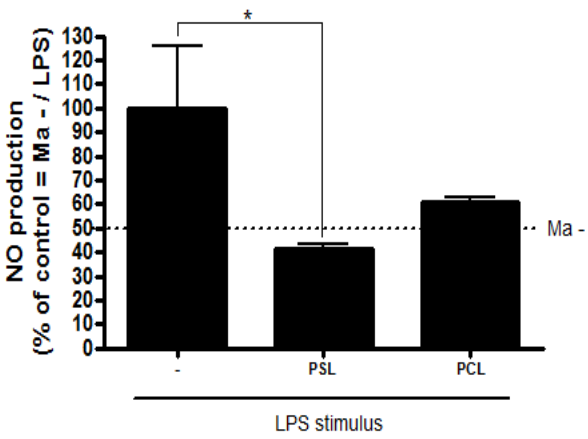

Fig. 11 PSL uptake suppresses NO production by LPS stimulated macrophages. Macrophages were incubated with PSL or PCL (100µg/mL) for 24h, followed by a 24h stimulus with LPS (100ng/mL) and analysis of nitric oxide levels in culture media using a test based on the Gries reaction. The uptake of PSL, but not PCL significantly suppressed NO production in macrophages. Data are presented as mean ± SEM of two experiments with quadruplicate measurements per experiment. *p<0.05 (One way ANOVA, Dunnett's Multiple Comparison Test).

3.7. The uptake of PSL induces an anti-inflammatory phenotype in macrophages *in vitro*

To assess the effect of PSL uptake on the expression of M1 and M2 associated genes of macrophages stimulated with LPS, gene expression patterns of ARG1, TGF-β1, TNFα and iNOS were analyzed with quantitative RT PCR. To this end, macrophages were incubated for 24h with PSL (100µg/mL) and cultured for another 24h with an LPS stimulus or no stimulus. As a control, DiI-labeled PCL (100µg/mL) were used.

Liposomes loaded with phosphatidylserine significantly suppressed iNOS, TNFα and TGF-β1 in LPS stimulated macrophages (Fig. 12). Moreover, they significantly upregulated the expression of the gene ARG1. Phosphatidylcholine liposomes induced, based on the expression of these genes, a less anti-inflammatory phenotype. Nonetheless, compared to LPS-stimulated M1 macrophages, they did induce a more "M2-like" phenotype.

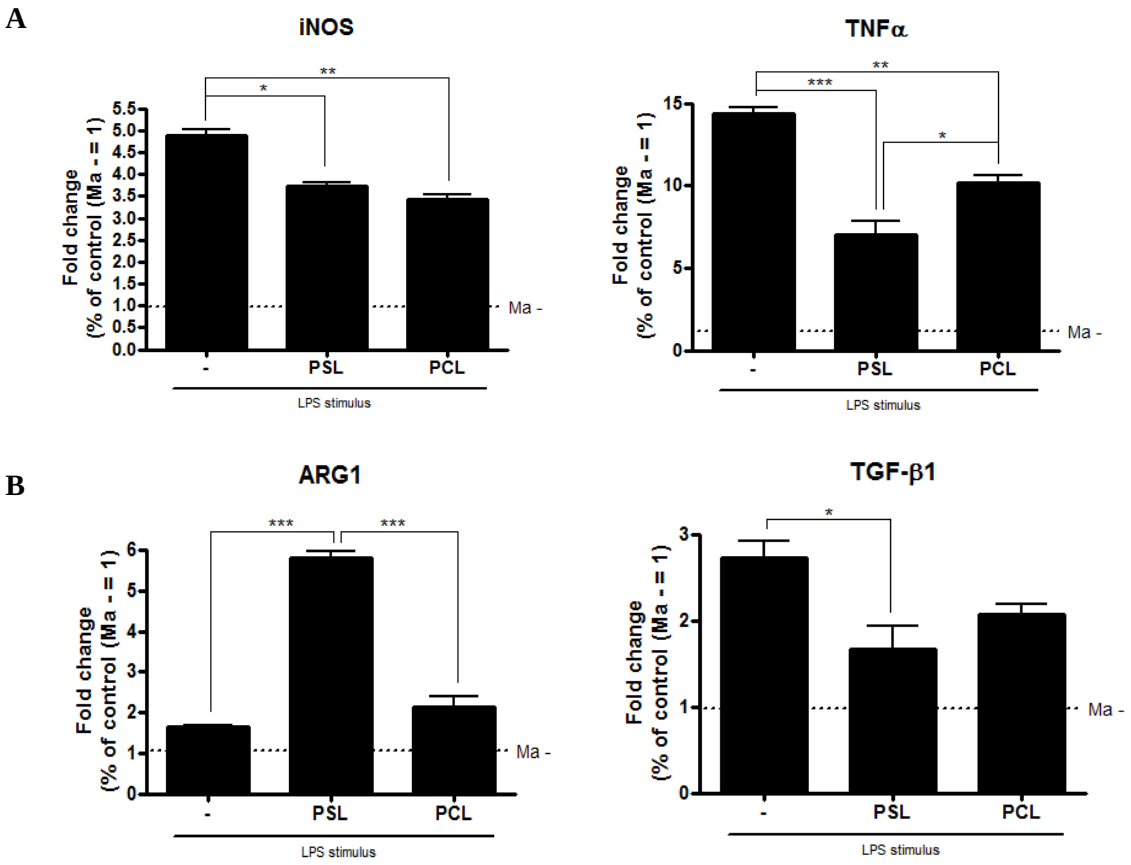

Fig. 12 The effect of PSL or PCL uptake by macrophages on gene expression in LPS stimulated macrophages. Macrophages were incubated with PS or PC liposomes (100µg/mL) for 24h, followed by a 24h stimulus with LPS (100ng/mL) or no stimulus (Ma -). mRNA was analyzed with real time quantitative polymerase chain reaction (RT PCR). (A) PSL and PCL incorporation significantly suppressed the expression of the pro-inflammatory genes TNFα and iNOS in LPS stimulated macrophages, as compared to macrophages without lipid incubation though stimulated with LPS (- / LPS). (B) The uptake of PSL by LPS stimulated macrophages significantly upregulated the expression of the gene anti-inflammatory gene ARG1 but significantly reduced the expression of TGF-β1, as compared to macrophages without lipid supplementation, though stimulated with LPS (Ma - / LPS). Data were calculated as a percentage of unstimulated cells (Ma - = 1). Data are presented as mean ± SEM of one experiment, with triplicate measurements per experiment, and representative of at least three independent experiments with similar results. *p<0.05, **p<0.01, ***p<0.001 (One way ANOVA, Tukey's Multiple Comparison Test).

3.8. PS loaded liposomes are taken up by macrophages *in vivo* and accumulate in the spleen in healthy rats

To verify whether macrophages could incorporate PS loaded liposomes *in vivo*, we intravenously injected PS-loaded, DiI labeled liposomes in the tail vein of healthy DA rats. As a control, we used i.v. injected DiI-labeled PCL. As a negative control, an untreated animal was used. As shown in Fig. 13, FACS analysis demonstrated that DiI-PCL and DiI-PSL were taken up by macrophages, and accumulated mainly in the spleen and the liver. However, we did observe fluorescence in the lungs,

lymph nodes and brain of both the PCL and PSL treated animals. The apparently reduced internalization of DiI-PSL liposomes can be explained by the fact that PSL were not labeled as extensively as the PCL liposomes (data not shown).

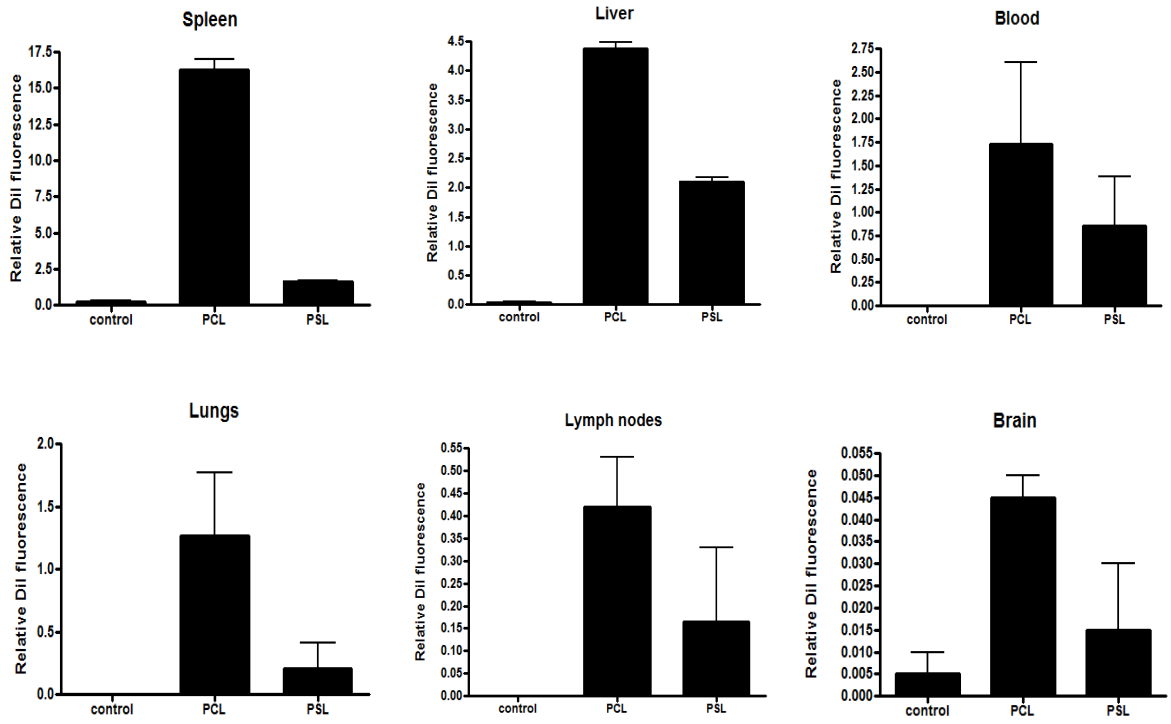

Fig. 13 The distribution of macrophages loaded with DiI-PCL or DiI-PSL in the organs of healthy rats. Rats were intravenously injected in the tail vein with DiI-PCL, DiI-PSL (5mg/kg in 400µL) or nothing. After 18h, the animals were sacrificed and the organs were collected and prepared for FACS analysis. Liposomes appeared to be mainly present in the spleen and the liver. Minor amounts of DiI fluorescence were present in the blood, the lungs, the lymph nodes and the brain. Data are presented as mean ± SEM of one experiment, with duplicate measurements per experiment. (One way ANOVA, Kruskal-Wallis test and Dunn's post hoc analysis).

To confirm the presence of DiI-PSL and DiI-PCL loaded liposomes in the spleen, cryosections were made and fluorescently stained with mouse anti-rat CD169 antibodies, or mouse-anti rat CD86 antibodies and detected with the secondary antibody goat anti-mouse Alexa Fluor 488 (Fig. 14). We found that the presence of PSL in the spleen was mainly seen in macrophages present in the marginal zone (MZ) and in the red pulp of the spleen. The MZ contains two types of macrophages, being Marginal Zone Macrophages (MZM) and Marginal Metallophilic Macrophages (MMM). Here we observed that whereas most MMM had not taken up any DiI-liposomes, several MZM had incorporated DiI-liposomes (red). Macrophages present in the red pulp of the spleen, stained with CD68 (green), contained a relatively higher number of liposome-DiI containing macrophages as compared to MZM or MMM.

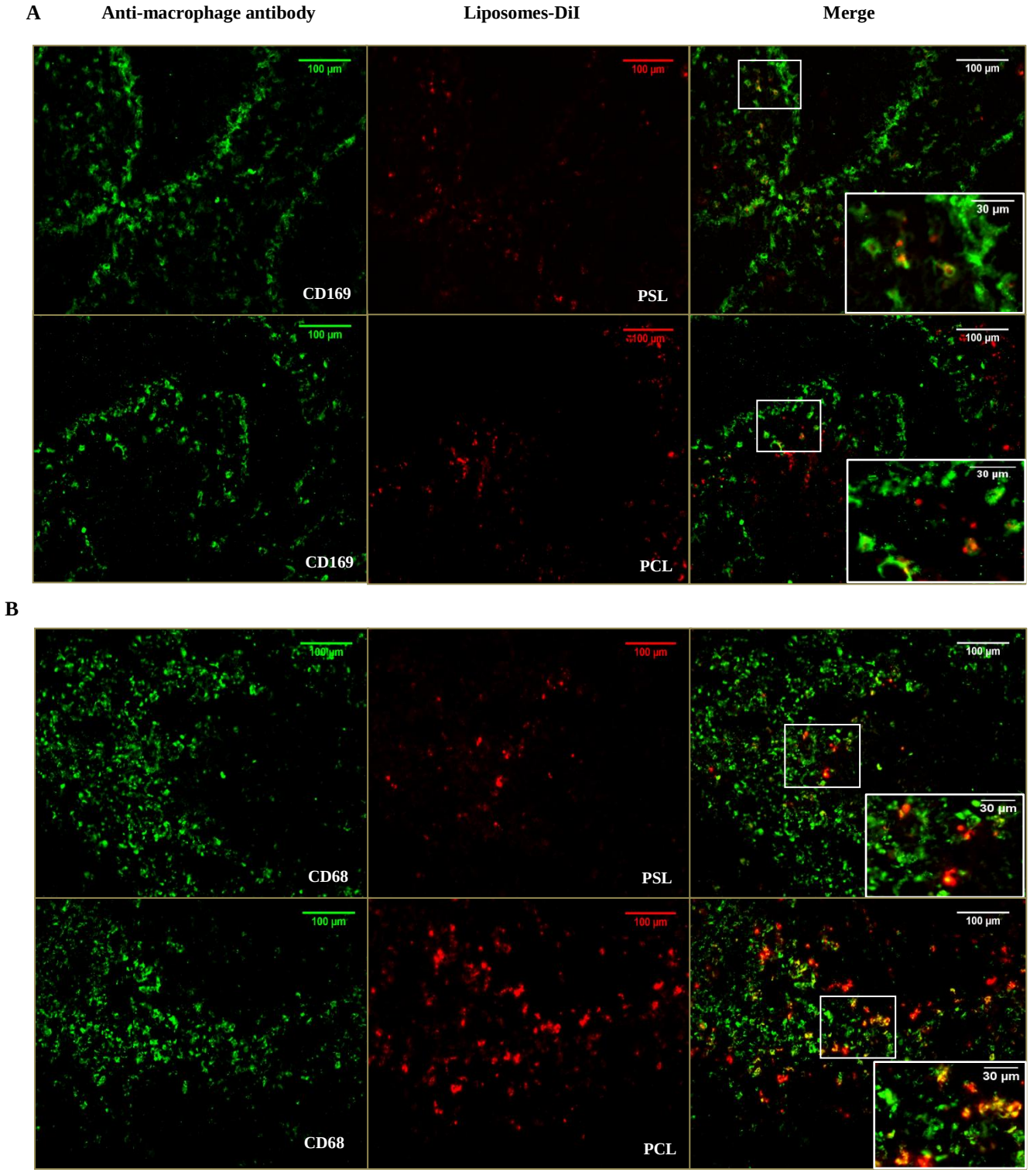

Fig. 14 The distribution of DiI-liposomes in the spleen of healthy rats. A healthy rat was intravenously injected with DiI-labeled PSL or PCL in the tail vein. As a control, an untreated animals was used. The animals were sacrificed after 18h, their organs were collected, cryosectioned and stained. (A) Under a fluorescent microscope, DiI-labeled liposomes (red) and CD169 positive Marginal Zone Macrophages (MZM) and Marginal Metallophilic Macrophages (MMM) (green) were additively combined. Whereas most MMM had not taken up any DiI-liposomes, several MZM had incorporated DiI-liposomes. (B) Macrophages present in the red pulp of the spleen, stained with CD68 (green), contained a relatively higher

number of liposome-DiI containing macrophages as compared to MZM or MMM. No DiI fluorescence was seen in the spleen of the untreated animal (data not shown). (Magnification x20, scale bars 100µm and 30µm for enlargements).

3.9. Intravenous PSL administration ameliorates EAE

To assess the effect of phosphatidylserine on EAE, we daily injected PSL (5mg/kg) in the tail vein of Dark agouti rats (n=9), starting from day 6, after immunization with MOG (day 0). Control animals (n=10) were injected with PBS. Weight and neurological scores (0-5) were closely monitored. EAE scores were determined according to the scoring system shown in Table 2 (*see:* Addendum). Higher EAE scores appeared to be associated with a decline in body weight. Moreover, PSL administration significantly ameliorated neurological scores at days post immunization (DPI) 10-12 (Fig. 15A) and significantly reduced weight loss at DPI 10-13 in animals with EAE (Fig. 15B). At day 13, rats in the control group started to remit, with lower neurological scores as a consequence.

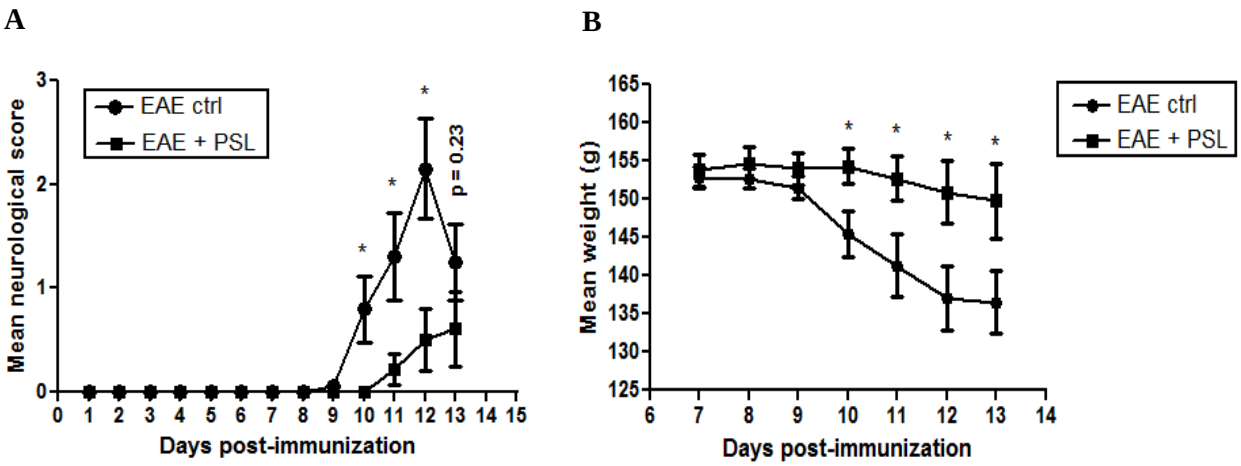

Fig. 15 The effect of PSL on EAE. Dark agouti rats were immunized on day 0 with MOG emulsified in complete Freund's adjuvant (CFA), or with a combination of MOG-CFA and PS liposomes (5mg/kg). From day 6 on, rats were injected intravenously in the tail vein once a day with PSL in PBS (5mg/kg in 400µL) (EAE + PSL, n=9, solid squares) or with PBS (EAE ctrl, n=10, solid circles). (A) Mean neurological scores are shown of control and treated animals. PSL administration significantly ameliorated neurological scores and weight loss in rats with EAE at DPI 10-12. At day 13, control rats started to remit with lower neurological scores as a result. (B) Shown are the mean weights of control and treated groups. PSL treated groups had significantly lower EAE induced weight loss at DPI 10-13. Data are presented as mean ± SEM of one experiment.*$p<0.05$ (T-test).

4. DISCUSSION

Injury in MS patients manifests as the formation of lesions in which degeneration of axons and removal of their surrounding myelin sheaths takes place. Remyelination can occur efficiently in primary lesions of MS patients. As the disease progresses, however, an increasing proportion of the areas of demyelination remains demyelinated. The question remains why the demyelination process is at times so thorough, and at other times fails to occur at all. Understanding the manifestation of remyelination is crucial for the development of new therapies in an attempt to convert areas of remyelination failure into areas of successful remyelination. A crucial step in an approach to find such a therapy, is to determine the prerequisites for remyelination per se.

A major inhibitor of remyelination in acute MS lesions is the production of pro-inflammatory molecules by activated macrophages/ microglia, which are extremely toxic to oligodendrocytes, neurons and myelin sheets. Moreover, the accumulation of myelin debris (61) inhibits the process of remyelination and axon regeneration. Although macrophages are mediators of CNS demyelination, they also deliver trophic factors and support myelin regeneration (62). It is well documented that activated macrophages/ microglia readily phagocytose myelin debris in MS and its animal model EAE (18, 53, 63-65). Several studies have shown that the incorporation of myelin by macrophages/ microglia (20, 21, 25) induces them to adopt an anti-inflammatory, 'foamy' phenotype. These 'foamy' macrophages may actively contribute to the resolution of inflammation in the CNS of MS patients.

Several reports (review (28)) indicate that the resolution of inflammation is an active process that is controlled by endogenous mediators which suppress the expression of pro-inflammatory genes, and induce inflammatory-cell apoptosis. Both of the previously mentioned processes are crucial to allow successful resolution of inflammation. Different studies (29, 30, 66) have pointed out lipids to contribute to the resolution of inflammation-associated diseases by inducing a more anti-inflammatory phenotype in macrophages. In this study, we hypothesized that certain lipids, present in myelin, can induce an anti-inflammatory phenotype in macrophages.

In this study we demonstrate that macrophages, cultured together with myelin or one of its derived lipids *in vitro*, contained numerous droplets that were stained with Oil red O (ORO) or Sudan Black B (SBB), suggesting that the particles surrounding the macrophages were taken up and stored as lipid droplets in the cells. These results are in agreement with several studies showing that macrophages, that had incorporated myelin, accumulate neutral lipids in their cell bodies and were thereby converted to foam cells *in vitro* (21, 67).

To determine the effect of myelin and the divergent lipids on the macrophage response we evaluated the production of ROS and NO.

In both MS and EAE, the incorporation of myelin by macrophages has been shown to induce the generation of ROS (18, 68).We confirm the upregulation of ROS by myelin-loaded macrophages *in vitro*. The effect that these free radicals have on the regulation of inflammation is still unclear. Nonetheless, the group of Hultqvist (60) reported that mice with EAE suffered a more severe disease course when carrying mutations in Ncf1, the gene encoding p47-phagocyte oxidase (PHOX), a key effector molecule of NADPH-oxidase. Consistent with this study, Liu et al. (25) have demonstrated that myelin-induced generation of ROS via p47-PHOX in macrophages, reduces the inflammatory response of microglia. Therefore, the observed increase in ROS production by myelin-loaded macrophages and microglia in the CNS may contribute to the resolution of inflammation in EAE and MS.

Nitric oxide is another free radical which is produced by macrophages upon certain stimuli. In macrophages, NO is generated by inducible NO synthase (iNOS). IFN-γ is an important factor for the priming of macrophages, and TNFα or LPS are strong inducers of NO production. NO limits injury to target molecules or tissues during events associated with excess production of reactive oxygen species. Nonetheless, NO plays an important role in the pathogenesis of MS (Fig. 16). In MS patients, the presence of NO and the upregulation of its associated gene iNOS is well documented. Analysis of the cerebrospinal fluid (CSF) (69) and the serum (70) from MS patients has shown the presence of higher levels of nitrite and nitrate in patients compared to normal controls. Although the high-output NO pathway probably evolved to protect the host from infection, simultaneous damage to other normal host cells occurs. It is believed that the toxic effects of NO in the process of neurodegeneration are related to its downstream metabolite, peroxynitrite ($ONOO^-$). Peroxynitrite is a highly reactive oxidant formed through the reactions of NO with superoxide radicals. *In vivo*, peroxynitrite leads to the disorganisation of the myelin structure and to axonal damage that shows similarities to the formation of MS lesions (71). Moreover, NO and $ONOO^-$ have also been implicated in BBB breakdown (72, 73), damage to astrocytes and to oligodendrocytes (74, 75), and may therefore contribute directly to demyelination *in vivo*.

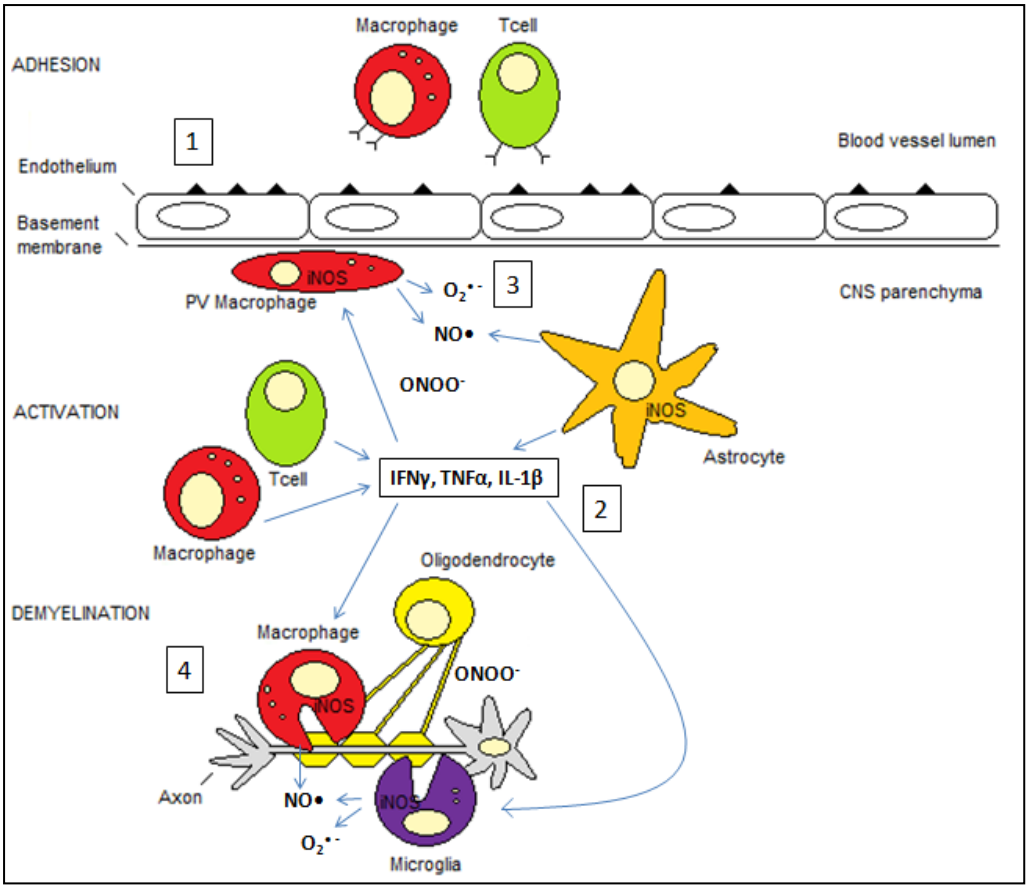

Fig. 16 The role of nitric oxide (NO) in the pathogenesis of MS and EAE. Activated T cells and circulating macrophages migrate from the blood vessel lumen into to the CNS parenchyma by means of chemotactic stimuli (not shown) and the expression of adhesion molecules on brain endothelial cells [1]. In the CNS parenchyma, T cells, macrophages and astrocytes contribute to the production of the inflammatory molecules IFN-γ, TNFα and IL-1β [2]. This pro-inflammatory environment activates perivascular (PV) macrophages, astrocytes, macrophages and microglia, and upregulates the expression of iNOS, the gene responsible for the synthesis of NO. Upon upregulation of iNOS, high amounts of $NO^{\bullet}$ and superoxide ($O_2^{\bullet -}$) are released by the cells, together forming the powerful oxidant peroxynitrite ($ONOO^-$). The oxidants released by PV macrophages and astrocytes exacerbate the loss of blood brain barrier function by promoting endothelial dysfunction [3]. Furthermore, oxidants released by phagocytic macrophages and microglia contribute to the demyelination process via damage to the myelin sheath of axons, and promote direct cell death of oligodendrocytes [4]. (Figure adapted from Parkinson et al., 1997 (76))

We evaluated the effect of a 24h, 48h or 72h incubation period with myelin or one of its derived lipids (e.g. PS, PE, PI, GA, SU) on nitric oxide production by macrophages. Effects were maximal after 24h, hence hereafter experiments were performed only for a 24h incubation period. Whereas myelin phagocytosis induced only a slight decline in macrophage NO production, PS significantly suppressed NO production by macrophages *in vitro*. This effect was seen, not only after LPS induction of macrophage cultures, but also in unstimulated macrophages. Furthermore, analysis of gene expression patterns showed the suppression of LPS induced iNOS, both after myelin and PS phagocytosis. Thus PS incorporation significantly suppresses the production of LPS induced NO production by macrophages both at transcriptional and translational level. Moreover, PS but not myelin incorporation

significantly upregulated the expression of the gene ARG1 in LPS stimulated macrophages. Since the suppression of iNOS and the simultaneous upregulation of ARG1 are two hallmarks of an M2 macrophage phenotype, it is presumptive that macrophages adopt an anti-inflammatory phenotype after the ingestion of PS. In view of the possible use of PS in an *in vivo* chronic EAE rat model, we saw liposomes as an efficient way of delivering PS to macrophages. It is well known that phagocytic cells such as macrophages preferentially take up liposomes composed of the negatively charged phospholipid phosphatidylserine (77). Initially, we co-cultured macrophages with PS liposomes (PSL) or PC liposomes (PCL) and found that macrophages readily incorporate the liposomes, and that the expression of the gene ARG1 is upregulated after PSL but not PCL incorporation in LPS stimulated macrophages. Furthermore, LPS-induced NO production is suppressed by PSL both at the transcriptional and translational levels. These results are consistent with those of a previous study of the group of Aramaki (78). They reported that the uptake of PSL, but not PC-liposomes by peritoneal mouse macrophages stimulated with LPS, suppresses their NO production *in vitro*, and that this inhibitory effect is due to a diminished iNOS induction. They also showed that the effect was dose-dependent, and a complete inhibition of NO was observed at 500µg PSL/mL. Furthermore, they reported that at least 21 hours of incubation with PSL were needed to decrease NO production by LPS-activated macrophages, and that preincubating the macrophages with liposomes seemed to be required in order to see this effect, since the addition of liposomes to macrophage cultures at the time of LPS stimulation did not interfere with their normal production of NO. Otsuka et al. (79) have also shown an inhibition in the production of NO by LPS stimulated PSL-loaded macrophages. Furthermore, the group of De Simone (80) described a strong reduction in NO production by LPS-activated microglia after the incorporation of PS liposomes. These effects, seen after PS incorporation in stimulated macrophages and microglia, are likely to be of great value for clinical research, since administration of such a molecule may contribute to the resolution of inflammation in diseases associated with an excess of NO, such as MS (81, 82).

Besides nitric oxide, the cytokine TNFα plays an important role in the pathogenesis of MS. Autopsies of MS patients have led to the discovery of elevated TNFα levels at the site of active MS lesions (83). Moreover, CSF and serum TNFα levels in patients with MS are elevated compared to healthy individuals, and these TNFα levels are correlated to the severity of the lesions (84, 85). To this end, we assessed macrophage TNFα gene expression of PS or myelin-loaded macrophages with RT PCR. We found that macrophages that had been incubated with myelin or PS significantly down regulated TNFα expression after a stimulus with LPS, compared to control cells (Ma - / LPS). The result that macrophage incubation with myelin following stimulation with LPS suppresses their production of TNFα, is consistent with the findings of Liu et al. (25). Furthermore, the group of Boven (21) also described a reduced expression of TNFα in foamy macrophages. When looking at the gene expression of TNFα of macrophages incubated with PSL, we also found a decreased TNFα expression,

comparable to that seen after PS incorporation. In literature, this suppressive effect of PSL on TNFα production has also been described for LPS-activated PSL-loaded microglia by the group of De Simone (80). According to literature, the reduction in TNFα production - seen after the uptake of PSL - is regulated by an upregulation of endogenously produced PGE_2. More specific, the inhibitory effect of PGE_2 is mediated by increasing levels of intracellular cAMP in macrophages, that selectively activates protein kinase A type I to inhibit gene transcription of TNFα (86). A reduction in the production of TNFα by PS, PSL or myelin-loaded macrophages and microglia, present in an MS lesion, may be relevant for the outcome of neurodegenerative diseases, since it may further contribute to the generation of an anti-inflammatory environment, needed for the resolution of inflammation and the regeneration of tissue.

To assess whether PS incorporation could also exert an effect on the recruitment of pro-inflammatory cells to the lesions, we analyzed the gene expression of the chemokine ligand 10 (CXCL10) in PS-loaded cells. Sorensen et el. (87) have suggested that CXCL10 is critical for the recruitment of T cells to the CNS in MS patients. Moreover, CXCL10 levels are elevated in the CSF of MS patients, and are associated with local demyelination in CNS tissue sections. We found that PS-loaded macrophages expressed significantly lower amounts of CXCL10 compared to control cells (Ma - / LPS). Myelin-loaded macrophages, however, significantly upregulated the expression of CXCL10. These results suggest that myelin-loaded macrophages do not completely match the M2 phenotype, and that the presence of such cells in the CNS might encourage the recruitment of even more pro-inflammatory cells to the lesion. The presence of PS-loaded macrophages in MS lesions, in contrast, may actually suppress the recruitment of pro-inflammatory cells.

Among the cytokines that are directly involved in the process of the regulation of inflammation, IL-11 plays an important role. The group of Gurfein (88) recently described that IL-11 is immunoregulatory and neuroprotective in EAE via immune system modulation as well as via trophic effects on oligodendrocytes. Furthermore, IL-11, like IL-10, accelerates the process from inflammation back to homeostasis, and its administration has been proven to be effective in resolving several other disease models associated with chronic inflammation (89). Moreover, i*n vitro*, IL-11 promotes myelin formation. Our data, indicate that PS but not myelin is able to upregulate IL-11 expression in LPS stimulated macrophages. Again, indicating that myelin-loaded macrophages have more of a mixed phenotype.

Analysis of the expression of the gene TGF-β1 indicated that neither PS, nor myelin uptake induces a significant difference in LPS stimulated macrophages, compared to control cells (Ma - / LPS). Moreover, PS liposomes suppressed TGF-β1 expression in activated macrophages. These results are in line with the findings of De Simone et al. (80), who described that TGF-β1 production by microglia stimulated with LPS is decreased after an incubation period with PS liposomes. However, this

decreased production of TGF-β1 is in contrast to the studies of Otsuka et al (90) and Matsuno et al. (91) that report an upregulation of TGF-β1 expression in macrophages after incubating them with PSL and stimulating them with LPS. Moreover, these studies demonstrate that the anti-inflammatory effect of PSL on macrophages is a results of an autocrine inhibitory effect of TGF-β1 on pro-inflammatory gene expression. These groups, however, both used only a selection of sizes of liposomes, indicating that the size of liposomes may have an impact on the gene expression induced upon phagocytosis of the liposomes. The main function of TGF-β1 in the immune system is to maintain tolerance through the regulation of lymphocyte proliferation, differentiation, and survival. Moreover, defects in TGF-β1 expression or in its signaling to T cells are correlated with the onset of several autoimmune diseases. In contrast, TGF-β1 also plays a role as a pro-inflammatory cytokine through the induction of IL-17 producing pathogenic T helper cells during an inflammatory response in which IL-6 is produced (92). Therefore, the neutral amount of macrophage TGF-β1 secretion by PSL- and myelin-phagocytosing macrophages in our experiments can either promote or inhibit the resolution of inflammation.

Finally, we analyzed expression of the gene Oncostatin M (OncoM). The OncoM protein is *in vitro* functionally related to IL-6 and IL-11 proteins that also influence immune and inflammatory functions (93). The *in vivo* properties and functions of OncoM, however, remain poorly defined. In the normal progression of inflammation, a self-limiting feedback system induces the production of acute phase proteins that attenuate the inflammatory response and allows wound healing and the return to tissue homeostasis. OncoM has been shown to regulate the expression of these acute phase proteins in rodents and non-human primates, indicating its importance in the resolution of inflammation (94). Furthermore, the administration of OncoM in mouse models of rheumatoide arthritis (RA) and MS suppressed inflammation (95). Consistent with these findings, we found an upregulation of OncoM after PS ingestion by LPS stimulated macrophages. Macrophage incubation with myelin, however, suppressed OncoM expression.

Altogether, whereas the macrophages that have incorporated phosphatidylserine particles adopt an M2 phenotype, myelin-loaded macrophages appear to adopt a phenotype that is clearly distinct from both the classical and the alternative phenotypes. However, more versatility among macrophages has been described in several different physiological and pathophysiological contexts (96, 97). Furthermore, as phosphatidylserine is only a minor constituent of myelin, the phenotype of myelin-phagocytosing macrophages is most likely the result of a combination of divergent stimuli delivered by multiple lipids and proteins present in myelin. Nevertheless, the M2 phenotype of PS or PSL loaded macrophages may be relevant for the resolution of inflammation in inflammatory, neurodegenerative diseases like MS.

The mechanisms whereby the pro-inflammatory NO and TNFα production are inhibited, and the anti-inflammatory ARG1 is upregulated in activated macrophages loaded with PS particles are still unclear.

Nonetheless, the uptake of phosphatidylserine-containing particles via specific receptors on macrophages can induce the expression of anti-inflammatory and immunosuppressive cytokines. Furthermore, a process which typically induces an immunological quiescence state in macrophages is the phagocytosis of apoptotic "self" cells during homeostatis (98-101). Therefore, the uptake of PS particles and the resulting M2 phenotype of the macrophages is possibly the result of "apoptotic mimicry" (Fig. 17). The presence of such macrophages in EAE or MS patients may contribute to the amelioration of the clinical state of disease. To this end, therapeutic strategies, like the administration of PSL that are specifically taken up by macrophages and mimic the incorporation of apoptotic cells may be useful for the treatment of inflammatory disorders.

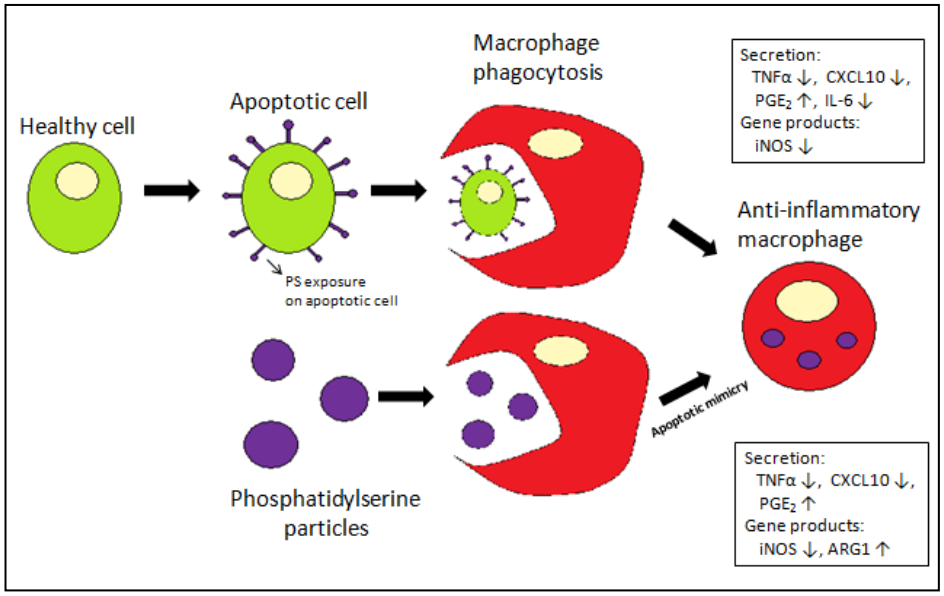

Fig. 17 PS particles mimic the effect of apoptotic cell incorporation by a process called "apoptotic mimicry". A healthy cell that receives certain signals (toxins, hormones, NO, cytokines, radiation) can induce the pathway of programmed cell death or apoptosis in the cell. Apoptotic cells externalize PS from the inner to the outer leaflet of the plasma membrane, which is then recognized by phagocytes that quickly take up the apoptotic cells before any content leakage of the apoptotic cell occurs. The macrophage that has taken up the apoptotic cell is subjective to a signaling cascade that induces it to adopt a more anti-inflammatory phenotype (e.g. TNFα ↓, CXCL10 ↓, PGE_2 ↑, iNOS ↓). Most of these characteristics are similarly seen after PS particle incorporation by macrophages, suggesting that the anti-inflammatory phenotype of macrophages - seen after the uptake of PS particles - is induced by a mechanism called "apoptotic mimicry".

Because PSL may be useful as a therapeutic agent in inflammatory diseases like MS, we assessed the effect of PSL-liposomes on EAE. Initially, we assessed pharmacokinetics of DiI-PS liposomes in healthy DA rats. When fluorescence-labeled PS liposomes were intravenously injected, labeled liposomes were mainly localized in the spleen and the liver, and minor amounts of PSL were present in the blood, the lymph nodes, the brain, the spinal cord and the lungs. The presence of PSL in the brain and the spinal cord suggests that resident macrophages of the CNS may directly take up approaching PSL, thereby adopting a more M2 phenotype. Accumulation of PS liposomes in the liver and the spleen of rats has also been described by the group of Palatini et al. (102). The spleen

functions as an extensively branched arterio-venous filter present in the bloodstream. As a consequence, a very large number of lymphocytes continuously passes through this organ. Lymphocytes that enter the spleen through the central arteriole leave the bloodstream in the marginal zone where small arterioles end in sinuses. Whereas the majority of the lymphocytes are carried along by the bloodstream into the red pulp and eventually leave the spleen by the venous bloodstream, a minority of the lymphocytes actively migrates from the marginal zone into the compartments of the white pulp (Fig. 18).

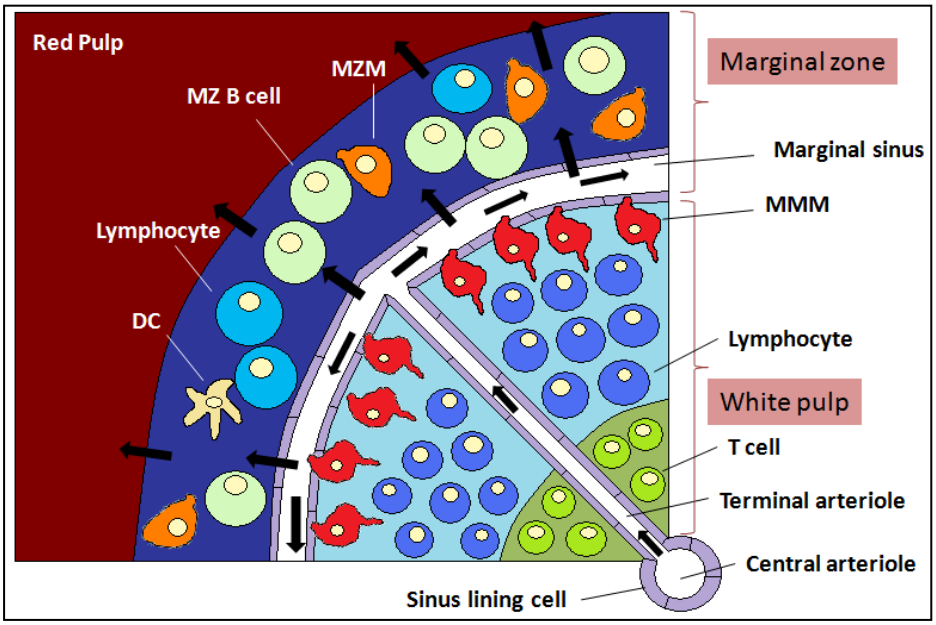

Fig. 18 The anatomic organization of cells in the spleen. A central arteriole branches into a terminal arteriole which opens into a sinus that is present at the margin of the white pulp, termed the marginal sinus. Different gaps exist between cells lining the sinus allowing blood (black arrows) to pass through these gaps, which is then filtered by marginal zone (MZ) B cells (green), macrophages (orange) and DCs (beige) on its way to the red pulp. The MZ contains two types of macrophages, being Marginal Zone Macrophages (MZM) and Marginal Metallophilic Macrophages (MMM). Whereas MMM are localized at the inner border of the MZ, adjacent to the white pulp, MZM can be found at the outer rim, close to the red pulp. Not shown are the many cells travelling from the blood into the red pulp and the minority of lymphocytes that travel from the marginal sinus into the white pulp. (Fig. adapted from Cyster, 2000 (103)).

We found that the presence of PSL in the spleen was mainly seen in macrophages present in the marginal zone (MZ) and in the red pulp of the spleen. The MZ contains two types of macrophages, being Marginal Zone Macrophages (MZM) and Marginal Metallophilic Macrophages (MMM) that can be identified with CD169 antibodies. In these cells, PS liposomes appeared to be mainly present in MZM. The cells present in the marginal zone are well equipped to constantly screen the blood for foreign particles, unusual debris and dying cells, because they are very well located at the interface between the lymphoid compartment of the spleen and the scavenging red pulp compartment. Nonetheless, relatively more DiI-liposome fluorescence was present in macrophages in the red pulp, which were detected with CD68 antibodies. Since the majority of lymphocytes that enters the spleen migrates towards the red pulp, it is likely that they will come across PS loaded, anti-inflammatory

macrophages. As a consequence, the anti-inflammatory mediators secreted by these macrophages may inhibit the responses of activated T cells during the effector phase of EAE. Moreover, MZM and macrophages in the red pulp of the spleen that have taken up PSL, may migrate to the CNS, and directly contribute to the resolution of inflammation herein. In literature, it has been described that MZM play an important role in the clearance of circulating apoptotic cells. MZM that phagocytose apoptotic cells expressing MOG can inhibit EAE in mice, by inhibiting MOG-specific T cells (104). These apoptotic cells, however, could not abolish EAE when PS on these apoptotic cells was masked, indicating that the PS signal is of paramount importance for MZM for the inhibition of T cells.

Given these findings, we studied the effect of PSL in a chronic EAE model of Dark Agouti rats. The intravenous administration of PSL at the time of MOG immunization (day 0) was followed by a series of injections once a day, starting from day 6 post immunization. The treatment with PSL resulted in significantly lower EAE scores and weight loss. These results are consistent with previous studies of PSL in other inflammatory diseases. Ma et al (105) reported that the administration of PSL in adjuvant arthritic (AA) rats suppresses inflammatory bone loss, and that this effect is mediated mainly through the upregulation of PGE_2. Furthermore, Wu et al. (106) described that the administration of PSL may have potential for pharmacological interventions for inflammatory and immune diseases through feedback mechanism utilizing PGE_2. Moreover, Ramos et al. (107) have shown that phosphatidylserine liposomes reduce inflammation through the activation of peroxisome proliferator-activated receptors (PPARs) *in vivo*. Altogether, the results described in this study strongly suggest that the administration of PSL actively contributes to diminished inflammation in EAE and possibly in MS .

5. CONCLUSION & SYNTHESIS

Current therapies for MS partially reduce new lesion development and prevent clinical disease activity to a certain degree, but none can halt the progression or cure the disease. Many current therapeutic strategies aim at an effective T cell inhibition, and most of these treatments also influence the myeloid compartment. Interestingly, recent evidence has indicated that myelin phagocytosis by infiltrated macrophages and activated microglia in the CNS of MS patients is not just a hallmark of multiple sclerosis, but also a key determinant of lesion development, disease progression, and most likely also disease resolution. We speculated that the severe complications, and/or the insufficient effectiveness of the current treatments necessitates the search for novel therapeutic targets, and postulated that these should aim at the manipulation of activated macrophages present in the CNS of MS patients.

Our hypothesis stated that certain lipid components of myelin can induce an anti-inflammatory phenotype in macrophages. Indeed, phosphatidylserine (PS), a myelin-derived lipid, induces the expression of the anti- inflammatory genes ARG1, IL-11 and OncoM, and simultaneously downregulates the production of the pro-inflammatory genes iNOS, CXCL10 and TNFα in LPS stimulated macrophages. Furthermore, PS liposomes accumulate in the MZM of the spleen in healthy rats, indicating that in a disease model, the anti-inflammatory mediators secreted by these macrophages may inhibit the responses of activated T cells during the effector phase of EAE. Moreover, macrophages loaded with PSL, may migrate to the CNS in EAE, and directly contribute to the resolution of inflammation herein. Our findings that the administration of PSL in an *in vivo* chronic EAE model significantly ameliorates neurological scores and weight loss, indicate that the use of PSL is likely to have a therapeutic effect in both EAE and MS. Nonetheless, a more profound study on the specific effects of PSL administration on possible neuroprotection, neurogenesis, remyelination and the plausible mechanism underlying these effects is needed.

Future experiments include a follow-up of the disease course (EAE scoring and weighing) of the animals that are being treated with PS liposomes at the moment compared to the control group. In the following experiments, different doses of PSL administration may be applied to animals with chronic EAE. Furthermore, when animals are sacrificed, the size of the germination centers in the lymph nodes, the amount of lesions (if present) in the CNS, and the amount of activated T cells will be analyzed. Isolation and co-culturing of these T cells with the immunization protein (MOG) may give a further indication of the amount of MOG-specific proliferation present in the animals. Moreover, immunohistochemistry on the brain and the spleen with antibodies directed against macrophages and M2 markers (e.g. ARG1, mannose receptor, etc…) will further elucidate the phenotypes of macrophages present in these locations.

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7. ADDENDUM

7.1. Supplemental Materials & Methods

Table 1 Primers used for quantitative RT PCR. Oncostatin M (OncoM), Tumor Necrosis Factor alpha (TNFα), inducible Nitric Oxide Synthase (iNOS), Tumor Growth Factor beta 1 (TGF-β1), Chemokine Ligand 10 (CXCL10), Arginase 1 (ARG1). All primers were provided by Eurogentec.

Gene	Species	Primer sequence (‘5-‘3)	Product size	Accession number
TNFα	*Rattus norvegicus*	F: GTCTGTGCCTCAGCCTCTTC R: CCCATTTGGGAACTTCTCCT	113 bp	NM_012675.3
iNOS	*Rattus norvegicus*	F: CACCAGGAGATGTTGAACTA R: GACTTTCCTGTCTCAGTAGCA	221 bp	NM_012611
TGF-β1	*Rattus norvegicus*	F: CTAATGGTGGACCGCAACAACG R: TCTGGCACTGCTTCCCGAATG	104 bp	NM_021578.2
CXCL10	*Rattus norvegicus*	F: GGGCCATAGGAAAACTTGAAATC R: CATTGTGGCAATGATCTCAACAT	71 bp	NM_139089.1
OncoM	*Rattus norvegicus*	F:AACATCCAAGGGATCAGGAAC R: GAAGACTCTCCCCACTGAACC	201bp	NM_001006961.1
ARG1	*Rattus norvegicus*	F: CAAGCTGGGAATTGGCAAAG R: GGTCCAGTCCATCAACATCAAA	101 bp	NM_017134

Table 2 Clinical scores of disease in EAE.

Score 0	**Normal**
Score 1	**Complete tail paralysis**
Score 2	**Complete hindlimb paralysis**
Score 3	**Complete paralysis of hindlimbs and midriff**
Score 4	**Complete hind and front limb paralysis**
Score 5	**Moribund**

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