2011
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GENEESKUNDE

master in de biomedische wetenschappen: klinische moleculaire wetenschappen

Masterproef

The immunomodulatory effects of phosphatidylserine containing liposomes in EAE rats

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

Maastricht University

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Jo Mailleux
June 9, 2012

ABBREVIATIONS

ANOVA = Analysis of Variance

APC = Antigen Presenting Cell

ARG1 = Arginase 1

BBB = Blood Brain Barrier

CD 4 = Cluster of Differentiation 4

CFA = Complete Freund's Adjuvant

CNS = Central Nervous System

CSF = Cerebrospinal Fluid

CXCL10 = C-X-C motif chemokine 10

DA = Dark Agouti (rats)

DAPI = 4,6'-diamidino-2-phenylindole

DMSO = Dimethyl sulfoxide

DNA = Deoxyribonucleic Acid

DPI = Days Post Immunization

EAE = Experimental Autoimmune Encephalomyelitis

EDTA = Ethylene Diamine Tetraacetic Acid

FACS = Fluorescence Activated Cell Sorting

FCS = Fetal Calf Serum

FITC = Fluorescein isothiocyanate

ICAM-1 = Inter-cellular adhesion molecule 1

IFN-γ = Interferon Gamma

IL-1 = Interleukin 1

IL-1R = Interleukin 1 Receptor

iNOS = inducable Nitric Oxide Synthase

LFA-1 = Lymphocyte function-associated antigen 1

LN = Lymph Nodes

LPS = Lipopolysaccharide

LXR = Liver X receptor

MBP = Myelin Basic Protein

MMM = Marginal Metallophilic Macrophages

MMP = metalloproteinase

MOG = Myelin Oligodendrocyte Glycoprotein

MS = Multiple Sclerosis

MZ = Marginal Zone

MZM = Marginal Zone Macrophages

NO = Nitric Oxide

OCT = Optimal Cutting Temperature

OPN = Osteopontin

PCR = Polymerase Chain Reaction

PC = Phosphatidylcholine

PCL = Phosphatidylcholine liposome

PBS = Phosphate Buffered Saline

PCL = Phosphatidylcholine liposomes

PLP = Proteolipid Protein

PS = Phosphatidylserine

PSL = Phosphatidylserine liposome

PPAR = Peroxisome Proliferator-activated Receptor

PPMS = Primary Progressive Multiple Sclerosis

PPRE = Peroxisome Proliferator Response Element

UFA = Unsaturated Fatty Acid

RNA = Ribonucleic Acid

ROS = Reactive Oxygen Species

RRMS = Relapsing-Remitting Multiple Sclerosis

ROS = Reactive Oxygen Species

RT-qPCR = Real Time Polymerase Chain Reaction

SC = Spinal Cord

SEM = Standard Error of Mean

SFA = Saturated Fatty Acid

SPMS = Secondary Progressive Multiple Sclerosis

TH1 = T Helper Cell 1 response

TCR = T cell Receptor

TGF-β = Transforming Growth Factor Beta

TNF-α = Tumor Necrosis Factor alpha

TRITC = Tetramethyl Rhodamine Iso-Thiocyanate

VCAM-1 = Vascular adhesion molecule 1

VLA-4 = Very Late Antigen 4

ABSTRACT

Multiple Sclerosis (MS) is an autoimmune disease, characterized by chronic inflammation and demyelination in the central nervous system (CNS). Macrophages play a central role in the disease process of MS and its animal model experimental autoimmune encephalomyelitis (EAE) by phagocytizing myelin and releasing inflammatory and toxic mediators in the CNS. Although traditionally regarded to be detrimental, it was recently demonstrated that myelin-laden macrophages present in MS and EAE lesions seem to exert an anti-inflammatory 'M2'-phenotype. However, it is still not completely clear by which mechanisms myelin affects the phenotype of macrophages and how this phenotype can influence lesion progression. Research at Biomed identified phosphatidylserine (PS), a phospholipid abundantly present in myelin that can induce an anti-inflammatory phenotype in macrophages. We therefore investigated the impact of PS-liposomes (PSLs) on the functional phenotype of macrophages *in vitro* and *in vivo* during EAE. We hypothesized that phosphatidylserine containing liposomes will ameliorate chronic EAE in rats through the polarization of macrophages into an anti-inflammatory phenotype.

In summary, this study shows that PSLs alter the functional properties of macrophages *in vitro* by activating PPAR-β/δ and PPAR-α. Moreover, systemic treatment of PSLs had both a prophylactic and therapeutic effect on EAE, which was paralleled by a reduced splenic lymphocyte proliferation, less CNS cell trafficking, a reduction in size of splenic follicles, and a decreased secretion of inflammatory mediators in the spleen. More importantly, the homing of PSLs to splenic macrophages following intravenous injections and the subsequent change in expression of TNF-α, iNOS, OPN and Arg-1, illustrates a crucial role for a PSL-mediated activation of PPAR-β/δ and PPAR-α in macrophages in the observed effects on EAE. Additionally, activation of PPAR-β/δ and possibly PPAR-α seems to be dependent on the composition and distribution of fatty acid chains in phosphatidylserine.

The results of our study suggest that increased apoptotic signals introduced by PSLs can promote an anti-inflammatory environment, and may encourage a further *in vivo* evaluation of PSLs regarding their potential therapeutic effect on inflammatory responses. 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. Expanding on the knowledge of PSLs, identification of anti-inflammatory constituents can lead to a new therapeutic approach, with the goal of inducing repair mechanisms in MS.

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

SAMENVATTING

Multiple sclerose (MS) is een chronische auto-immuun aandoening van het centrale zenuwsysteem (CZS) gekenmerkt door chronische inflammatie en demyelinisatie. Macrofagen spelen een belangrijke rol in het ziekteproces van zowel MS als het dierenmodel experimentele auto-immuun encefalomyelitis (EAE) door de fagocytose van myeline en secretie van inflammatoire en toxische componenten in het CZS. Macrofagen werden in MS en EAE oorspronkelijk louter als schadelijk aanzien. Desondanks heeft recent onderzoek aangetoond dat myeline-beladen macrofagen aanwezig in MS en EAE laesies een anti-inflammatoir 'M2'-fenotype aannemen. Echter, het is nog steeds niet duidelijk hoe myeline dat fenotype van macrofagen en bijgevolg de progressie van laesies kan beïnvloeden. Onderzoek op Biomed heeft fosfatidylserine (PS) geïdentificeerd, een fosfolipide dat prominent aanwezig is in myeline en tevens een anti-inflammatoir fenotype in macrofagen kan induceren. Bijgevolg hebben we onderzocht hoe fosfatidylserine liposomen (PSLs) het functioneel fenotype van macrofagen *in vitro* en *in vivo* tijdens EAE kunnen beïnvloeden. Onze hypothese stelt dat fosfatidylserine liposomen chronische EAE in ratten onderdrukt door de polarisatie van macrofagen naar een meer anti-inflammatoir fenotype.

Kort samengevat toont deze studie aan dat PSLs de functionele eigenschappen van macrofagen *in vitro* kunnen beïnvloeden door de activatie van PPAR-β/δ en PPAR-α. Daarnaast hebben PSLs zowel een profylactisch als therapeutisch effect op EAE, wat wordt gekenmerkt door een verminderde lymfocyt proliferatie in de milt, minder CNS-infiltratie van immuun cellen en een verkleining van de follikels en een verlaagde productie van inflammatoire componenten in de milt. De migratie van PSLs naar macrofagen in de milt, gevolgd door een verandering in expressie van TNF-α, iNOS, OPN en Arg-1, betekent dat PSL-gemedieerde activatie van PPAR-β/δ en PPAR-α in macrofagen een belangrijke rol speelt in de effecten tijdens EAE. Bovendien lijkt de activatie van PPAR-β/δ en mogelijk PPAR-α afhankelijk van de samenstelling en verdeling van vetzuurketens in fosfatidylserine.

De resultaten van deze studie tonen aan dat verhoogde apoptotische signalen aangevoerd door PSLs een anti-inflammatoire omgeving kunnen promoten, en sporen verdere *in vivo* evaluatie aan met betrekking tot mogelijke therapeutische effecten op inflammatoire responsen. Huidige therapieën voor MS verminderen de ontwikkeling van nieuwe laesies gedeeltelijk, en verhinderen de klinische ziekteactiviteit tot een bepaalde graad, maar geen enkele therapie verhindert de progressie van de ziekte of geneest ze. Identificatie van anti-inflammatoire componenten is daarom belangrijk, en kan leiden tot een nieuwe therapeutische strategie met als doel het induceren van herstelmechanismen in MS.

Sleutelwoorden: *Multiple sclerose, fosfatidylserine, M2-macrofagen, anti-inflammatoir*

1. INTRODUCTION

1.1 Multiple Sclerosis

Multiple Sclerosis (MS) is a chronic inflammatory and neurodegenerative disease of the central nervous system (CNS) characterized by demyelination, focal T cell and macrophage infiltrates, oligodendrocyte loss and axonal damage [1]. MS is an auto-immune disorder which generally manifests between the age of 20 and 40 and affects women twice as often as men. Symptoms include impairment of motor, sensory, visual and autonomic systems [2].

MS is generally categorized as being either relapsing-remitting (RRMS) or primary-progressive (PPMS). Patients with RRMS tend to experience a series of attacks that result in varying degrees of disability followed by complete or partial remission. Eventually, 40% of RRMS patients will change to a progressive form, known as secondary progressive (SPMS). The PPMS subtype lacks the acute form and is characterized by gradual clinical decline with no real or distinct periods of remission [1].

MS was first described in the early nineteenth century by Cruveilhier and Carswell, but the systematic clinical and pathological characterizations of the disease were provided by Charcot in 1868 [3]. Since then, advances in neurophysiology and microscopic techniques have resulted in a thorough analysis of inflammation and demyelination and this has eventually led to a better understanding of the disease. Today, there is substantial evidence that both genetics (e.g. Interleukin-7 Receptor A (IL-7RA), IL-1R and IL-1β) and environmental factors like pathogens, viruses, smoking and vitamin D intake can determine a person's susceptibility to MS and play a role in modulating the disease [4-11]. However, the exact etiology of the disease is currently still unknown.

1.2 Pathogenesis and Treatment

Early pathogenesis of MS is characterized by activation of autoreactive T cells in the periphery (Fig. 1). Peripheral T cell activation is believed to result from functional recognition of antigenic peptides by T cell receptors (TCRs) present on the surface of $CD4^+$ T cells. Myelin-reactive T cells are found in the blood, cerebrospinal fluid (CSF), and brain tissue of MS patients. The nature of these antigenic peptides, or autoantigens, is currently unknown. However, they may be derived from myelin-associated proteins like myelin basic protein (MBP), myelin oligodendrocyte glycoprotein (MOG) and proteolipid protein (PLP). Experimental autoimmune encephalomyelitis (EAE) an animal mode of MS, can be induced in a variety of animals (e.g. rats, mice, rabbits, guinea pigs and rhesus monkeys) by adoptive transfer of myelin reactive T cells or by generating a T cell mediated immune response against CNS antigens like MOG, MBP or PLP [12]. Peripheral T cells could cross-react with these self-antigens through a process of molecular mimicry. Several

other mechanisms like the bystander effect, or the presence of viral and bacterial superantigens that could activate myelin-specific T cells have also been proposed [13].

Traditionally, MS was regarded as a $CD4^+$ T helper 1 (TH1) mediated autoimmune disorder driven by IL-12. However, recent evidence has established that IL-17 producing $CD4^+$ T cells (TH17 cells) and cytotoxic $CD8^+$ T cells play a cardinal role in MS and EAE. Additionally, pro-inflammatory cytokines like osteopontin (OPN), interleukin-23 (IL-23) and interferon gamma (IFN-γ) play an important role in the pathogenesis, all of which are reported to activate TH1/TH17 immune responses, and up-regulate the production of their downstream inflammatory cytokines [14-23]. Upon activation, T lymphocytes migrate to the CNS where they cross the blood-brain barrier (BBB) via adhesion molecules like very late antigen-4 (VLA-4), lymphocyte function-associated antigen 1 (LFA-1), vascular cell adhesion protein 1 (VCAM-1) and inter-cellular adhesion molecule 1 (ICAM-1), helped by the secretion of various matrix metalloproteinases (MMP-2/9) [13, 24, 25].

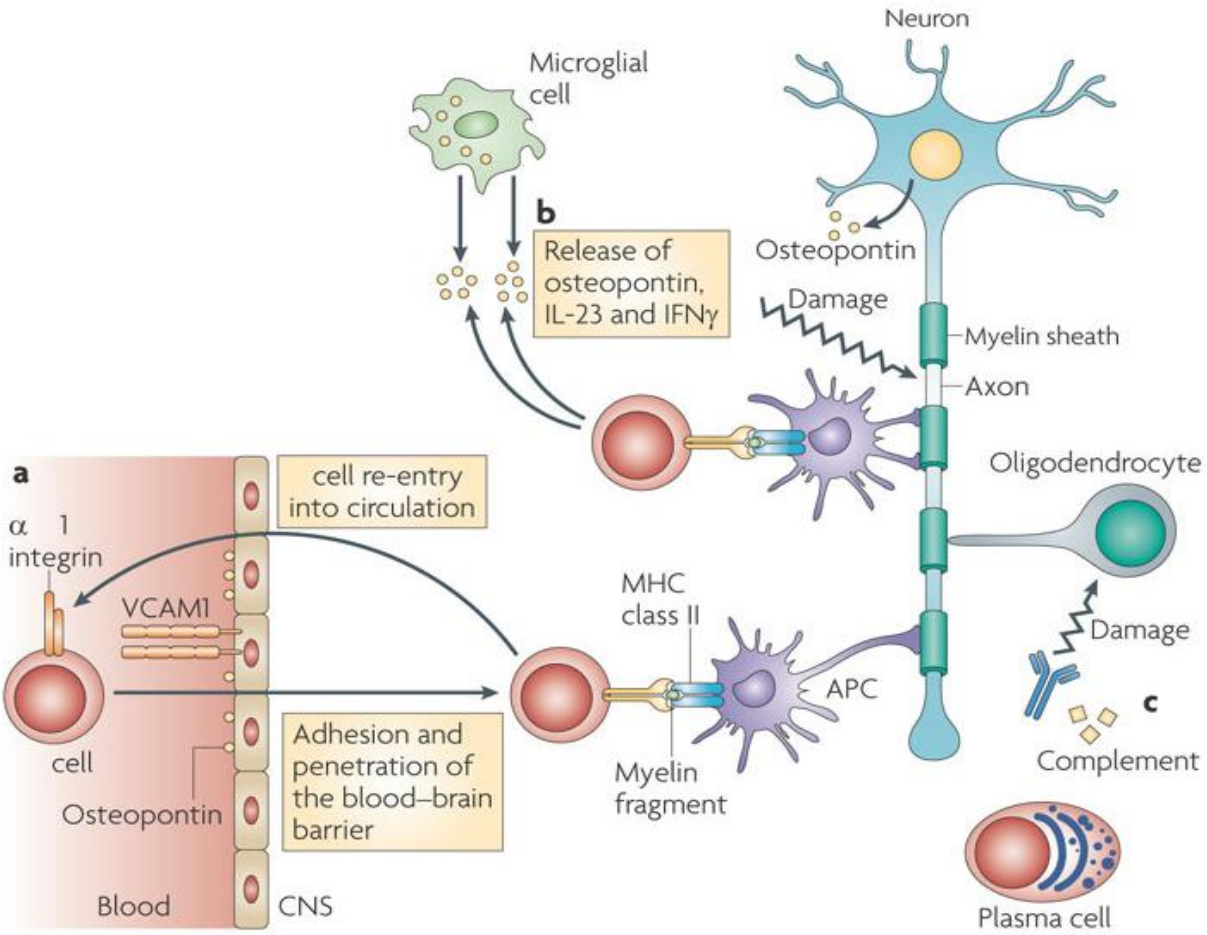

Figure 1 | The pathogenesis of multiple sclerosis [26]. **a)** Autoreactive T cells become activated in the periphery and migrate towards the CNS. T cells expressing α1 integrins (VLA-4) bind to ligands like vascular adhesion molecule 1 (VCAM-1) and osteopontin (OPN) on the endothelial cells that line the venules. Through diapedesis T cells traverse the perivascular cuff and enter the CNS. **b)** Inside the brain, the release of inflammatory cytokines such as OPN, IL-23 and IFN-γ damages oligodendrocytes, which produce myelin. **c)** As inflammation continues plasma cells will produce myelin-specific antibodies that result in further damage to the myelin sheath. (Fig. adapted from Nature Reviews Immunology 9, 440-447; June 2009)

Once in the CNS, T lymphocytes are reactivated by local antigen presenting cells (APCs), like microglia, presenting myelin antigens. In turn, reactivated T cells will release proinflammatory cytokines IFN-γ which will subsequently recruit macrophages and B cells into the CNS. This will lead to the production of tumor necrosis factor α (TNF-α), reactive oxygen species (ROS), nitric oxide (NO), OPN, complement, antibodies and other detrimental mediators. These immune interactions will eventually result in the vicious circle of local CNS inflammation seen in MS patients [27, 28].

Treatment options in MS have been focusing largely on suppressing or altering the activity of the immune system. First-line agents include interferon beta-1b (IFN-β1b), intramuscular or subcutaneous interferon beta-1a (IFN-β1a), and glatiramer acetate. However, these drugs all have modest efficacy. Two agents, mitoxantrone and natalizumab are more potent but typically are second line because of potential safety concerns. In addition, some patients treated with IFN-β or natalizumab develop neutralizing antibodies that abolish efficacy. Hence, more research is needed to develop new strategies for MS treatment [29].

1.3 A dual role for macrophages

Besides T and B lymphocytes, macrophages play a central role in the degenerative process of MS, since depletion of macrophages inhibits the manifestation of acute or chronic EAE in Lewis rats. [30, 31]. Macrophages produce pro-inflammatory cytokines like TNF-α, IL-1β, IL-6 and IL-12. In addition, macrophages produce ROS which can lead to oxidative damage resulting in necrosis or apoptosis, BBB disruption and DNA damage leading to neurodegeneration [32, 33]. Besides the release of pro-inflammatory cytokines and toxic mediators, macrophages also mediate myelin phagocytosis. They directly contribute to myelin damage through receptor mediated phagocytosis of myelin involving scavenger, complement and Fc-receptors [34].

Macrophages have a marked heterogeneity. The most extreme subtypes are M1 or classically activated macrophages, and M2 or alternatively activated macrophages. Classically activated M1 macrophages are activated by lipopolysaccharide (LPS), TNF-α or IFN-γ and can produce NO and other pro-inflammatory cytokines. Alternatively activated M2 macrophages are activated by transforming growth factor beta (TGF-β), IL-4 or IL-13 and can produce IL-10 and other anti-inflammatory cytokines [35, 36]. For a long time, it was thought that macrophages could solely adapt to a pro-inflammatory phenotype in MS. However, recent experiments have shown that the internalization of myelin can lead to an anti-inflammatory subset of macrophages [36, 37]. Moreover, myelin-phagocytosing macrophages have been described to inhibit autoreactive T cell proliferation [38]. These foamy macrophages are abundantly present in the center of MS lesions and have a phenotype resembling M2 macrophages characterized by the high levels of HLA-DR, CD11b, CD163 expression and the secretion of anti-inflammatory cytokines like IL-10, IL-4 and TGF-β. More importantly, these macrophages lack the expression pro-inflammatory cytokines such as TNF-α or IFN-γ

[36]. The polarization of pro-inflammatory into anti-inflammatory macrophages in MS is interesting, as it could provide a possible explanation for the self-limiting nature of the lesions and the remitting phase of MS. In spite of these studies, it is not completely clear which myelin constituent has the greatest anti-inflammatory inducing effect on the phenotype of macrophages.

Because myelin consists for ~80% of lipids, it was anticipated that one or more myelin-derived lipids could be responsible for the induction of this M2-like phenotype in myelin-phagocytosing macrophages. Indeed, previous research in Biomed has shown that a myelin-mediated liver X receptor (LXR) activation is involved in skewing macrophages towards an anti-inflammatory phenotype [39]. LXRs are oxysterol activated nuclear receptors that have recently been described to control both cholesterol homeostasis and inflammation [40-42].

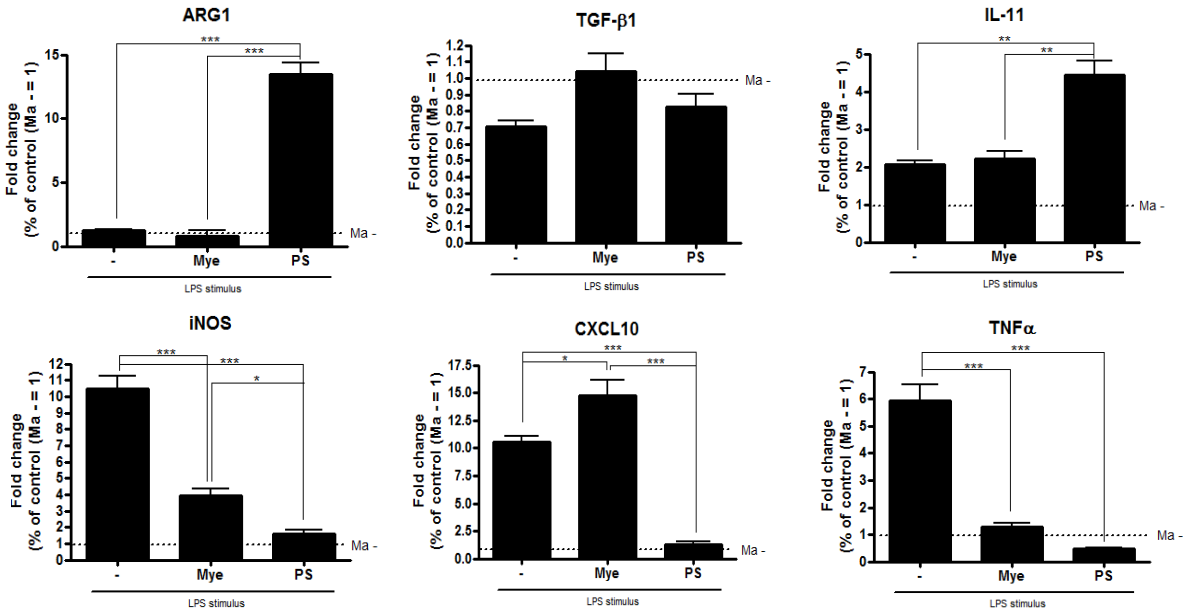

Figure 2 | The effect of PS or myelin uptake by macrophages on gene expression in LPS stimulated macrophages. PS (250 µg/ml) and myelin (100 µg/ml) incorporation significantly suppressed the expression of pro-inflammatory genes iNOS and TNF-α and significantly upregulated the expression of anti-inflammatory genes Arg-1 and IL-11, as compared to macrophages without lipid supplementation, though stimulated with LPS. Data are presented as mean +- SEM of one experiment. * $p < 0,05$; ** $p < 0,01$ and *** $p < 0,001$ (One Way ANOVA).

Although myelin-derived cholesterol clearly plays a crucial role in the phenotype of myelin-phagocytosing macrophages, preliminary data indicate that phosphatidylserine (PS), a major myelin phospholipid, is even more potent in inducing an anti-inflammatory phenotype in macrophages. Notably, PS-treated macrophages suppressed their NO production and up- and downregulated genes characteristic for respectively M2 (Arg-1 and IL-11) and M1 (iNOS, CXCL10 and TNF-α macrophages, *in vitro* (Fig. 2). Additionaly, a pilot study has shown that combinatorial intravenous and subcutaneous phosphatidylserine-containing liposome (PSL) treatment ameliorates disease score in EAE animals (Fig. 3).

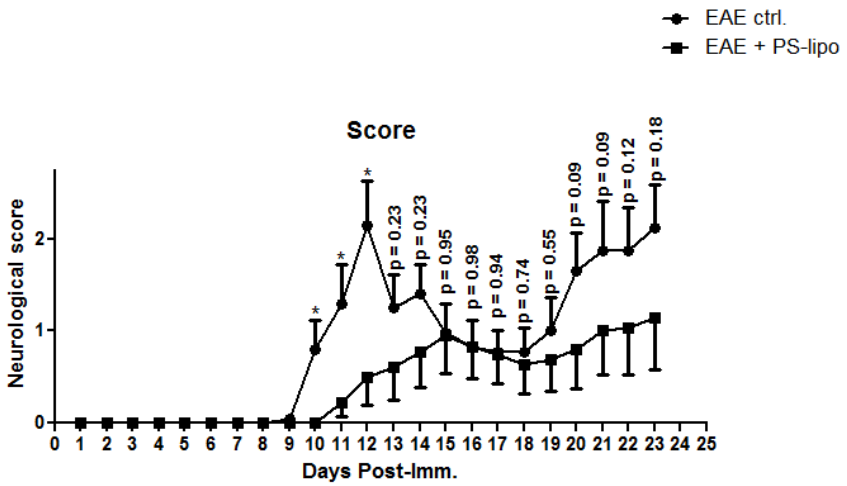

Figure 3 | The effect of PSL on EAE. PSL administration (5 mg/kg) significantly ameliorated neurological scores and weight loss in rats with EAE at DPI 10-12. At day 13, control rats started to remit with lower neurological scores as a result. Data are presented as mean +- SEM of one experiment. * $p < 0,05$ (t-test).

1.4 Phosphatidylserine

Phosphatidylserine, 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. On a molar basis, PS is a relatively minor component of most biological membranes. However, this is outweighed by its physiological importance: PS is known to be involved in the onset of apoptosis, it signals the internalization of viruses by host cells and triggers phagocytosis and blood clotting [39].

Phosphatidylserine species were first identified in whole brain lipid extracts by Folch and coworkers in 1942 [40]. Similar to other phospholipids, PS contains two acyl chains at the sn-1 and sn-2 positions of the glycerol moiety, with the polar headgroup attached to position sn-3 (Fig. 4). Although PS is distributed widely among animals, plants and microorganisms, it is usually less than 10% of the total phospholipids, the greatest concentration being in myelin from brain tissue [39].

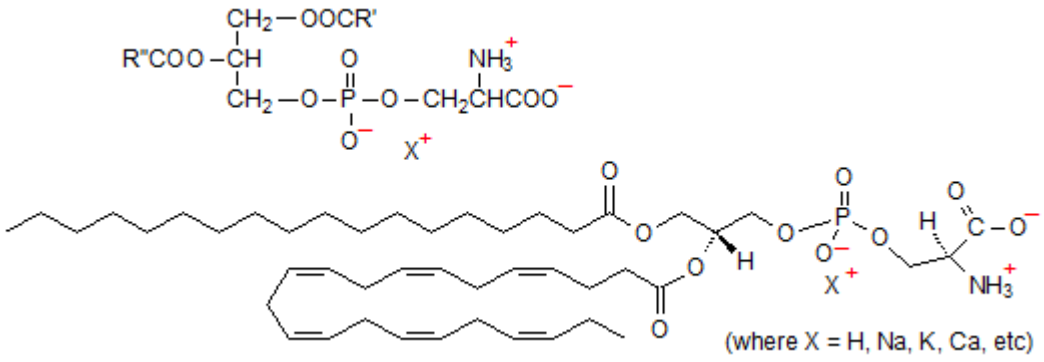

Figure 4 | Structure of phosphatidylserine in the brain. Phosphatidylserine is an acidic (anionic) phospholipid with three ionizable groups: the phosphate moiety, the amino group and the carboxyl function. The 1-octadecanoyl-2-docosahexaenoyl molecular species, which may be especially important in brain tissue, is illustrated here.

PS is also an essential factor in the recognition and clearance of apoptotic cells. When cells undergo programmed cell death, the asymmetric distribution of phospholipids of the plasma membrane gets lost, and PS is translocated to the outer leaflet of the plasma membrane. There, the exposure of PS acts as an 'eat me' signal that ensures efficient recognition and uptake of apoptotic cells by phagocytes. More importantly, PS recognition of activated macrophages induces the secretion of anti-inflammatory cytokines like IL-10 and TGF-β [41]. The importance of PS on inflammation *in vivo* is illustrated by the fact that intravenous injections of apoptotic but not necrotic cells can prevent the development of EAE and that marginal zone macrophages (MZM) are involved [42, 43]. Moreover, ingestion of apoptotic cells by macrophages also induces the secretion of IL-10 and TGF-β [44].

The mechanism of this anti-inflammatory response has not been fully elucidated, but several researchers have highlighted the importance of PS. Notably, PS-containing liposomes (PSLs) can recapitulate anti-inflammatory signaling, and blockade of PS recognition shifts the balance of immune responses from tolerance towards (auto)immunity [45, 46]. Interestingly, MS patients have reduced PS serum levels compared to healthy subjects, and deficiencies in cellular communication are present in brain lesions. Moreover, PS already has several clinical applications in neurodegenerative diseases including Parkinson and Alzheimer's disease [47, 48]. Expanding on the knowledge of PSLs, we will try and unravel the mechanism by which PSLs modulate the immune response. Since PS contains two fatty acyl chains at the sn-1 and sn-2 positions of the glycerol moiety and native or modified fatty acids are natural ligands for peroxisome proliferator-activated receptors (PPARs), we will explore whether PSLs alter the functional properties of macrophages by activating PPARs.

1.5 Peroxisome proliferator-activated receptors

Peroxisome proliferator-activated receptors (PPARs) are nuclear membrane-associated transcription factors and members of the steroid hormone nuclear receptor superfamily. They play an important role in the regulation of lipid metabolism, energy balance, atherosclerosis and glucose control [49]. To date, three subtypes of PPARs have been identified in various species, PPAR-α, PPAR-β/δ, and PPAR-γ. Whereas PPAR-β/δ is ubiquitously expressed in a wide range of cells, PPAR-α is found in tissues with high fatty acid catabolism such as brown adipose tissue, live, heart, kidney and skeletal muscle, and PPAR-γ is mainly expressed in the brown and white adipose tissue [50].

Like other nuclear receptor family members, PPARs have distinct functional domains which are responsible for ligand binding, DNA binding and co-activator / co-repressor binding. PPARs regulate gene expression by binding to specific DNA sequences, peroxisome proliferator response elements (PPRE), in the promoter regions of target genes. PPREs were identified as a direct repeat 1 motif (DR-1), which consists of two

copies of a core motif sequence, AGGTCA, separated by one nucleotide. Prior to DNA binding, PPAR forms a heterodimer with the retinoid X receptor (RXR), another member of the nuclear receptor super family [51].

As PPARs are considered to be nutritional sensors, they are bound and activated by fatty acid intermediates and can thus translate lipid levels into altered gene transcription. PPAR-γ binds nitrosylated fatty acids as well as certain eicosanoids and prostaglandins [52] whereas PPAR-β/δ and PPAR-α have overlapping specificities for long chain unsaturated fatty acids [53, 54]. All three subtypes have recently been shown to play an important role in regulating inflammation. In immune cells, PPARs have a second function of negative regulation of activator protein 1 (AP-1) and nuclear factor KappaB (NF-κB) dependent transcriptional activity, a mechanism which has been termed ligand-dependent transrepression [55]. Furthermore, the functional expression of PPARs by several immune cell types suggests that these receptors may play a very important role in regulation of immune responses [56].

AIMS

Previous research has predominantly focused on the detrimental effects of macrophages in MS. This led to the development of new therapies to counteract these harmful processes. However, recent evidence suggests that the internalization of myelin can lead to an anti-inflammatory subset of macrophages. The new approach of a possible protective role of macrophages in MS is a very recent and innovative concept.

In this project, we will study the effects of phosphatidylserine-containing liposomes (PSLs) on the functional phenotype of macrophages *in vitro* and *in vivo* during EAE. For specific delivery to macrophages we will use liposomes because they are taken up naturally by cells of the mononuclear phagocytic system (MPS), in particular by macrophages in the spleen when injected intravenously. We hypothesize that **phosphatidylserine containing liposomes will ameliorate chronic EAE in rats through the polarization of macrophages into an anti-inflammatory phenotype**.

In a first objective, we will validate and extent results obtained from the pilot study. Our second objective is to study the *in vivo* polarization of macrophages in control, empty phosphatidylcholine liposomes (PCL) and PSL-treated animals. We will look at macrophage polarization using immunohistochemistry, and quantitative RT-qPCR (RT-qPCR) will be used to determine gene expression differences in PSL-treated animals. Additionally, lymphocyte proliferation and polarization will be determined. The third objective is to study the possible immunomodulatory capabilities of PSL-loaded macrophages *in vitro*, and determine the mechanisms underlying this immune modulation. For this, we will study the role of peroxisome proliferator-activated receptors (PPARs) regarding the anti-inflammatory effects of PSLs *in vitro*.

Results from this study will increase the knowledge about a possible protective role of macrophages in MS and EAE. Furthermore, they will provide more information about the exact role of PS in the polarization of

pro-inflammatory macrophages into a more protective, anti-inflammatory phenotype. Interestingly, recent studies have shown that intravenous injections of PSLs can improve infarct repair and inflammatory bone loss by modulating macrophages [57, 58].

Multiple sclerosis is a major health problem that affects 2.5 million people worldwide. There is currently no therapy available to cure this auto-immune disorder. New insight into possible anti-inflammatory mechanisms of the myelin-derived phospholipid PS can lead to new applications to control lesion development in MS. Since currently no therapy is available to halt or cure the disease, identification of anti-inflammatory constituents can lead to a new therapeutic approach, with the goal of inducing repair mechanisms in MS.

2. MATERIALS & METHODS

2.1 Cell culture

Alveolar macrophage-derived NR8383 macrophages were cultured in RPMI 1640 (Lonza) supplemented with 10% fetal calf serum (FCS) (Hyclone) and 50 U/ml penicillin and 50 U/ml streptomycin (Invitrogen). Cell cultures were incubated in humidified atmosphere at 37°C and 5% CO_2. Cells were treated for 24 hours with 100 µg/ml myelin, 250 µg/ml PC, 250 µg/ml PS, 250 µg/ml PSLs or 250 µg/ml PC-containing liposomes (PCLs) in 96-well plates (1.5 x 10^5 cells/well) or in 24-well plates (7.5 x 10^5 cells/well), unless otherwise stated. Subsequently, cells were stimulated with 100 ng/ml lipopolysaccharide (LPS; Sigma-Aldrich) for 9 hours for RNA isolation or 18 hours for analysis of culture supernatants (LPS; Sigma-Aldrich). To evaluate the involvement of peroxisome proliferator-activated receptors (PPARs), macrophages were pretreated for 2 hours with antagonists for PPAR-α (GW6471, 10 µM), PPAR-β/δ (GSK0660 10 µM) and PPAR-γ (GW9662, 1 µM) (all from Sigma-Aldrich).

2.2 Isolation of peritoneal rat macrophages

Three days prior to macrophage isolation, rats were injected intraperitoneally with 10 ml 3% thioglycolate (Sigma Aldrich). Peritoneal macrophages were isolated by peritoneal lavage using PBS (Lonza) supplemented with 5 mM ethylene diamine tetraacetic acid (EDTA). Cells were cultured for 2 hours in RPMI 1640 supplemented with 10% FCS (Hyclone) and 0,5% P/S (Invitrogen). After 2 hours incubation at 37°C with 5% CO_2 non-adherent cells were washed away.

2.3 Thymidine incorporation

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

2.4 Liposome preparation

Liposomes were made solely from phosphatidylcholine (PCL; Sigma-Aldrich), or a combination of PC:PS at a molar ratio of 7:3 respectively (PSL). Briefly, the phospholipids PC or PC:PS, stored in a solvent of chloroform or chloroform/methanol (95:5) respectively, were dried under nitrogen. Dried lipids were resolved in PBS and vigorously vortexed. Liposomes were formed using a sonicating bath with ice, for 12

min. Prepared liposomes were stored at 4°C for a maximum of 7 days. In some experiments, liposomes were labeled with 1,1”-diotadecyl-3,3,3’,3’,-tetramethylindocarbocyanide perchlorate (DiI; Sigma-Aldrich). For this, liposomes were incubated with DiI for 10 min at 37°C, after which liposomes were centrifuged to remove non-encapsulated DiI. Flow cytometry was used to assess labeling efficacy and the degree of DiI-liposome uptake. The DiI-liposomes were stored at 4°C for a maximum of 7 days.

2.5 Rats

Dark Agouti (DA) rats (Harlan Laboratories) were used as a chronic animal model for MS. The rats were housed under specific pathogen-free conditions in an animal facility. For anesthesia, rats were subdued in a transparent chamber with 3% isofluorane gas. At the onset of drowsiness, the animal was removed from the chamber with its nose and mouth placed within an anesthesia mask. Rats continued spontaneous breathing and the anesthesia depth level was maintained throughout the entire experiment. Rats were also placed under a red infrared lamp to keep their body temperature at 37.5 °C ± 0.5 °C. Experiments were conducted in accordance with institutional guidelines and approved by the local Ethical Committee for Animal Experiments of Hasselt University.

2.6 The effect of PS liposomes on chronic EAE

Dark agouti rats were immunized subcutaneously at the base of the tail with 140 µg of recombinant human myelin oligodendrocyte glycoprotein (MOG) emulsified in incomplete Freund's adjuvant (Sigma-Aldrich) supplemented with 500 µg of heat-inactivated Mycobacterium tuberculosis (DIFCO). Immunized animals were treated daily with PBS, 5 mg/kg PCLs or 5 mg/kg PSLs beginning 5 days post-immunization or at disease onset. An end volume of 400 µl, containing liposomes or PBS, was injected intravenously in the tail vein. In parallel, to determine the tracking of liposomes healthy and immunized animals were injected with 5 mg/ml of DiI-labeled liposomes. Weight and neurological scores were closely monitored (clinical EAE scoring system 0-5, see addendum table 1) and compared to controls.

On days 10 and 30 post immunization, 4 rats from each group were sacrificed and spleen, inguinal and popliteal lymph nodes, brains and spinal cord were collected. Half of the organ tissue was used for RT-qPCR. Lymph node and spleen tissue were homogenized using liquid nitrogen, followed by RNA extraction. The remaining half of the organ tissue was used for immunohistochemistry.

2.6.1 RNA Isolation – cDNA synthesis – Real Time PCR

Total RNA from cultures and tissues was prepared using respectively the RNeasy mini kit or RNeasy lipid tissue mini kit (Qiagen), according to the manufacturer’s instructions. The RNA concentration and quality was determined with a NanoDrop spectrophotometer (Isogen Life Science). RNA was converted to cDNA using the reverse transcription system (Promega). In brief, RNA was supplemented with $MgCl_2$ (25 mM),

RTase buffer (10x), dNTP mixture (10 mM); RNasin (20-40 U/µl); AMV RTase (20 U/µl) Oligo(dt) 15 primer and nuclease free water. The reverse transcription reaction was performed on 42°C for 60 minutes, 95°C for 5 minutes, using a My Cycler PCR apparatus (BIORAD). Following cDNA synthesis, nuclease free water was added to the PCR mixture to achieve a concentration of 5 ng). Samples were stored at -20°C. RT-PCR was performed in a StepOnePlus™ Real-Time PCR System (Applied Biosystems). The PCR reaction consisted of fast SYBR green master mix (Applied biosystems), 10 µM of forward and reverse primers, RNase free water and 12.5 ng template cDNA in a total reaction volume of 10 µl. Results were analyzed using the relative quantification ($\Delta\Delta C_T$) method. Normalization of gene expression was achieved by determining the most stable household genes. For this, expression of various household gene candidates was assessed by RT-qPCR and analyzed with GeNorm [59]. PCR products were loaded on 1.5% agarose gels to confirm specificity of amplification and the absence of primer dimer formation. Primers were chosen according to literature or designed using Primer-Express (http://www.ncbi.nlm.nih.gov/tools/primer-blast). Details of primers used are shown in addendum table 1.

2.6.2 Immunohistochemistry

Tissues were embedded in optimal cutting temperature (OCT) compound Tissue-Tek (Sakura Finetek) and frozen in liquid nitrogen for cryosectioning and immunohistochemistry. The lymph nodes, spleen, brains and spinal cord of rats were cryosectioned at 7 µm using a cryostat (Leica) and stored at -20°C. Spinal cord sections were stained with T cell marker mouse anti-rat CD3 IgM 1:100 (AbD Serotec) or macrophage marker mouse anti-rat CD68 1:250 (AbD Serotec) and a secondary antibody goat anti-mouse IgM 1:300 (Invitrogen) and goat anti-mouse Alexa Fluor 555 (Invitrogen) respectively. Other sections were stained with mouse anti-rat arginase 1:200 (BD Biosciences), mouse anti-rat CD169 (Santa Cruz) or goat anti-rat OX2R (CD200) 1:200 (Santa Cruz) and a secondary antibody goat anti-mouse Alexa Fluor 555 1:600 (Invitrogen) and donkey anti-goat Alexa Fluor 488 1:250 (Invitrogen).

In short, frozen sections were first dried for 30 min at room temperature followed by fixation in ice cold acetone for 10 min. Slides were shortly rinsed with 1x PBS / Tween (0.05%; Merck) and blocked for 20 min with 10% serum. Sections were then incubated for 2h with a primary antibody diluted in 1x PBS / Tween (0.05%). Following 2 wash steps (5 min.) with 1x PBS, sections were incubated for at least 1.5h with the secondary antibody diluted in 1x PBS with 10% serum. Slides were then washed 2 times with 1x PBS. Sections were mounted in fluorescent mounting medium (Dako) and coverslipped. Sections of rats treated with PBS and PCL were used as negative controls. Sections stained with secondary but not primary antibody were used to verify the absence of non-specific binding. Spleen sections required an extra antigen retrieval protocol: following acetone fixation, sections were immersed in a citrate buffer (10mM Citric Acid, 0.05% Tween 20, pH 6.0) and heated in a microwave oven (2x 1 min, 1000W) with intermittent cooling of 5 min.

2.7 Nitric Oxide Assay

Nitric Oxide (NO) levels were measured using the Griess Reagent System (Promega). 50 µl of each experimental sample was added to a 96 well plate. An equal amount of sulfanilamide solution was added to each well and incubated for 10 minutes at room temperature, protected from light. Hereafter, 50 µl of N-1-naphthylethylenediamine dihydrochloride (NED) was added to each well and incubated for 10 minutes at room temperature, protected from light. Absorbance was measured within 30 minutes, at 550 nm using a microplate reader (BIORAD).

2.8 Enzyme-Linked Immunosorbent Assay

Supernatants were collected and used to determine cytokine levels produced by NR8383 cells in the presence or absence of PSL and PCL (100 µg/ml). Levels of TNF-α were measured using an enzyme-linked immunosorbent assay (ELISA) kit (eBioscience), according to the manufacturer's instructions. Experimental samples were diluted to obtain optimal results. Absorbance was measured at 450 nm using a microplate reader (BIORAD). Cytokine concentrations were derived from a standard curve and multiplied by their respective dilution factor.

2.9 Cell viability

Cell viability assays were performed using an 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Cells were cultured in 96-well plates. After removing the supernatants, 12.5 µl MTT and 100 µl culture medium were added to each well. Subsequently, plates were incubated for 4 hours at 37°C and 5% CO_2. Next, medium was removed and a solution of 150 µl dimethyl sulfoxide (DMSO) and 25 µl 0.1 M glycine were added to each well. Absorbance was measured using a microplate reader at 550 nm (BIORAD).

2.10 Statistical analysis

Results were statistically analyzed with Graph Pad Prism v5.0.1 software and are expressed as mean values ± standard error (SEM) for 'n' experiments. D'Agostino and Pearson omnibus normality test was used to test normal distribution. An analysis of variances (ANOVA) or two-tailed unpaired student T-test (with Welch's correction if necessary) was used for normally distributed data sets. The Kruskal-Wallis or Mann-Whitney analysis was used for data sets which did not pass normality. EAE scores were analyzed using either a two-tailed unpaired student T-test or the Kruskal-Wallis and repeated measures ANOVA test. *P<0.05, **P<0.01 and ***P<0.001.

3. RESULTS

The goal of this study was to investigate the effects of PSLs on the functional phenotype of macrophages *in vitro* and *in vivo* during EAE. We hypothesize that PSLs will ameliorate chronic EAE in rats through the polarization of macrophages into an anti-inflammatory phenotype. First, we validated and extended the results obtained from the pilot study. Second, using RT-qPCR and immunohistochemistry we show that PSLs home primarily to the spleen where they affect expression of TNF-α, iNOS, OPN and Arg-1. Third, we found that PSLs suppress T cell proliferation and decrease the mean follicle surface area in the spleen. Moreover, PSLs were found to influence immune cell trafficking towards the CNS. Lastly, we show that PSLs can modulate the phenotype of macrophages by acting as ligands for PPAR-α and PPAR-β/δ.

3.1 Prophylactic and therapeutic administration of PSLs suppress EAE

3.1.1 Phosphatidylserine containing liposomes.

To confirm results obtained from the pilot study, phosphatidylserine containing liposomes were constructed. The presence of PS on liposome surface was validated by FACS analysis using fluorescein isothiocyanate (FITC)-annexin A5 (Biolegend). As expected, only PSLs were shown to express phosphatidylserine compared to PCL controls (Fig. 5).

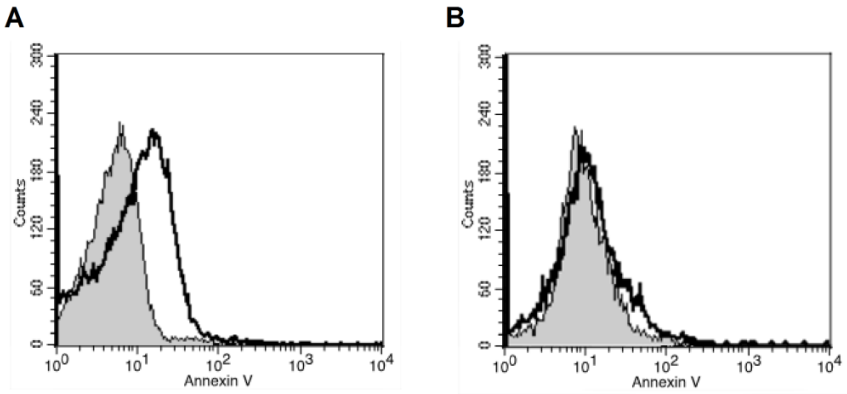

Figure 5 | FACS analysis liposomes. FACS analysis of PS-presenting (A) and PS-lacking (B) liposomes. Annexin A5 specifically targets phosphatidylserine, resulting in a curve shift to the right. PS-lacking liposomes did not produce this effect.

3.1.2 Prophylactic administration of PSLs suppresses EAE

Immunization with MOG (day 0) emulsified in CFA induces chronic EAE in dark agouti rats. From day 6 on, rats were injected daily in the tail vein with PSL (5 mg/kg in 400 µl PBS) (n=12), PCL (5 mg/kg in 400 µl PBS) (n=12) or PBS (n=12). Weight and neurological scores were closely monitored (scoring system; see addendum table 2). Higher EAE scores appeared to be associated with a decline in body weight (Fig. 6A and

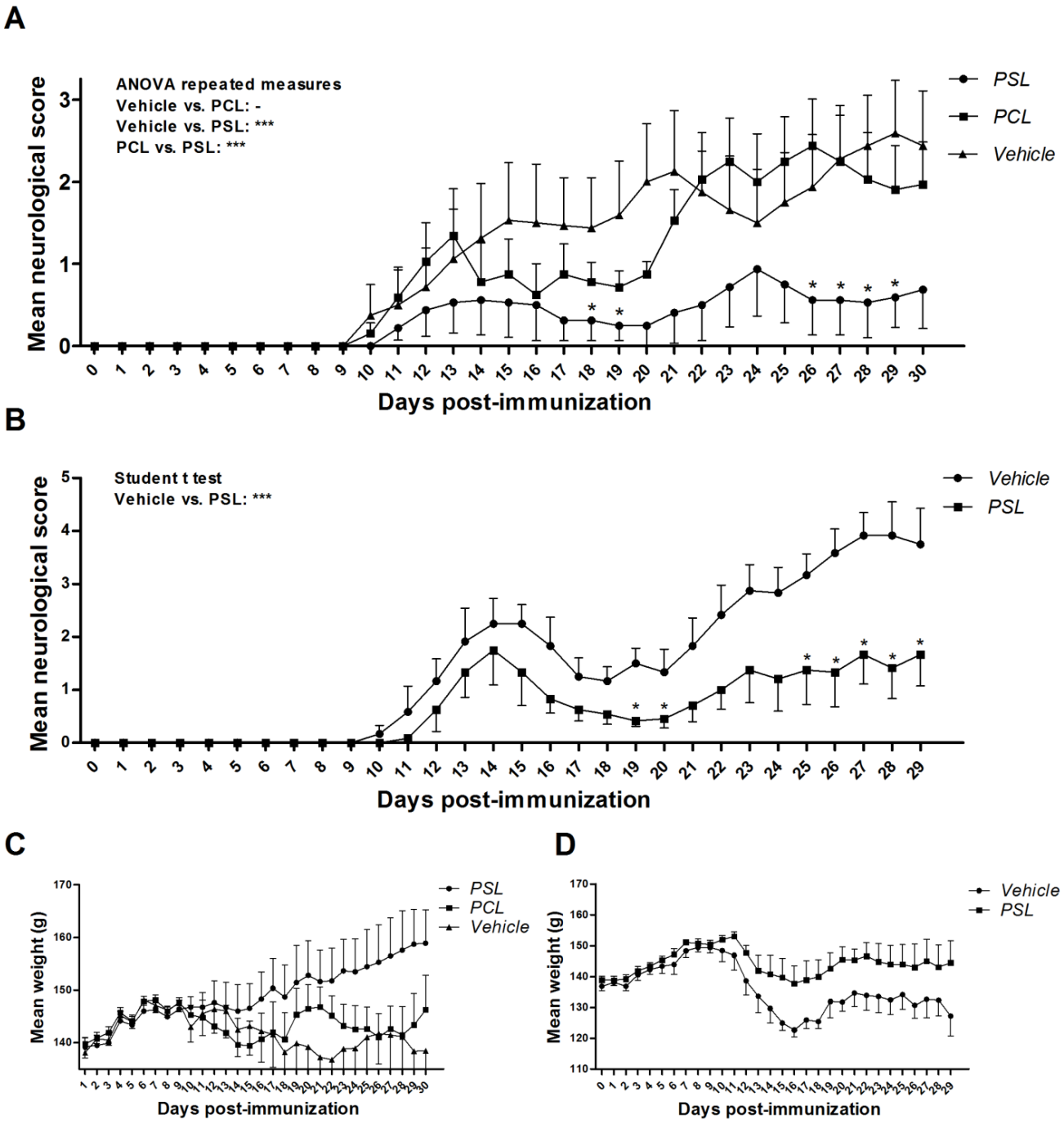

Figure 6 | The prophylactic (A, C) and therapeutic (B, D) effect of PSLs on EAE. Dark agouti rats were immunized on day 0 with MOG emulsified in CFA. **(A)** From day 6 on, rats were injected intravenously in the tail vein once a day with PSL (5 mg/kg in 400 µl PBS) (EAE + PSL, n=12, solid circles), with PCL (5 mg/kg in 400 µl PBS) (EAE + PCL, n=12, solid squares) or with PBS (EAE control, n=12, solid triangles). Mean neurological scores are shown of control, PCL and PSL-treated animals. **(B)** Rats were treated daily after onset of EAE (score >= 1) with PSL (5 mg/kg in 400 µl PBS) (EAE + PSL, n=6, solid squares) or with PBS (EAE control, n=6, solid circles). Mean neurological scores are shown of control and PSL-treated animals. **(C, D)** Mean weights of control and prophylactic (C) or therapeutic (D) PSL-treated groups. Data are presented as mean ± SEM of one experiment * $p < 0,05$; ** $p < 0,01$ and *** $p < 0,001$.

6C). Moreover, PSL administration significantly ameliorated neurological scores ($p < 0.001$) (DPI: 26-28 vs. PCL; DPI: 18,19,26-29 vs. EAE control) and significantly reduced weight loss ($p < 0.001$) (DPI: 22 vs. EAE

control) in rats with EAE. As shown in figure 6A, EAE was induced in PBS- and PCL-treated rats with an average maximum clinical score of 3.09 and 3.13 respectively. In striking contrast, prophylactic daily injections of PSLs significantly suppressed development of chronic EAE with an average maximum clinical score of 1.4.

3.1.3 Therapeutic administration of PSLs suppresses EAE

When considering clinical applications of PSLs to treat autoimmune diseases, it is important to examine the therapeutic potentials of PS-containing liposomes. For this, PSLs (5 mg/kg in 400 µl PBS) (n=6) were intravenously injected into the tail vein of individual rats upon disease onset (defined as a score of 1 or more). EAE control rats were injected with PBS (n=6). Weight and neurological scores were closely monitored (scoring system; see addendum table 2). Higher EAE scores appeared to be associated with a decline in body weight (Fig. 6B and 6D). Moreover, PSL administration significantly ameliorated neurological scores ($p < 0.001$) (DPI: 19,20,25-29 vs. EAE control) and significantly reduced weight loss ($p < 0.001$) in rats with EAE. As shown in figure 6B, EAE was induced in PBS-treated rats with an average maximum clinical score of 4.4. Interestingly, PSLs significantly suppressed development of chronic EAE with an average maximum clinical score of approximately 2.5. Taken together, results from these rat studies indicate that prophylactic and daily PSL-injections following disease onset suppress EAE in rats.

3.2 PSLs home primarily to the spleen

To determine the homing properties of PSLs, immunized rats were injected with Dil-labeled liposomes. FACS analysis showed that injected liposomes were primarily retrieved in the spleen, liver and lungs (Fig. 7). Immunohistochemistry further demonstrated that splenic $CD68^+$ red pulp and $CD169^+$ MZ/MM macrophages internalized most of the liposomes (Fig. 8A and 8B). In healthy rats, Dil-labeled liposomes were absent in the CNS, suggesting that liposomes are incapable of crossing an intact BBB (data not shown).

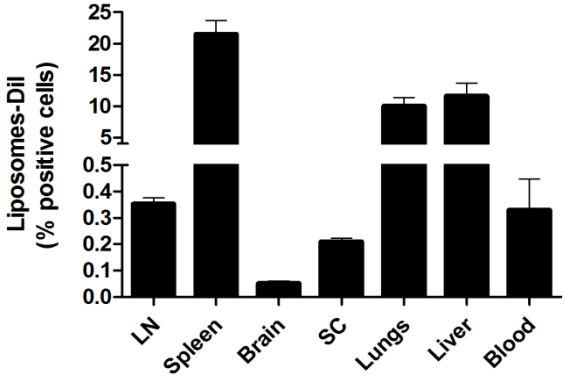

Figure 7 | FACS analysis of distribution of Dil-labeled liposomes in EAE rats. DA rats were intravenously injected in the tail vein with Dil-PSL (5 mg/kg in 400 µl PBS) one day following EAE onset. After 6 days, animals were sacrificed and the organs were collected and prepared for FACS analysis. Liposomes were primarily retrieved in the spleen, liver and lungs. Data are presented as ± SEM of two experiments, with triplicate measurements per experiment (LN = lymph nodes, SC = spinal cord).

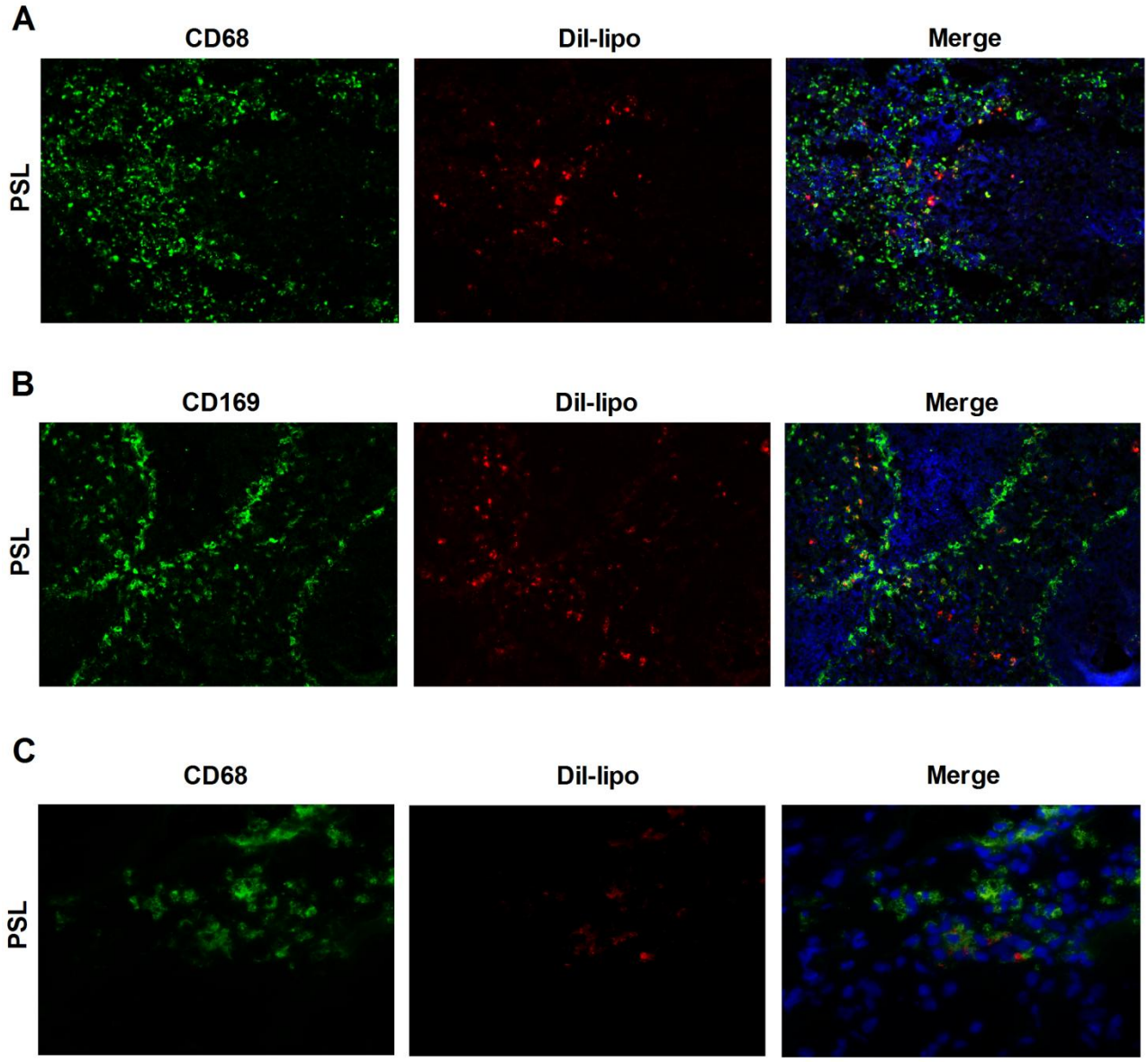

Figure 8 | Dil-labeled liposomes in the spleen and spinal cord of EAE rats. DA rats were intravenously injected in the tail vein with Dil-PSL (5 mg/kg in 400 µl PBS) one day following EAE onset. After 6 days, animals were sacrificed and the organs were collected, cryosectioned and stained. **(A)** Splenic $CD68^+$ red pulp macrophages (green) internalize Dil-labeled liposomes (red). Cells were stained using DAPI (blue). Original magnification, 20x. **(B)** Splenic $CD169^+$ MM and MZ macrophages (green) internalize Dil-labeled liposomes (red). Cells were stained using DAPI (blue). Original magnification, 20x. **(C)** Dil-liposomes (red) could sporadically be tracked back in the spinal cord in the context of $CD68^+$ macrophages (green). Original magnification, 40x.

However, when rats were injected one day following EAE onset, Dil-liposomes could sporadically be tracked back in the spinal cord in the context of $CD68^+$ macrophages (Fig. 8C). Thus, these results indicate that PSLs migrate primarily towards splenic $CD68^+$ red pulp and $CD169^+$ MZ/MM macrophages following intravenous injection. Additionally, they demonstrate that liposomes can migrate towards the CNS when the integrity of the BBB is compromised.

3.3 PSLs affect T cell proliferation and splenic expression of TNF-α, iNOS, OPN and ARG-1

To determine whether PSLs have an effect on lymphocyte proliferation, cognate antigen specific proliferation of splenic cultures 10 days post-immunization was assessed. Splenic lymphocyte cultures from PSL-treated animals showed a significantly reduced MOG reactivity, compared to both vehicle- and PCL-treated animals (Fig. 9A). We next investigated the splenic expression of TNF-α, iNOS and OPN. PSL-treated animals showed a reduced gene expression of TNF-α and iNOS compared to both vehicle- and PCL-treated animals (Fig. 9B and 9C). In concordance, gene expression (data not shown) and production of TNF-α and NO was reduced in macrophage cultures treated with PSLs *in vitro* (Fig. 9E and 9F). OPN was also reduced in PSL-treated animals compared to both vehicle- and PCL-treated animals, albeit not statistically significant. (Fig. 9D).

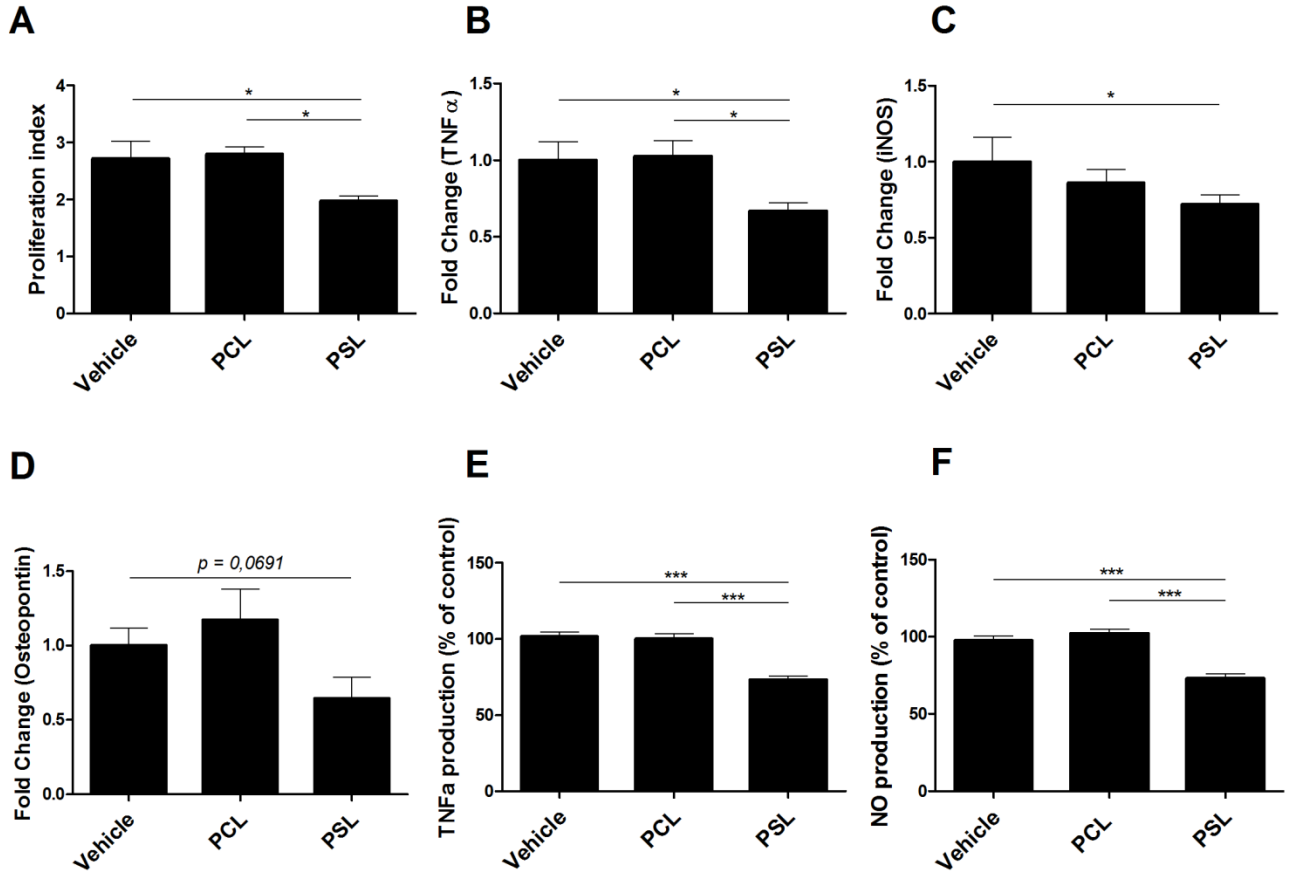

Figure 9 | Gene and protein expression of TNF-α, iNOS and OPN. (A) Isolated spleen T cells from PSL-treated animals 10 DPI were cultured with or without MOG. Following 48h of incubation, 1 µCi [3H]thymidine was added to the culture. Next, cells were harvested and uptake of radioactivity was measured (n=3). **(B-C)** Splenic gene expression of TNF-α (B), iNOS (C) and OPN (D) from PSL-treated animals 10 DPI was determined using RT-qPCR and compared to control and PCL-treated animals (n=4; OPN n=3). **(E, F)** NR8383 rat macrophages were incubated with 250 µg/ml PCL or PSL for 24 hours. Following 18 hours of LPS stimulation (100 ng/ml), (E) cytokine levels of TNF-α were determined in the supernatants of macrophage cultures using sandwich ELISA (n=4). (F) NO production was determined using the Griess Reagent System (n=9). Error bars represent standard error of the mean (SEM). Data were statistically analyzed by one way ANOVA (Kruskal-Wallis/Bonferroni's posttest). * $p < 0,05$; ** $p < 0,01$ and *** $p < 0,001$.

Previous research at Biomed has demonstrated that PSL-treated macrophages increase the expression of ARG-1 *in vitro*. Although splenic Arg-1 gene expression was unaffected (data not shown), the total fluorescent intensity of splenic Arg-1 expression was significantly increased in animals treated with PSLs (Fig. 10). Moreover, in agreement with the homing of Dil-liposomes towards $CD68^+$ red pulp and $CD169^+$ MZ/MM macrophages, the induction of Arg-1 expression was most prominent in splenic regions harboring these macrophage subtypes. Collectively, these results demonstrate that PSLs suppress T lymphocyte proliferation. Furthermore, they show that PSLs decrease and increase the expression of respectively iNOS and TNF-α, and Arg-1 *in vivo* in similar manner than PSL-macrophages did *in vitro*. These immunosuppressive and -regulatory properties of PSLs might underlie the observed reduced clinical score in PSL-treated animals.

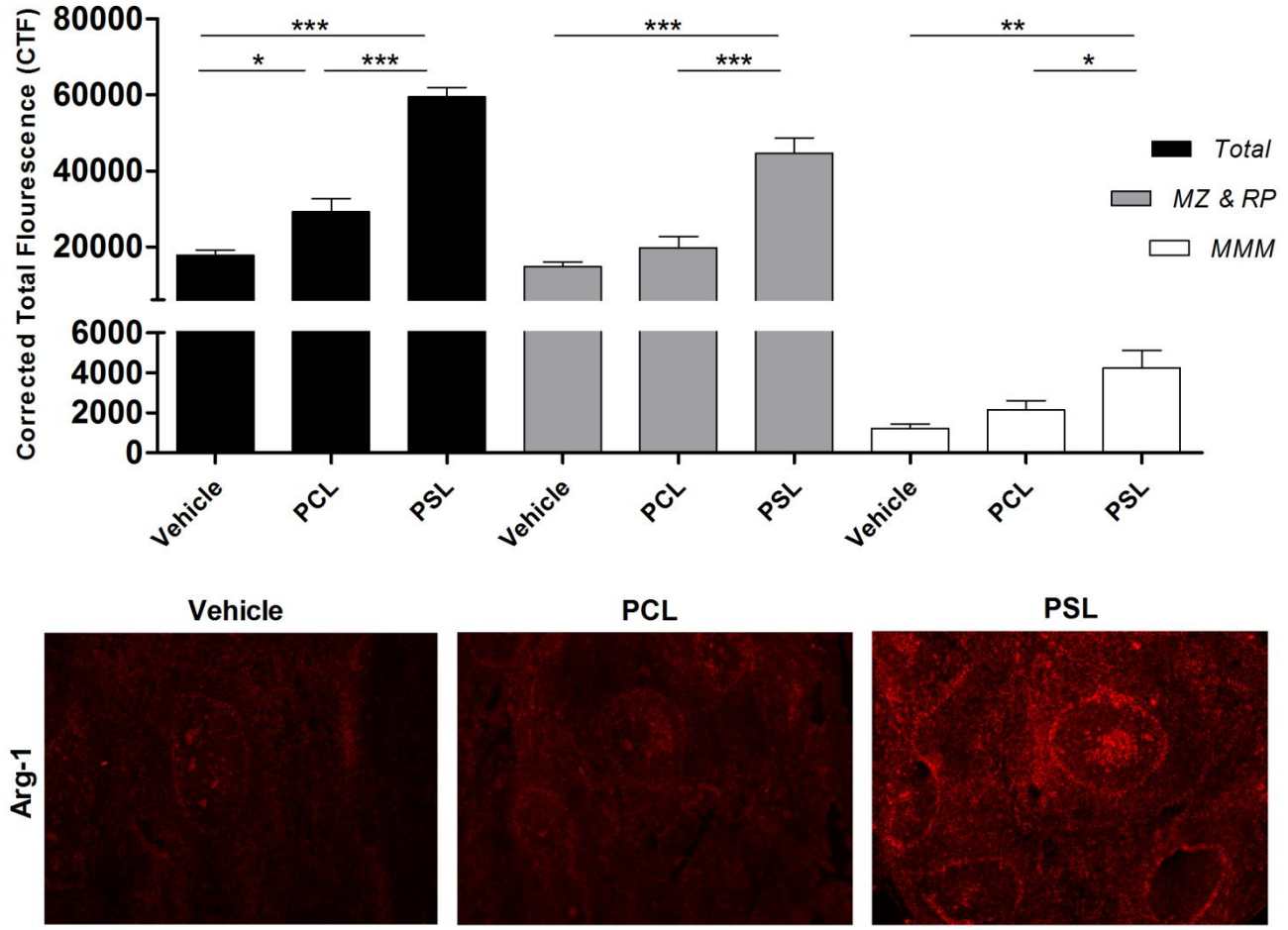

Figure 10 | Arg-1 expression in the spleen of EAE rats. At 10 days post-immunization, control, PCL- and PSL-treated animals were sacrificed and the spleens were collected, cryosectioned and stained for Arg-1 expression. Using ImageJ software we determined the fluorescence intensity for Arg-1 as described previously [60] (n=4). Error bars represent standard error of the mean (SEM). Data were statistically analyzed by one way ANOVA (Bonferroni's posttest). * $p < 0,05$; ** $p < 0,01$ and *** $p < 0,001$.

3.4 PSLs decrease mean follicle surface area in the spleen

In the previous section it was shown that PSLs have a suppressive effect on cognate antigen specific T cell proliferation. To further study the *in vivo* immunomodulatory capacity of PSL-loaded macrophages, we determined the mean follicle surface area in the spleen of control, PCL- and PSL-treated animals 10 days post-immunization. By measuring the region surrounded by marginal metallophilic macrophages, we found that the mean follicle surface area was significantly reduced in PSL-treated animals compared to both vehicle- and PCL-treated animals. (Fig. 11). These results further demonstrate the immunosuppressive properties of PSLs that might underlie the observed reduced clinical score in PSL-treated animals.

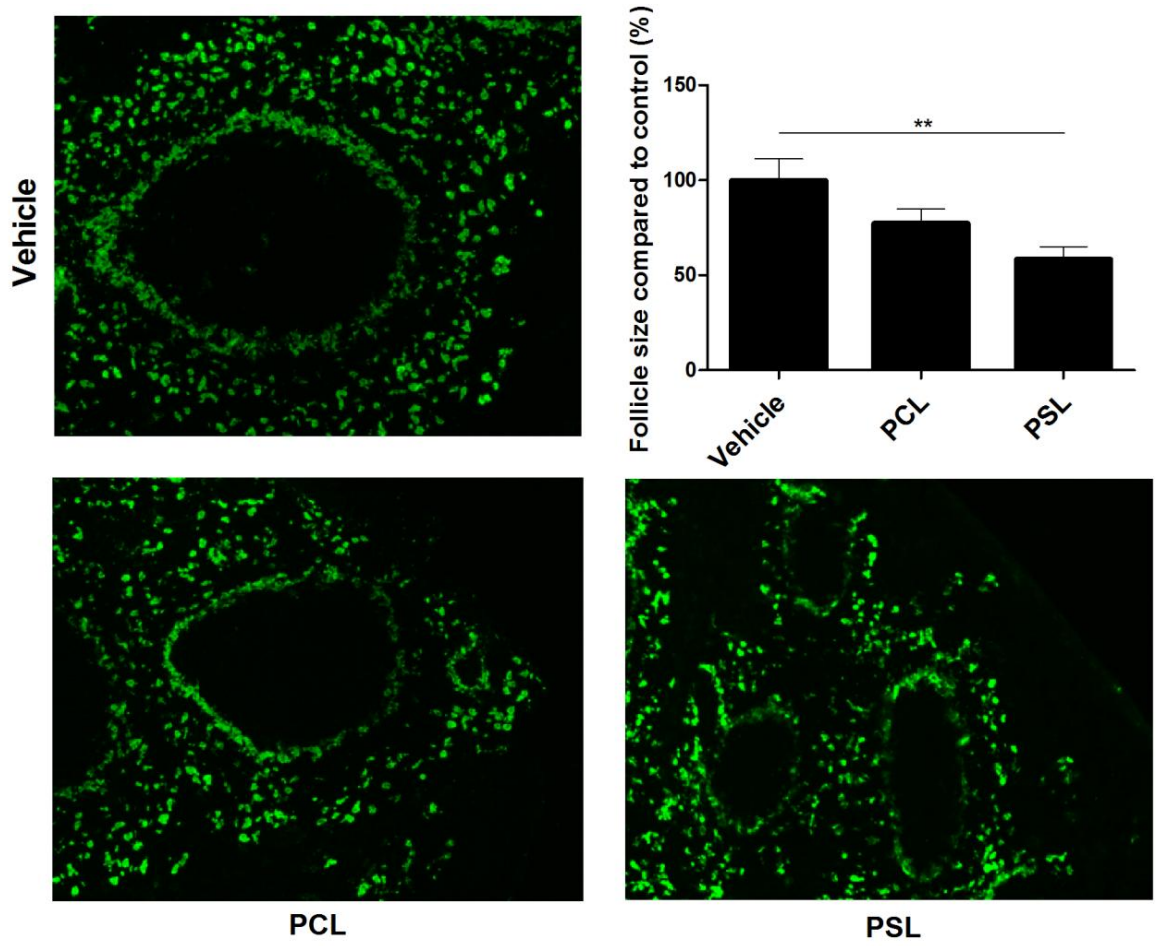

Figure 11 | PSLs decrease mean follicle surface area in the spleen. At 10 days post-immunization, control, PCL- and PSL-treated animals were sacrificed and the spleens were collected, cryosectioned and stained for CD200 expression. ImageJ software was used to measure the region surrounded by MM (n=4). Original magnification, 20x. Error bars represent standard error of the mean (SEM). Data were statistically analyzed by one way ANOVA (Kruskal-Wallis posttest). * $p < 0,05$; ** $p < 0,01$ and *** $p < 0,001$.

3.5 PSLs lower infiltration of CD68+ macrophages and CD3+ T cells towards the CNS

Because EAE is characterized by massive infiltration of immune cells towards the CNS, we investigated whether PSLs could influence this process. Vehicle-, PCL- and PSL-treated rats were sacrificed 30 days post-immunization and the spinal cords were collected, cryosectioned and stained for $CD68^+$ macrophages and $CD3^+$ T cells (Fig. 12A).

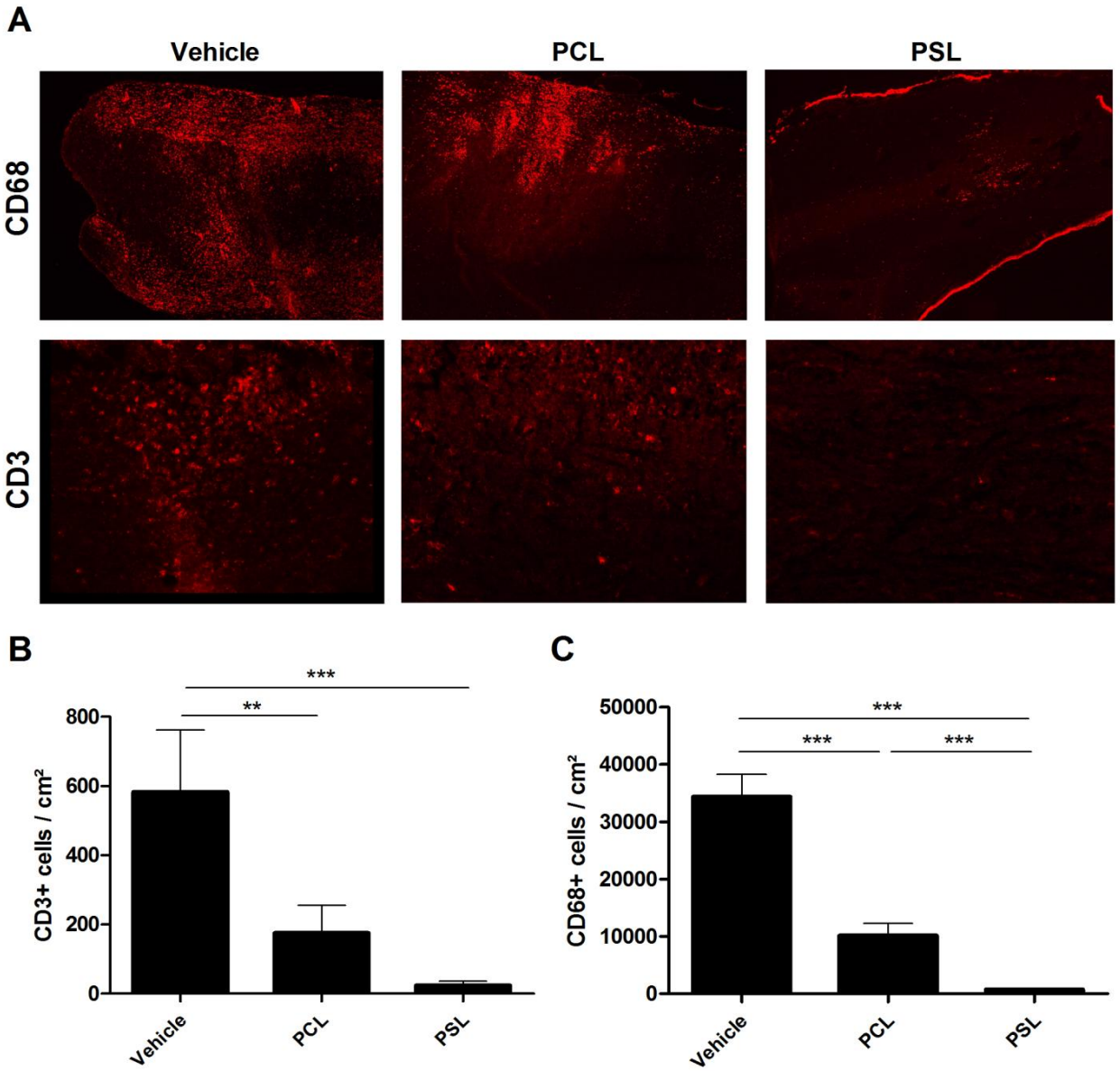

Figure 12 | PSLs lower infiltration of $CD68^+$ macrophages and $CD3^+$ T cells towards the CNS. (A) At 30 days post-immunization, control, PCL- and PSL-treated animals were sacrificed and the spleens were collected, cryosectioned and stained for CD68 and CD3 expression. Original magnification, 4x (CD68) 20x (CD3). **(B)** Using NIS-Elements 4.0 software we calculated the number of infiltrated $CD68^+$ macrophages or $CD3^+$ T cells in the spinal cord (n=4). Error bars represent standard error of the mean (SEM). Data were statistically analyzed by one way ANOVA (Kruskal-Wallis posttest). * $p < 0,05$; ** $p < 0,01$ and *** $p < 0,001$.

We next calculated the number of infiltrated $CD68^+$ macrophages or $CD3^+$ T cells per square centimeter (Fig. 12B and C). Results show that a negligible amount of macrophages and T cells were present in the CNS of

PSL-treated animals. Notably, PCL-treated animals also demonstrated a reduced infiltration of immune cells in the spinal cord compared to vehicle-treated animals, but to a lesser than PSL-treated animals. These results indicate that PSLs suppress EAE by influencing immune cell trafficking towards the CNS.

3.6 PSLs modulate the phenotype of macrophages by activating PPARs *in vitro*

As mentioned previously, PPARs are a group of nuclear receptor proteins that are activated by fatty acids and their derivatives. Three subtypes have been described, namely PPAR-α, PPAR-β/δ and PPAR-γ, which all can regulate the differentiation of macrophages towards an anti-inflammatory phenotype following activation [61-64]. Because PS contains two fatty acyl chains at the sn-1 and sn-2 positions of the glycerol moiety and PPARs have been known to be activated by native or modified fatty acids [65, 66], we sought to determine whether a PS-mediated activation of PPARs affects the observed anti-inflammatory phenotype of PSL-macrophages.

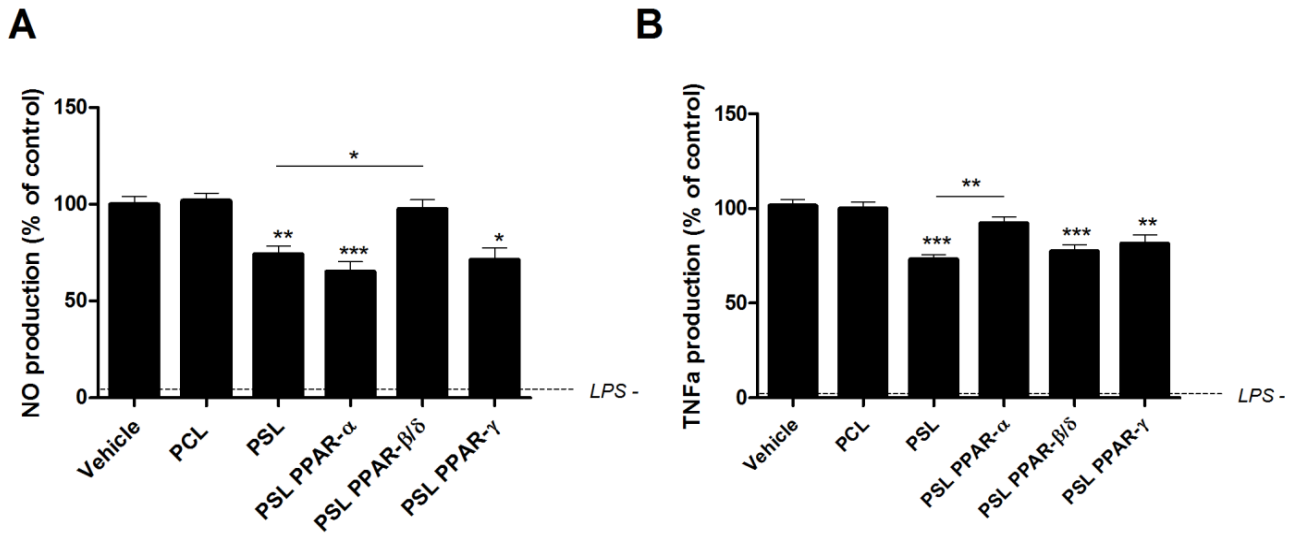

Figure 13 | PSLs modulate the phenotype of macrophages by activating PPARs. NR8383 rat macrophages were incubated with 250 µg/ml PCL or PSL in the presence of PPAR-α, PPAR-β/δ (10 µM) or PPAR-γ (1 µM) antagonists. Cells were pre-incubated for 2 hours with PPAR antagonists before adding PCL or PSL for 24 hours. After 18 hours of LPS stimulation (100 ng/ml), supernatants were collected. **(A)** NO production was determined using the Griess reagent system (n=9). **(B)** Sandwich ELISA was used to determine cytokine levels of TNF-α (n=4). Error bars represent standard error of the mean (SEM). Data were statistically analyzed by one way ANOVA (Tukey's posttest). * $p < 0,05$; ** $p < 0,01$ and *** $p < 0,001$.

Prior to liposome administration (250 µg/ml; 24 hours), macrophages were treated for 2 hours with antagonists for all three subtypes. When using a concentration of 250 µg/ml PSLs lowered NO production without negatively affecting cell viability (data not shown). After 18 hours of LPS stimulation, supernatants were collected and NO and TNF-α production were determined. Whereas PSL-macrophages treated with PPAR-α or PPAR-γ antagonists still suppressed NO production, those treated with PPAR-β/δ antagonist

completely reversed the inhibitory effect (Fig. 13A). Interestingly, PSL-macrophages treated with PPAR-β/δ or PPAR-γ antagonists still suppressed TNF-α production, whereas those treated with PPAR-α antagonist completely reversed the inhibitory effect (Fig. 13B).

In additional experiments we demonstrated that sn-2-18:1 lyso-PS, containing only the oleic acid chain, and not the sn-1-18:0 lyso-PS, containing only the stearic acid chain, was able to suppress NO production (Fig. 14). We did see a similar trend when determining the effect of lyso-PSLs on TNF-α production, albeit not statistically significant (data not shown). Collectively, these results indicate that PSLs alter the functional properties of macrophages by activating PPAR-β/δ and PPAR-α. Moreover, we demonstrate that this activation depends on the composition and distribution of fatty acid chains in PS.

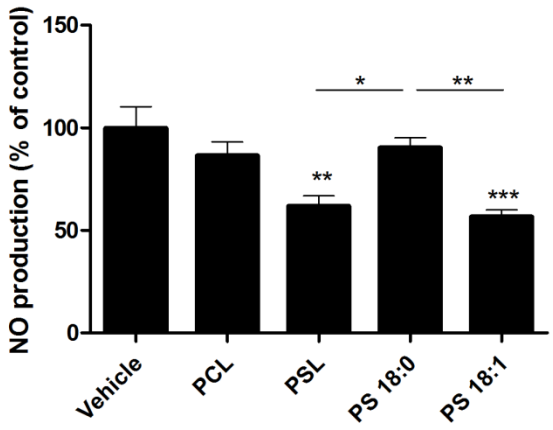

Figure 14 | PS-liposomes and Lyso-PS 18:0 suppress NO production. NR8383 rat macrophages were incubated with 250 µg/ml PCL or PSL or 100 µg/ml lyso-PS sn-1-18:0 or sn-2-18:1 for 24 hours. After 18 hours of LPS stimulation (100 ng/ml), supernatants were collected. NO production was determined using the Griess reagent system (n=3). Error bars represent standard error of the mean (SEM). Data were statistically analyzed by one way ANOVA (Bonferroni's posttest). * $p < 0,05$; ** $p < 0,01$ and *** $p < 0,001$.

3.7 Myelin partially affects the phenotype of macrophages via PPARs *in vitro*

Previous research at Biomed has demonstrated an important role for myelin-derived cholesterol in activating LXRs and subsequently affecting the phenotype of macrophages [67]. Particularly, the suppression of IL-6 production by macrophages following myelin internalization was cancelled out in LXR-β-deficient macrophages. Nonetheless, in contrast to IL-6, the reduced NO secretion was unaffected in both LXRα- and LXRβ-KO macrophages, indicating that NO is regulated in a distinct manner.

PS is a phospholipid that is highly expressed in myelin (Fig. 15A). Moreover, we know that PSLs alter the phenotype of macrophages via PPARs. By incubating macrophages with PPAR antagonists prior to administrating myelin, we demonstrated that the suppressed NO production by myelin-treated macrophages was controlled by PPAR-β/δ (Fig. 15B). As expected, the reduced IL-6 secretion by myelin-treated macrophages was independent of a myelin-mediated PPAR-β/δ activation (Fig. 15C). However, myelin-treated macrophages did not suppress TNF-α production, thereby eliminating any effect on PPAR-α (data not shown). Collectively these results indicate that both myelin and PSLs alter the functional properties of macrophages by activating PPAR-β/δ.

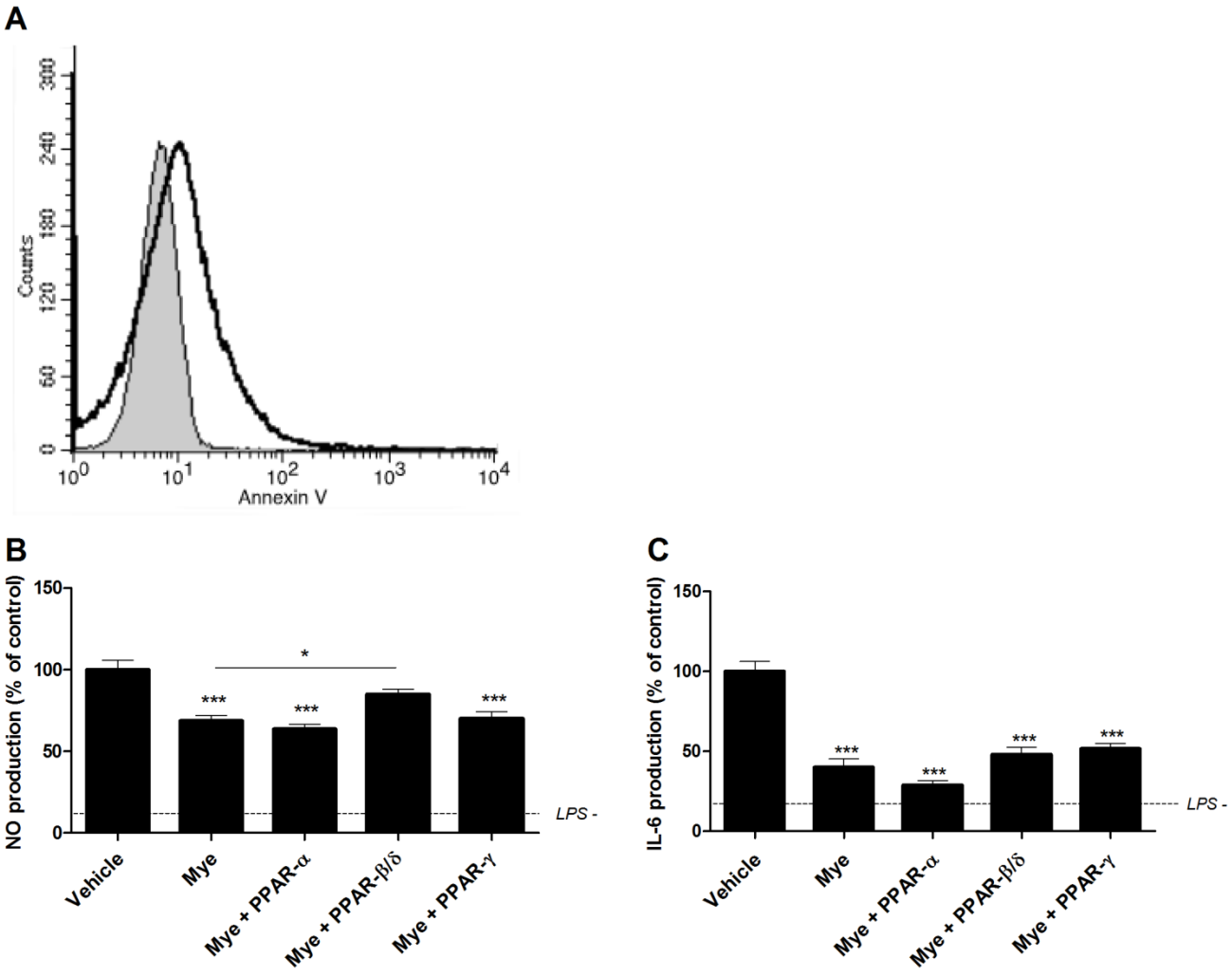

Figure 15 | Myelin partially affects the phenotype of macrophages *in vitro*. (A) FACS analysis of PS-presence in myelin. Annexin A5 specifically targets phosphatidylserine, resulting in a curve shift to the right. **(B, C)** NR8383 rat macrophages were incubated with 100 µg/ml myelin in the presence of PPAR-α, PPAR-β/δ (10 µM) or PPAR-γ (1 µM) antagonists. Cells were pre-incubated for 2 hours with PPAR antagonists before adding myelin for 24 hours. After 18 hours of LPS stimulation (100 ng/ml), supernatants were collected. **(B)** NO production was determined using the Griess reagent system (n=3). **(C)** Sandwich ELISA was used to determine cytokine levels of IL-6 (n=3). Error bars represent standard error of the mean (SEM). Data were statistically analyzed by one way ANOVA (Bonferroni's posttest). * $p < 0,05$; ** $p < 0,01$ and *** $p < 0,001$.

4. DISCUSSION

The goal of this study was to investigate the effects of PSLs on the functional phenotype of macrophages *in vitro* and *in vivo* during EAE. We hypothesized that PSLs will ameliorate chronic EAE in rats through the polarization of macrophages into an anti-inflammatory phenotype. The main finding of this study is that intravenously injected PSLs ameliorate EAE severity. Moreover, PSLs showed both a prophylactic and therapeutic effect on EAE. Interestingly, the homing of PSLs to splenic CD68 and CD169 positive macrophages following intravenous injections and the subsequent change in expression of TNF-α, iNOS, OPN and Arg-1 illustrates a crucial role for PSL-macrophages in the observed effect on EAE pathogenesis. Additionally, we demonstrated an important role for a PSL-mediated activation of PPAR-α and PPAR-β/δ in changing the phenotype of macrophages *in vitro*. Moreover, activation of PPAR- β/δ and possibly PPAR-α seems to be dependent on the composition and distribution of fatty acid chains in phosphatidylserine.

To evaluate the effect of PSLs on EAE pathogenesis, we performed both a prophylactic and therapeutic study on EAE rats. As expected, results indicate that PSL administration significantly ameliorates neurological scores and significantly reduces weight loss in EAE rats. These results are consistent with previous studies of PSLs in other inflammatory diseases: PSLs have already been described to promote resolution of inflammation and elicit repair by modulating macrophage functions in a myocardial infarction and inflammatory bone loss model [57, 58]. As macrophages play an important role in neuroinflammation, modulation of their functional properties can potentially impact EAE pathogenesis [68]. As previously reported, we demonstrated that intravenously injected Dil-PSL-liposomes home primarily to the spleen, liver and lungs. Liposome particles larger than 3 µm can become stuck in small blood vessels e.g. lung capillaries which can explain the relatively high percentage of Dil-labeled PSLs present in the lungs. Accumulation of PS liposomes in the liver and spleen of rats has also been described by Palatini *et al* [69]. Whereas liposomes are broken down in the liver, the spleen functions as an extensively branched arterio-venous filter present in the bloodstream. As a consequence, a very large number of macrophages, lymphocytes and other immune cells continuously come into contact with the bloodstream where they filter out pathogens and foreign particles (Fig. 16).

We found that $CD68^+$ red pulp and $CD169^+$ marginal zone macrophages were responsible for most of the internalization of liposomes in the spleen. $CD169^+$ MZ macrophages are well equipped to continuously screen the blood because they are very well located at the interface between the lymphoid compartment of the spleen and the scavenging red pulp compartment. Of note, Miyake *et al.* showed that MZ macrophages regulate the clearance of circulating apoptotic cells and that this regulation plays an important role in the induction of tolerance to cell-associated antigens, which is one of the principle mechanisms for the maintenance of self-tolerance. Interestingly, when PS was masked on these apoptotic cells, the aforementioned effect was completely abolished, again highlighting the importance of PS [43].

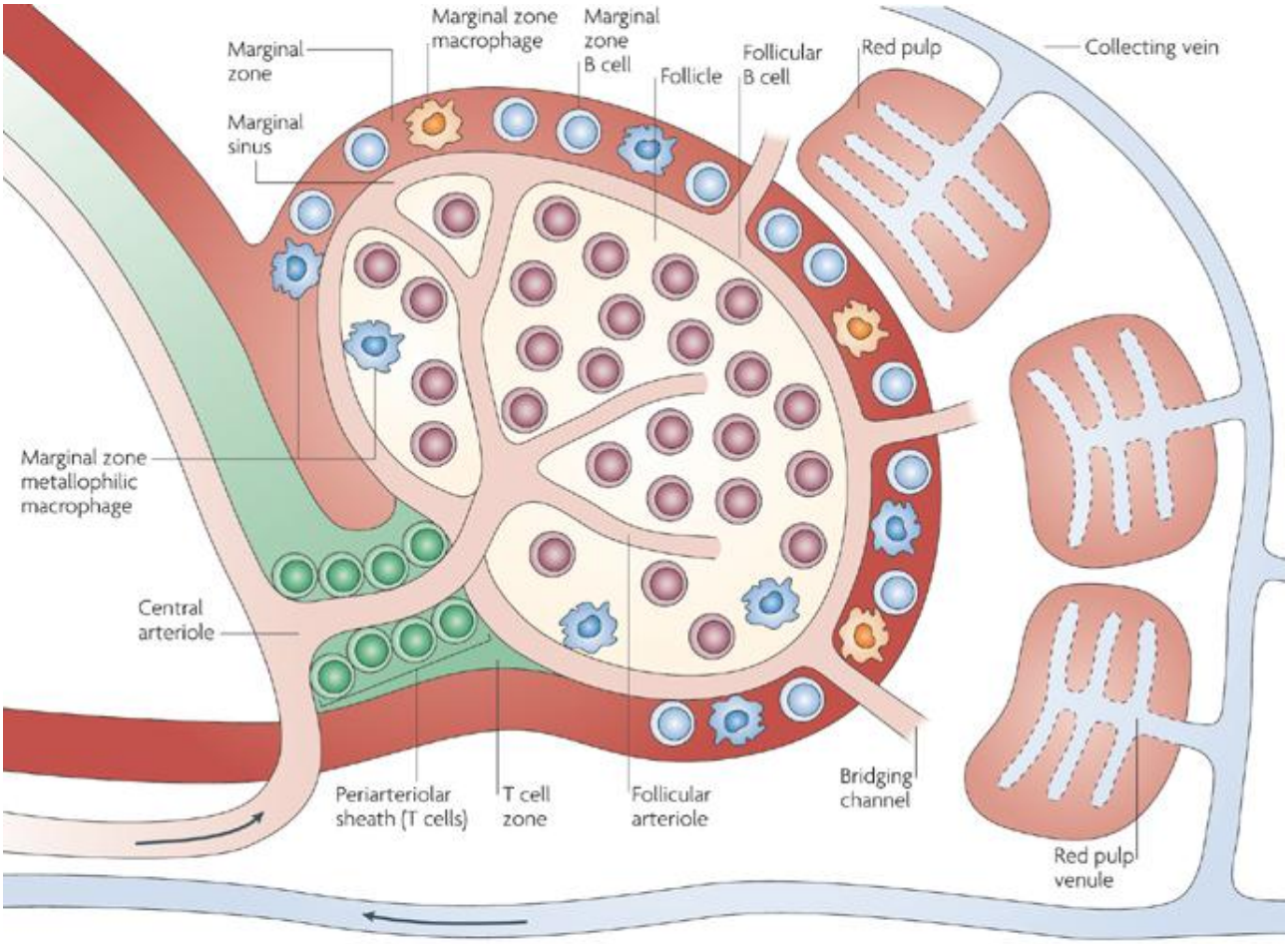

Figure 16 | The anatomic organization of cells in the spleen. A central arteriole branches into follicular arterioles which cross the marginal zone (MZ) and reach the red pulp as bridging channels. Here, blood is filtered by T cells, MZ B cells and macrophages on its way to the red pulp. The MZ contains two types of macrophages: Marginal Zone Macrophages (MZM) and Marginal Metallophilic Macrophages (MMM). Whereas MMM are located 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 (Fig. adapted from Nature Reviews Immunology 9, 767-777; November 2009).

In healthy rats, Dil-liposomes were absent in the CNS, suggesting that liposomes are incapable of crossing an intact BBB. However, when rats were injected one day after EAE onset, liposomes could also be located in a small number of $CD68^+$ macrophages in the spinal cord, indicating that liposomes can migrate into the CNS when the integrity of the BBB is compromised. Whether liposomes enter in the context of infiltrating macrophages or can freely cross the BBB remains to be clarified. However, In 2009 Cavaletti *et al.* studied the binding selectivity of charged liposomes to the spinal cord of EAE rats. Positively and negatively charged liposomes were injected into the tail vein of rats, and BBB targeting was determined. Accumulation in spinal cord was observed for cationic, but not for anionic, liposomes and only in EAE but not in healthy rats [70]. Since PS-liposomes are negatively charged because of PS being an anionic phospholipid, it is very unlikely that PSLs freely cross the BBB. And thus it is more likely that PSLs are first internalized by $CD68^+$ macrophages before entering the CNS.

In literature, PSLs have been described to regulate T cell activity through modulation of cytokine secretion [71]. To determine an impact of PSLs on lymphocyte proliferation, cognate antigen specific proliferation of splenic cultures 10 days post-immunization was assessed. Splenic cells from PSL-treated animals showed a significantly reduced MOG reactivity, compared to both vehicle- and PCL-treated animals. Although previous research has shown that infusion of apoptotic spleen cells and subsequent engulfment by macrophages induces the expansion of regulatory T cells via TGF-β [72], we were unable to detect any differences in the gene expression of transcription factors and cytokines specifying T cell subsets: T-bet / IFN-γ (TH1), GATA-3 / IL-4 (TH2), ROR-γt / IL-17a (TH17) and Foxp3 / IL-10 (Tregs) (see addendum Fig. 1A, 1B). The unaffected gene expression of TGF-β could explain the fact that Tregs did not expand in PSL-treated animals (see addendum Fig. 1C). In concordance, the mean follicle surface area in the spleen, which was determined by measuring the region surrounded by marginal metallophilic macrophages, was reduced in animals treated with PSLs. This is in line with previous findings by Hoffmann *et al.* who showed that PSLs can reduce the formation and size of germinal centers in the spleen in EAE mice [73].

To further investigate how PSLs impact EAE pathogenesis, the splenic expression of TNF-α, iNOS, OPN and Arg-1 was assessed. PSLs have been known to inhibit both TNF-α and NO production [74], and like the *in vitro* cultures with PSL-macrophages, a reduced gene expression of iNOS and TNF-α was observed in PSL-treated animals. Although splenic Arg-1 gene expression was unaffected, the total fluorescent intensity of splenic Arg-1 expression was significantly increased in animals treated with PSLs. Furthermore, in agreement with the homing of DiI-liposomes towards $CD68^+$ red pulp and $CD169^+$ marginal zone macrophages, the induction of Arg-1 expression was most prominent in splenic regions harboring these macrophage subtypes.

Additionally, gene expression of OPN, also known as early T lymphocyte activation (ETA-1), was reduced compared to both vehicle- and PCL-treated animals, albeit not significantly. Although there was a very clear trend in OPN suppression, there was not enough time to reproduce the results to obtain significant results. Nonetheless, OPN is a multifunctional protein that has been linked to many physiological and pathological events, including bone remodeling, cancer and inflammation [75, 76]. The involvement of OPN in autoimmunity has been emphasized in research on EAE. Osteopontin-deficient mice were resistant to progressive EAE and had frequent remissions, and myelin-reactive T cells in $OPN^{-/-}$ mice produced more IL-10 and less IFN-γ than in $OPN^{+/+}$ mice [77]. Moreover, OPN has been found to be highly expressed in MS lesions and plasma levels are increased during relapses in relapse-onset MS patients [21]. Because PSLs home primarily to the spleen and OPN is expressed by macrophages and other parafollicular cells in the spleen [78], it is possible that internalization of PS by splenic macrophages may have a direct effect on OPN gene expression, possibly by activating PPAR-α, but this will be discussed further on.

To assess whether PSLs have an effect on immune cell trafficking towards the CNS, vehicle-, PCL- and PSL-treated rats were sacrificed 30 days post-immunization and the spinal cords were collected, cryosectioned and stained for $CD68^+$ macrophages and $CD3^+$ T cells. Reduced numbers of macrophages and T cells were present in PSL- treated animals at day 30. Of note, PCL-treated animals also demonstrated a reduced infiltration of immune cells in the spinal cord compared to vehicle-treated animals, but to a lesser than PSL-treated animals. The latter is in contrast with the observed disease course, since both PCL and vehicle-treated animals did not differ in their neurological score at day 30. However, similar to our results, empty PC-containing liposomes have already been described to affect inflammation and neuronal cell death in a mouse retinal ischemia model [79]. We were unable to detect immune cell infiltration in spinal cord tissue 10 days post-immunization, probably because 10 day post-immunization was too early for the EAE to clinically manifest in the CNS.

Apart from modulating the macrophage phenotype and inflammation in a myocardial infarction and inflammatory bone loss model, PSLs have been described to resolve the delayed phase of carrageenan mouse paw edema [80]. Here, PPAR-γ antagonists partially prevented the anti-inflammatory effects of PSL administration. In this study we demonstrated that, instead of altering the functional properties of macrophages by activating PPAR-γ, PSLs acted as ligands for PPAR-α and PPAR-β/δ in macrophages *in vitro*. By incubating macrophages with PPAR antagonists prior to administrating PSLs, we demonstrated that the suppressed NO and TNF-α production by PSL-macrophages was controlled by PPAR-β/δ and PPAR-α respectively. PPAR-β/δ has previously been described as a regulator for the alternative activation of macrophages [63, 81, 82]. Moreover, Mukundan *et al.* have recently shown that PPAR-β/δ activation following apoptotic cell clearance by macrophages induces an immunosuppressive phenotype [61], like we observed for PSL-macrophages. Additionally, these results are in line with previous findings by Gallardo-Soler *et al*. who showed that PPAR-β/δ regulates Arg-1 expression in macrophages [83]. In macrophages and many other cell types, L-arginine is used as a substrate by both nitric oxide synthase (NOS) and arginase to produce NO and urea, respectively [84]. Thus, PPAR-β/δ most likely inhibits NO production by inducing Arg-1 which in turn competes with NOS for their common substrate. This is supported by the strong increase in Arg-1 expression in PSL-treated animals we observed. In concordance, PPAR-α also plays a role in immunomodulation. Previous findings show that PPAR-α can suppress OPN expression in macrophages [85]. Interestingly, TNF-α is known to stimulate OPN expression in macrophages [86], thus making it possible that the decrease in splenic OPN expression in PSL-treated animals is the result of PPAR-α activation and the subsequent decrease of TNF-α production. Moreover, results are in line with previous findings that PPAR-α can suppress TNF-α production through the inhibition of NF-kB [87, 88]. In addition, macrophages were also treated with antagonists but without PSLs. Results show no difference in NO or TNF-α production, indicating the observed effect is solely due to PPAR activation by PSLs (data not shown).

PS contains two acyl chains at the sn-1 and sn-2 positions of the glycerol moiety, with the polar headgroup attached to position sn-3. saturated fatty acids are concentrated in position sn-1 and unsaturated in position sn-2. PS used in our experiments consists of saturated stearic (40%) and unsaturated oleic acid (29%). To determine which fatty acid chain is responsible for suppressing NO production, sn-1-18:0 and sn-2-18:1 lyso-PS lacking respectively oleic and stearic acids were administrered to macrophages (Fig. 17). Although at present we cannot directly exclude other possibilities, it is most likely that lyso-PS exerts this effect by activating PPAR-β/δ, similar to PSLs. Interestingly, 18:1 sn-2-lyso-PS lacking the stearic chain but not 18:0 sn-1-lyso-PS lacking the oleic chain is responsible for activating PPAR-β/δ. We did see a similar trend regarding PPAR-α, albeit not statistically significant. However, the observed effect cannot be explained by the oleic acid chain alone as both PCL and PSL used in our experiments share the same amount of oleic acid (31% and 29% respectively) suggesting headgroup specificity is important. Macrophages are known to specifically take up cells when their membranes are exposed to negatively charged phospholipids [89], indicating that the combination of an anionic serine headgroup together with unsaturated fatty acid chains are necessary to activate PPARs. Moreover, the process of apoptosis is often accompanied by generation of ROS, which brings about rapid oxidation of fatty acids in PS. Indeed, it is now apparent that only molecular species of phosphatidylserine with an oxidatively truncated sn-2 fatty acyl group that incorporates unsaturated acyl moieties are recognized by scavenger receptors in macrophages as a prerequisite for engulfment of apoptotic cells [90-92].

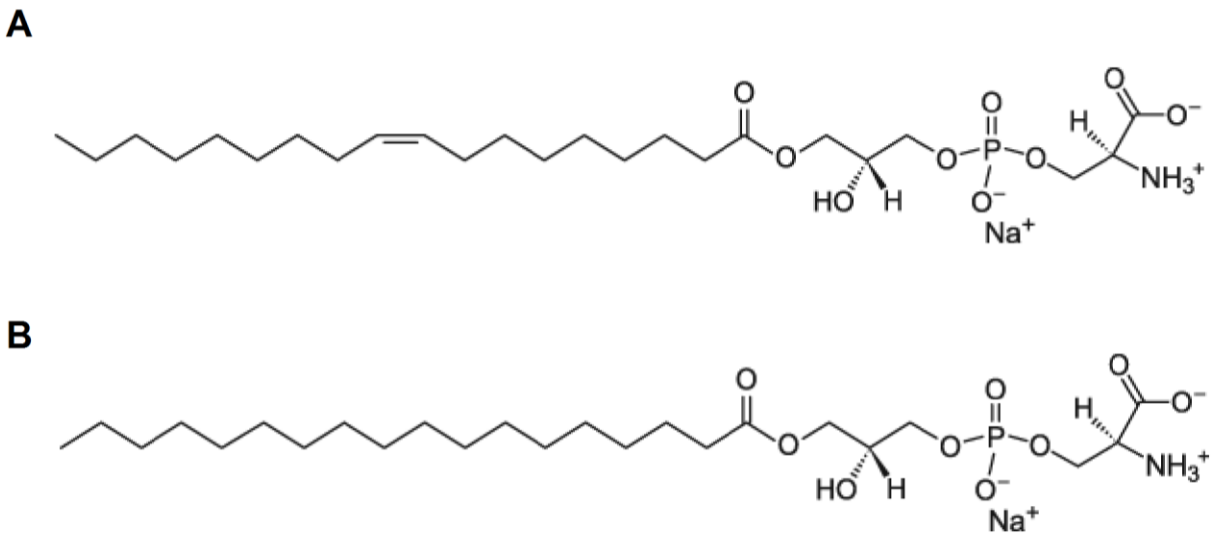

Figure 17 | Lyso-PS 18:1 (A) and 18:0 (B). sn-2-18:1 lyso-PS contains only an oleic fatty acid chain. Oleic acid is classified as an monounsaturated omega-9 fatty acid. sn-1-18:0 lyso-PS (top) contains only an stearic fatty acid chain. Stearic acid is classified a saturated fatty acid.

Results obtained in this study are in line with previous findings showing that lyso-PS has a role in the resolution of inflammation [93]. More importantly, in Schistosome infections, lyso-PS from the parasite is believed to be a key activator molecule in the host. Whereas the sn-2-lyso-PS stimulates degranulation of mast cells, most other lysophospholipids have no such activity. Furthermore, the capacity of the oleic acyl

moiety to specifically activate PPAR-β/δ and PPAR-α is in agreement with its described capacity to efficiently bind to PPAR-β/δ and PPAR-α at low concentrations [94]. Additionally, Ravnskjaer *et al*. showed that oleic acid, by itself, is capable of activating the peroxisome proliferator response element (PPRE), resulting in PPAR-β/δ, and to a lesser extend PPAR-α activation [95]. Further studies should determine whether sn-2-18:1 lyso-PSLs can alter the functional properties of macrophages by activating PPAR-α or PPAR-β/δ.

As we previously identified PS as a myelin-derived phospholipid, we sought to determine whether myelin-treated macrophages suppress NO in a similar way PSL-treated macrophages do. Research at Biomed has demonstrated that suppression of IL-6 production by macrophages following myelin internalization was cancelled out in LXR-β-deficient macrophages. However, the reduced NO secretion was unaffected in both LXRα- and LXRβ-KO macrophages, indicating that NO is regulated in a distinct manner. In contrast to IL-6, whose secretion is regulated via myelin-mediated LXR activation [67], the reduced NO production by macrophages following myelin internalization was counteracted by a PPAR-β/δ antagonist. These results show that myelin-treated macrophages suppress NO production through PPAR-β/δ activation, similar to macrophages treated with PSLs. In addition, macrophages were also treated with antagonists but without myelin. Results show no difference in NO production, indicating the observed effect is solely due to PPAR activation by myelin (data not shown). Collectively, these results show that both cholesterol and PS, and the nuclear receptors they activate, play a cardinal role in determining the phenotype of myelin-phagocytosing macrophages.

5. CONCLUSION & SYNTHESIS

The goal of this study was to investigate the effects of PSLs on the functional phenotype of macrophages *in vitro* and *in vivo* during EAE. We hypothesized that PSLs will ameliorate chronic EAE in rats through the polarization of macrophages into an anti-inflammatory phenotype. In summary, this study shows that PSLs alter the functional properties of macrophages *in vitro* by activating PPAR-β/δ and PPAR-α. Moreover, systemic treatment of PSLs had both a prophylactic and therapeutic effect on EAE, which was paralleled by a reduced splenic lymphocyte proliferation, less CNS cell trafficking, a reduction in size of splenic follicles, and a decreased secretion of inflammatory mediators in the spleen. More importantly, the homing of PSLs to splenic macrophages following intravenous injections and the subsequent change in expression of TNF-α, iNOS, OPN and Arg-1, illustrates a crucial role for a PSL-mediated activation of PPAR-β/δ and PPAR-α in macrophages in the observed effects on EAE. Additionally, activation of PPAR-β/δ and possibly PPAR-α seems to be dependent on the composition and distribution of fatty acid chains in phosphatidylserine.

The results of our study suggest that increased apoptotic signals introduced by PSLs can promote an anti-inflammatory environment, and may encourage a further *in vivo* evaluation of PSLs regarding their potential therapeutic effect on inflammatory responses. As PS is a naturally occurring cell membrane constituent and PSLs have been described to have no side effects in humans, PSLs may potentially be used to treat inflammatory disorders like MS. 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. Expanding on the knowledge of PSLs, identification of anti-inflammatory constituents can lead to a new therapeutic approach, with the goal of inducing repair mechanisms in MS.

In the future, PSLs could be modified for oral administration. Key points include stability under high acid environment (stomach) and absorbability into the intestinal wall and into the bloodstream. Furthermore, as particulate carriers, liposomes naturally target cells of the mononuclear phagocytic system, particularly macrophages. The goal for targeting macrophages using liposomes not only includes drug delivery but can also be used to deliver immunomodulatory factors (like IL-4) in an attempt to strengthen the immunomodulatory properties of PSLs. Moreover, the composition and the distribution of fatty acids in phosphatidylserine and its effect on PPAR activation could be further elucidated. Whereas oleic acid is a known activator of PPARs, other fatty acids like arachidonic acid are much more potent in activating PPARs. Therefore, 1-stearoyl-2-arachidonoyl-phosphatidylserine molecular species could be used in future experiments.

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7. ADDENDUM

Table 1 | Primers used for quantitative RT-qPCR. Tumor Necrosis Factor alpha (TNF-α), inducible Nitric Oxide Synthase (iNOS) and Osteopontin (OPN). All primers were provided by Eurogentec.

Gene	Species	Primer sequence (5'->3')	Product size	Accession nummber
TNF-α	*Rattus norvegicus*	Fw: CTTATCTACTCCCAGGTTCTCTTCAA Rv: GAGACTCCTCCCAGGTACATGG	200 bp	NM_012675.3
iNOS	*Rattus norvegicus*	Fw: GCATCCCAAGTACGAGTGGT Rv: TGTTGTAGCGCTGTGTGTCA	176 bp	NM_012611.3
OPN	*Rattus norvegicus*	Fw: CTCGGAGGAGAAGGCGCATTA Rv: CCATCGTCATCGTCGTCGTCA	207 bp	NM_012881.2

Table 2 | Clinical scores of disease in EAE.

EAE Score	Condition
Score 0	Clinically normal
Score 1	Complete Tail paralysis
Score 2	Complete hind limb paralysis
Score 3	Complete paralysis of hind limbs and midriff
Score 4	Complete hind and front limb paralysis
Score 5	Moribund

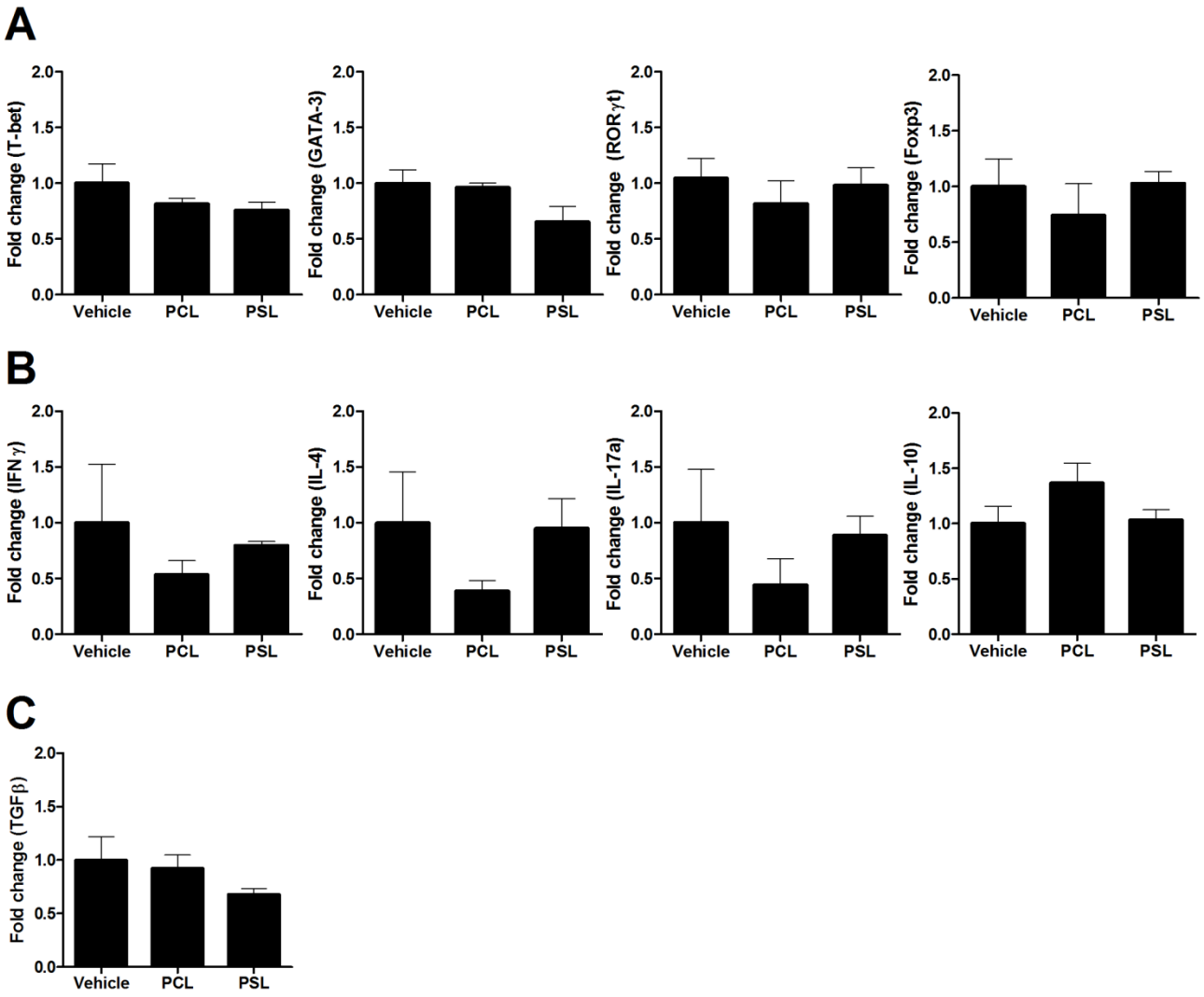

Figure 1 | RT-qPCR of gene expression of transcription factors and cytokines specifying T lymphocyte subsets. Splenic gene expression of T-bet, IFNγ, GATA-3, IL-4, RORγt, IL-17a, Foxp3, IL-10 and TGF-β from PSL-treated animals was determined using RT-qPCR and compared to control and PCL-treated animals (n=3). Error bars represent standard error of the mean (SEM).

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