The role of liver X receptors in the macrophage response after myelin phagocytosis

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Abbreviations

ABC:	ATP binding cassette transporter
ANOVA:	Analysis of variance
AP-1:	Activator protein 1
ApoE:	Apolipoprotein E
BBB:	Blood brain barrier
CNS:	Central nervous system
COX-2:	Cyclo oxygenase 2
CR3:	Complement receptor 3
CSF:	Cerebrospinal fluid
Cyp7a1:	Cholesterol 7α hydroxylase
DHR:	Dihydrorhodamine
DiI:	1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate
DMCA:	N,N-dimethyl-3β-hydrocycholenamide
DMSO:	Dimethylsulfoxide
EAE:	Experimental autoimmune encephalomyelitis
EDTA:	Ethylenediamine tetraacetic acid
ELISA:	Enzyme linked immunosorbent assay
FACS:	Fluorescent activated cell sorting
FCS:	Fetal calf serum
GAPDH:	Glyceraldehyde 3-phosphate dehydrogenase
GLUT-4:	Glucose transporter 4
GW:	GW9662
HC:	22(S)-hydroxycholesterol
HMBS:	Hydroxymethylbilane synthase
IFNγ:	Interferon gamma
IL:	Interleukin
iNOS:	Inducible nitric oxide synthase
LA:	Lipoic acid
LDL:	Low density lipoprotein
LDL-R:	Low density lipoprotein receptor
LPL:	Lipoprotein lipase
LPS:	Lipopolysaccharide
LXR:	Liver X receptor
LXRE:	Liver X receptor responsive element
MHC-II:	Major histocompatibility complex class 2
MMP-9:	Matrix metalloprotease 9
MS:	Multiple sclerosis
MTT:	Thiazolyl blue tetrazolium bromide
Mye:	Myelin
NADPH:	Nicotinamide adenine dinucleotide phosphate

Ncf1:	Neutrophil cytosolic factor 1
N-CoR:	Nuclear receptor corepressor
NED:	N-1-napthylethylenediaminedihydrochloride
NFAT:	Nuclear factor of activated T cells
NFκβ:	Nuclear factor Kappa Bèta
NO:	Nitric oxide
NOX 2:	Nicotinamide adenine dinucleotide phosphate complex
NPC:	Niemann pick C
P/S:	Penicilline/streptomycin
p47-PHOX:	p47 domain of phagocyte oxidase
PBS:	Phosphate buffered saline
PGK1:	Phosphoglycerate kinase
PMA:	Phorbol 12-myristate 13-acetate
PPARγ:	Peroxisome proliferator activated receptor gamma
PP-MS:	Primary progressive Multiple Sclerosis
PPRE:	PPAR response element
ROS:	Reactive oxygen species
RR-MS:	Relapsing remitting Multiple Sclerosis
RT-PCR:	Real-time quantitative polymerase chain reaction
RXR:	Retinoid X receptor
SEM:	Standard error of means
SMRT:	Silencing mediator of retinoic acid and thyroid hormone receptor
SP-MS:	Secondary progressive Multiple Sclerosis
SREBP-c:	Sterol regulatory element binding protein c
STAT:	Signal transducers and activators of transcription protein
SUMO:	Small ubiquitin-related modifier
TBP:	Tata box binding protein
TGFβ:	Transforming growth factor beta
Th1:	T helper 1
TLR-4:	Toll like receptor 4
TNFα:	Tumour necrosis factor alpha
β-ME:	β-mercaptoethanol
$\Delta\Delta C_t$ method:	Comparative C_t method

Abstract

Multiple Sclerosis (MS) is a chronic inflammatory disease of the central nervous system in which macrophages play an important role. Classically, macrophages are hypothesized to be only detrimental in MS by phagocytosing myelin and secreting toxic mediators like nitric oxide (NO), reactive oxygen species (ROS) and inflammatory cytokines. However, recent evidence suggests that macrophages may also be neuroprotective in MS, as myelin-laden macrophages in the centre of MS lesions seem to demonstrate an anti-inflammatory phenotype. Nonetheless, underlying mechanisms inducing this protective phenotype still remain to be clarified. One such possible mechanism could be the activation of liver X receptors (LXRs) in macrophages. LXRs are nuclear receptors with important roles in cholesterol homeostasis, glucose metabolism and inflammation. LXRs are activated by cholesterol derivatives. Interestingly, since myelin contains cholesterol, LXRs could be involved in the macrophage response after myelin phagocytosis. Therefore, the role of LXRs in the macrophage response after myelin phagocytosis was investigated in this study. We hypothesized that LXRs are activated after myelin phagocytosis and induce a protective, anti-inflammatory phenotype in macrophages. First, the activation of LXR response genes, after myelin phagocytosis, was investigated in rat peritoneal macrophages by RT-PCR. Next, macrophage production of inflammatory mediators, after myelin phagocytosis and LXR activation, was investigated with ELISA, NO-assays and DHR-assays. First results showed that both T09 and myelin increased the expression of LXRα, apoE, ABCA1 and LDL-R. These results indicate that LXRs are most likely activated after myelin phagocytosis and could therefore be involved in the macrophage response after myelin phagocytosis. Secondly, both T09 and myelin increased ROS production by macrophages, indicating that the myelin-induced increase in ROS is probably mediated via LXR signalling. Thirdly, both myelin and T09 decreased NO production by macrophages, indicating that the myelin-induced decrease in NO is probably mediated via LXR signalling. Finally, myelin decreased TNFα production by macrophages, suggesting that myelin phagocytosis induces a protective phenotype in macrophages. On the other hand, only low concentrations T09 decreased TNFα production. These results indicate that multiple mechanisms, probably including LXR signalling, are involved in inducing a protective phenotype in macrophages after myelin phagocytosis. In the future, additional mechanisms by which myelin modulates the phenotype of macrophages will be investigated. Furthermore, LXR knock-out animals will be used to confirm the obtained results. Future in vivo studies are required to elucidate the role of myelin-phagocytosing macrophages in EAE/MS. This study has led to new views in MS pathogenesis and could direct the development of new, more effective therapies for the disease.

Keywords: *Multiple Sclerosis, myelin, macrophages, liver X receptor, anti-inflammatory.*

1 Introduction

1.1 Multiple Sclerosis

Multiple Sclerosis (MS) is a chronic inflammatory disease of the central nervous system (CNS), characterized by demyelination, focal T cell and macrophage infiltrates, axonal injury and loss of neurological function. MS is an autoimmune disorder affecting more than two million people worldwide and has been recognized as the major cause of neurological disability in young adults. Symptoms include impairment of bladder and bowel function, sexual difficulties, sensory loss, impairment of vision and weakness or paralysis of extremities. The major cause of most symptoms is thought to be an axonal conduction block, resulting from demyelination and inflammation. This conduction block prevents an efficient salutatory conduction, leading to a decreased conduction velocity. MS is generally categorized as relapsing remitting (RR) or primary progressive (PP). The RR form is characterized by series of attacks from which patients recover partly or completely. Ultimately, this disease course will change to a progressive form, known as secondary progressive (SP) MS. The PP subtype is characterized by a gradual clinical decline, without remissions [1]. The cause of MS is currently still unknown. However, according to current data the disease seems to develop in genetic susceptible individuals and requires additional exposure to environmental factors [2]. Genetic factors, like interleukin (IL)-1β, IL-1r, immunoglobulin Fc receptor and apolipoprotein E (apoE) genes have been demonstrated to have an effect on MS susceptibility. In addition, divergent association studies and genome wide linkage screens have elucidated a strong correlation between the HLA-DR2 haplotype and MS susceptibility. Environmental factors could include pathogens, hormones, chemicals and ultraviolet radiation [3].

1.1.1 Pathogenesis

The pathological hallmark of MS is the formation of sclerotic plaques. These plaques represent the end stage of a process involving inflammation, demyelination, remyelination, oligodendrocyte depletion, gliosis and neuronal and axonal degeneration [2]. These inflammatory lesions are characterized by the infiltration of cellular and soluble mediators of the immune system, including T cells, B cells, macrophages, microglia, cytokines, chemokines, antibodies, complement and other toxic substances [4]. Demyelination is the result of an immune cell mediated destruction of the myelin sheath and the oligodendrocyte itself. This immune attack involves T cells, which are directed against myelin and oligodendrocytes, but also macrophages, which mediate antigen presentation, myelin phagocytosis and secrete toxic mediators. Also B cells, which secrete anti-myelin antibodies are involved in the immune attack. Remyelination can occur in the sclerotic plaque, but is generally incomplete and characterized by thinner myelin sheaths than the original myelin [4]. Traditionally, MS was considered to be a CD4+ T helper 1 (Th1) mediated autoimmune disorder. However, recent evidence suggests a pivotal role for CD8+ T cells and T helper 17 cells as effectors in the disease [5, 6]. Activation of myelin reactive CD4+ T cells in the periphery is hypothesized to be one of the first events in the pathogenesis of MS. After activation, lymphocytes expand and migrate across the blood brain barrier (BBB) to enter the CNS. The adherence and

migration of T cells is mediated by adhesion molecules expressed on the surface of both T cells and endothelial cells. Initial adherence is mediated by selectins, expressed on T cells, and the subsequent strong adhesion and transmigration is mediated by integrins. Autoreactive T cells are reactivated in the CNS after binding to their specific myelin antigen, presented by resident antigen presenting cells. Reactivation of autoreactive T cells results in the release of pro-inflammatory cytokines like tumour necrosis factor α (TNFα) and interferon γ (IFNγ). This will trigger an inflammatory cascade leading to the recruitment of macrophages and B cells into the CNS. These cells will produce detrimental mediators like reactive oxygen species (ROS), nitric oxide (NO), auto-antibodies and complement factors, which will ultimately lead to demyelination and axonal damage [7].

1.1.2 Treatment

Treatment options in MS have extended over the past decade: immunosuppressants, like mitoxantrone, as well as immunomodulatory drugs, like beta-interferons and glatiramer acetate, are nowadays frequently applied. However, these drugs are only partially effective and often associated with considerable long-term toxicity. Moreover, the risk-to-benefit ratio is not yet entirely clear and most of these compounds are targeted against the inflammatory component of the disease process, while demyelination and axonal injury remain insufficiently treated. Consequently, there is still research needed to develop new compounds for future MS therapy [8].

1.2 Macrophages

Macrophages are the most important effector cells in MS and its equivalent animal model, experimental autoimmune encephalomyelitis (EAE). Both resident brain macrophages and peripheral blood macrophages are involved in the pathogenesis of MS. Three different macrophage populations are present in the normal brain. These populations include microglia, perivascular cells and meningeal macrophages [9]. Microglia are located inside the neural parenchyma and are highly ramified cells with extensive branches and a very slow turnover rate. They are thought to originate from circulating monocytes which populate the CNS during embryonic, fetal or perinatal stages or from mesenchymal progenitor cells. Perivascular macrophages are located in the perivascular space, outside the neural parenchyma and are often wrapped around blood vessels. These macrophages are small, elongated cells with short but broad processes. Unlike microglia, perivascular macrophages show a high turnover and are thought to be regularly replenished by monocytes from the peripheral circulation [10]. Meningeal macrophages are located outside the neural parenchyma, between meningeal epithelium and basal membranes. They are large rounded cells, characterized by a fast turnover from circulating monocytes. Blood-borne macrophages, on the other hand, are not present in the normal brain, but are recruited from the periphery in certain inflammatory conditions like EAE and MS. These different subpopulations are related cell types that perform a variety of different functions, but together they are considered to be involved in the brain pathology in MS [10].

1.2.1 Macrophages in the pathogenesis of MS

In normal physiologic conditions, the brain contains resting microglia, perivascular macrophages and meningeal macrophages. In pathological conditions, like MS, all these cell types are activated.

Additionally, BBB disruption and local production of chemoattractant agents in the CNS stimulates the migration of peripheral blood monocytes to the site of inflammation [9, 10]. In early MS lesions, this cell invasion is localized around blood vessels. However as the disease progresses, a gradual migration into the brain parenchyma is observed [11]. After infiltration, bloodmonocytes differentiate into mature effector macrophages supporting ongoing inflammation. About 50 % of mononuclear cells present in lesions of EAE animals are blood-derived macrophages. Furthermore, active plaques of MS are characterized by impressive numbers of macrophages that are mainly located in areas of ongoing demyelination [12]. Resident and infiltrated macrophages are responsible for the release of numerous detrimental mediators, like pro-inflammatory cytokines, ROS, NO, glutamate and proteases. This will eventually lead to an increased inflammation, demyelination and axonal damage.

Macrophages produce pro-inflammatory cytokines like TNFα, IL-1 and IL-6. TNFα is neurotoxic in vitro, increases axonal vulnerability by enhancing demyelination via oligodendrocyte killing and induces NO production [13]. In addition, macrophages produce ROS. ROS form little thread in low concentrations, as cells contain various defence and repair mechanisms protecting them from minor oxidative stress. However, during inflammation, the respiratory burst of macrophages increases the release of ROS. High ROS concentrations cause oxidative damage to proteins, lipids and nucleic acids resulting in cell death by apoptosis or necrosis [13]. In addition, ROS can induce BBB disruption and a subsequent extravasation of leukocytes into the CNS. Furthermore, ROS induce DNA damage leading to neurodegeneration and ROS have been implicated as mediators of demyelination and axonal damage. Free radicals can also activate certain transcription factors, like nuclear factor κβ (NFκβ), which upregulate the expression of many genes involved in the pathogenesis of MS and EAE, such as TNFα and inducible NO synthase (iNOS). Interestingly, the anti-oxidant defence mechanisms in the CNS, which have been described to be beneficial, are weakened during MS, illustrating that ROS could indeed play an important role in pathological processes underlying MS pathogenesis [3, 14]. Furthermore, macrophages produce large amounts of NO. NO is a free radical found at elevated concentrations in inflammatory MS lesions. Moreover, markers of NO production, like nitrate and nitrite are raised in the cerebrospinal fluid (CSF), blood and urine of MS patients. NO may be involved in the development of several pathological features of MS, as it has been described to cause vasodilatation and BBB disruption, leading to the entry of inflammatory cells and toxic mediators in the CNS. Other effects of NO include demyelination, axonal degeneration, oligodendrocyte injury and neuronal apoptosis. Finally, it has been reported that iNOS inhibitors ameliorate EAE in mice [13, 15]. Moreover, the excitatory neurotransmitter glutamate is secreted in large quantities by macrophages. An excess of glutamate causes excitotoxicity in neurons, resulting in cell death. Similarly oligodendrocytes are very vulnerably to high glutamate levels both in vitro and in vivo [13]. Treatment with riluzole, an antagonist of glutamate neurotransmission, reduces clinical severity, inflammation, demyelination and axonal damage in EAE mice. Furthermore, there is evidence for an association between glutamate levels in the CSF of MS patients and the severity of the disease [3]. Finally, macrophages produce proteases. Proteases are a family of proteolytic enzymes that have important roles in cell development and physiology. The normal CNS contains a limited number of proteases, however they are upregulated in certain inflammatory conditions, like MS and EAE. This upregulation results

in a cascade of detrimental effects in the CNS. Proteases degrade the extracellular matrix and in this way they facilitate the migration of inflammatory cells into the CNS. Furthermore, they cause axonal damage, neuronal dysfunction and neuronal apoptosis [13, 16].

Besides the release of inflammatory mediators, macrophages mediate myelin phagocytosis. Macrophages contribute to myelin damage either directly by phagocytosis of myelin or indirectly by the production of inflammatory mediators that damage the myelin sheath. Brain myelin is composed of 70-75 % lipid and 20-30 % protein. Lipids include cholesterol, phospholipids and glycolipids. Proteins include proteolipid protein and myelin basic protein. During myelin phagocytosis, myelin lamellae are attached to coated pits on the macrophage surface and ingested by receptor mediated phagocytosis. This receptor mediated phagocytosis is mediated through Fc-receptors, complement receptors and scavenger receptors. Fc-receptors, present on the macrophage surface, bind to the Fc portion of immunoglobulins and for that reason may be considered as the main instrument for antibody mediated phagocytosis. Complement receptors, like complement receptor 3 (CR3), are also present on macrophages and are able to phagocytose myelin. Scavenger receptors have a high affinity for anionic phospholipids, like phosphatidylserine. Myelin damage, caused by a series of detrimental mediators, can cause an externalization of phosphatidylserine. This phospholipid could then serve as a ligand for the scavenger receptor present on macrophages [11]. Phagocytosis of myelin triggers the release of pro-inflammatory cytokines, NO and ROS. This suggests that myelin phagocytosis could enhance neuroinflammation [17]. Furthermore, depletion of macrophages suppresses the expression of clinical signs of EAE. This suppression was accomplished by a reduction of infiltrated macrophages in the CNS, suggesting that macrophages could play a crucial role in the effector phase of the disease [18].

1.2.2 Macrophages as mediators of protective immunity

Since MS lesions are self limiting and do not expand indefinitely it is likely that local mechanisms restrict CNS inflammation and may also promote tissue repair. Furthermore, the relapsing-remitting nature of MS suggests that there is a possible counter-regulatory mechanism present that keeps the disease under control [19]. One such possible mechanism involves the inhibition of T cell responses by regulatory T cells [20]. Anther possible mechanism involves the presence of myelin-laden macrophages in demyelinating lesions in MS. Macrophages were first thought to be only detrimental in MS, but recent evidence suggests that myelin-laden macrophages may also mediate protective effects. For instance, myelin-containing foam cells in the centre of MS lesions express a series of anti-inflammatory molecules, like IL-1ra and IL-10, while they lack pro-inflammatory cytokines, like TNFα and IL-1β [19]. Furthermore, it was shown that low-density lipoprotein (LDL) uptake by macrophages inhibits TNFα expression [21] and induces IL-10 expression [22]. Myelin-laden macrophages, present in active demyelinating MS lesions have been shown to contain plasma LDL [23]. Recent evidence suggests an anti-inflammatory reaction of macrophages phagocytosing myelin upon contact with damaged nerve tissue [24]. In support of this view, other data suggests that the macrophage response to toxin-induced demyelination influences the growth factor environment, thereby affecting the behaviour of oligodendrocyte precursor cells and hence the efficiency of remyelination [25].

In response to cytokines and microbial products, macrophages express specialized and polarized functional properties (figure 1). Classically activated M1 macrophages are activated by lipopolysaccharide (LPS), TNFα or IFNγ. Alternatively activated M2 macrophages, on the other hand, are activated by IL-4, IL-10 or transforming growth factor β (TGFβ). M1 macrophages are characterized by a high production of NO and by the production of pro-inflammatory cytokines, like TNFα, IL-12 and IL-23. Furthermore, they are involved in Th1 cell responses and in the killing of micro-organisms and tumour cells. In addition, the expression of co-stimulatory molecules and major histocompatibility complex class 2 (MHC-II) is upregulated, which results in an increased antigen presentation. M2 macrophages are involved in Th2 cell responses, scavenging of debris and the promotion of tissue remodelling and repair. Furthermore, they express anti-inflammatory molecules, like IL-1ra and IL-10. The terms M1 and M2 are used to refer to two extremes of a spectrum of possible forms of macrophage activation. Macrophages may be able to switch from one phenotype into another in response to changing inflammatory conditions. Currently, there is little known about the phenotype of macrophages present in MS lesions. In the onset of disease, they probably display a M1 phenotype, leading to increased inflammation. Myelin-laden foamy macrophages, in contrast, seem to resemble M2 macrophages and could therefore have anti-inflammatory effects and be involved in the remitting phase of MS [19, 26, 27].

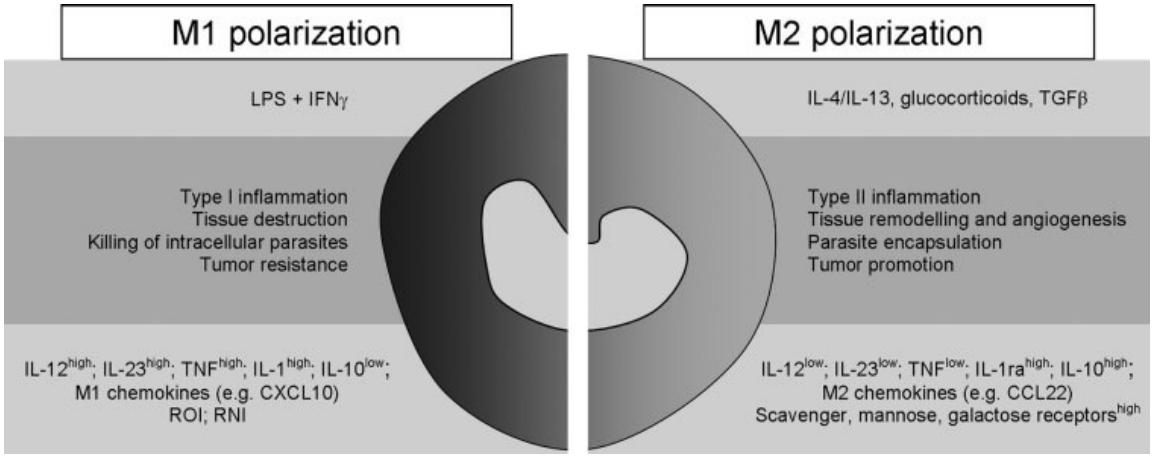

Figure 1: Macrophage polarization. Macrophages can be categorized in being either classically activated M1 macrophages or alternatively activated M2 macrophages.

1.3 Liver X receptors

Liver X receptors (LXRs) are ligand-dependent transcription factors, activated by oxidized forms of cholesterol (oxysterols) or by certain intermediates of the cholesterol biosynthetic pathway. Since myelin contains cholesterol, LXRs may play a role in the macrophage response after myelin phagocytosis. LXRs are members of the nuclear receptor superfamily and form heterodimers with the retinoid X receptor (RXR). Additionally, LXRs are bound to corepressors, like silencing mediator of retinoic acid and thyroid hormone receptor (SMRT) and nuclear receptor corepressor (N-CoR). The LXR-RXR-corepressor complex is interconnected with a LXR responsive element (LXRE) in the nucleus. After ligand binding, the corepressors are replaced by coactivators and transcription of target genes takes place. Two LXR subtypes exist, LXRα and LXRβ, both of which are expressed in the CNS. LXRα is highly expressed in the liver and at lower levels in the adrenal glands, intestine,

adipose tissue, macrophages, lung and kidney. LXRβ, in contrast, is almost ubiquitously expressed [28]. There are three LXRα isoforms which can all bind to response elements. However, $LXR\alpha_2$ shows reduced transcriptional activity and $LXR\alpha_3$ is transcriptionally inactive [29].

1.3.1 Functions of LXRs

LXRs have been described to play an important role in cholesterol metabolism. Cholesterol accounts for 99 % of all sterols in mammals and plays multiple biological roles. Cholesterol is an essential component of cellular membranes, where it is required to establish proper membrane permeability and fluidity [29]. Furthermore, cholesterol serves as a precursor for steroid hormones and bile acids. An appropriate balance should be maintained between cholesterol biosynthesis, dietary ingestion of cholesterol and cholesterol elimination [30]. LXRs function as cholesterol sensors that are activated in response to elevated intracellular cholesterol levels. After activation, LXRs induce the expression of genes involved in cholesterol metabolism. This results in the uptake, transport and catabolism of cholesterol to prevent excess accumulation. A first target of the LXR is cholesterol 7α hydroxylase (Cyp7a1), an enzyme involved in the metabolisation of cholesterol into bile acids to prevent an excess accumulation of cholesterol esters [31]. LXRs also regulate the expression of genes that participate in the transport of cholesterol. ATP binding cassette transporter (ABC) A1, ABCG1, ABCG5, ABCG8 and ApoE mediate the efflux of cholesterol from the cell interior to the periphery. The induction of target genes ABCG5 and ABCG8 promote the bilary and fecal excretion of cholesterol [32]. The activation of LXRs also leads to an upregulation of the Low Density Lipoprotein Receptor (LDL-R), which results in the uptake of cholesterol into the liver [33]. In addition to their ability to modulate cholesterol metabolism, LXRs also play a role in hepatic lipid formation. This lipogenic activity of LXRs results from the upregulation of Sterol Regulatory element Binding Protein c (SREBP-c), fatty acid synthase, acyl-CoA carboxylase and stearoyl-CoA desaturase 1. Finally, glucose metabolism is also influenced by LXR activity, as LXR activation improves glucose tolerance by mediating the repression of the hepatic gluconeogenic genes phosphoenolpyruvate carboxykinase, glucose-6-phosphotase and glucokinase. These changes result in a decreased hepatic glucose output and an increased hepatic glucose utilization. The LXR mediated induction of glucose transporter 4 (GLUT4) results in an increased uptake and utilization of glucose by white adipose fat [28]. As mentioned here, LXRs are a family of transcription factors with pleiotropic functions. They have important roles in cholesterol and glucose metabolism, and even play a pivotal role in liver lipid formation. Interestingly, LXRs have been linked to metabolic disorders, like atherosclerosis and diabetes type 2 [34]. Moreover, as cholesterol is the primary component of myelin, LXRs may even be involved in demyelinating disorders of the CNS.

1.3.2 LXRs in macrophages

Several studies have demonstrated the presence and activation of LXRs in macrophages [35]. LXRs can be activated in macrophages by several mechanisms. First, scavenger receptor mediated uptake of oxidized lipoproteins, leads to the transcriptional activation of LXRs present in macrophages [36]. Second, ligands may also be generated intracellular from accumulated cholesterol by the action of mitochondrial cyp27. For example, the intracellular production of 24-(S), 25-epoxycholesterol in macrophages was recently reported [28]. A primary function of LXRs in

macrophages is to maintain cellular cholesterol homeostasis. LXRs regulate macrophage cholesterol efflux, to reduce the intracellular cholesterol burden, by inducing ABCA1, ABCG1 and ABCG4 gene expression. These ABC transporters are critical for the ability of LXRs to enhance efflux to cholesterol acceptors, like ApoE. In addition, LXRs induce the expression of Niemann pick C 1 (NCP1) and NCP2. This results in the mobilisation of cholesterol to the plasma membrane, where it becomes available for efflux [35]. In addition to their role in cholesterol homeostasis, LXRs have emerged as regulators of inflammatory gene expression in macrophages. LXR activation decreases the induction of inflammatory genes like iNOS, cyclo oxygenase 2 (COX-2), Matrix metalloprotease 9 (MMP9) and various chemokines in response to LPS, TNFα and IL-1β stimuli. Furthermore, LXR activation positively regulates the expression of arginase II, a gene that has anti-inflammatory effects through antagonism of NO signalling [37]. The mechanism underlying this inhibition of inflammatory signalling by LXRs is poorly understood. This repression might be accomplished by the inhibition of the NFkB signalling pathway [28, 38]. Finally, LXR activation leads to an improved macrophage survival through the induction of anti-apoptotic genes, like Spα, Bcl-XL and Birc1a. Furthermore, LXR activation results in the inhibition of pro-apoptotic elements, like caspases and Fas ligand (figure 2) [28, 29].

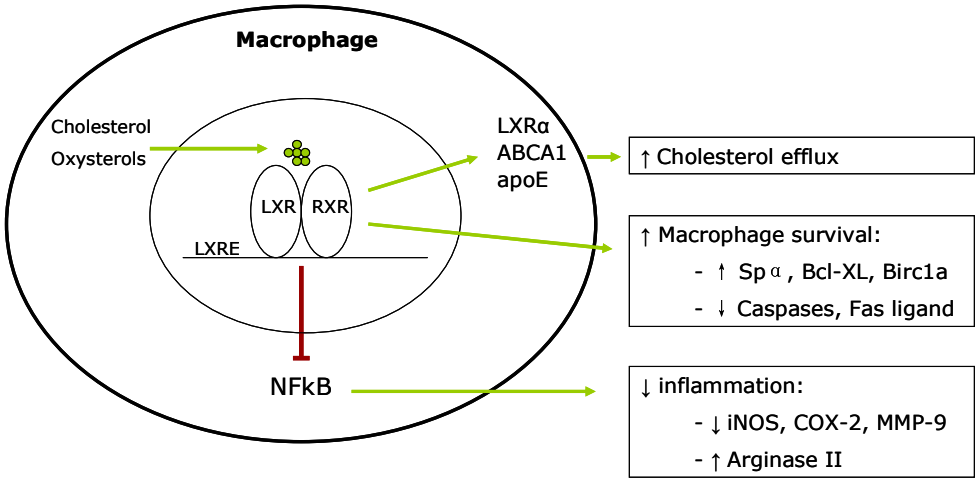

Figure 2: The role of LXRs in macrophages. LXRs are activated by oxysterols or oxydized cholesterol derivatives. After activation, they regulate macrophage cholesterol efflux by the induction of LXRα, ABCA1 and apoE. Furthermore, LXRs increase macrophage survival by the induction of anti-apoptotic genes, like Spα, Bcl-XL and Birc1a and the inhibition of pro-apoptotic elements, like caspases and Fas ligand. Finally, LXRs decrease macrophage inflammation by the inhibition of NFkB.

1.3.3 LXR agonists as treatment

The ability of LXRs to integrate metabolic and inflammatory signalling makes them attractive targets for the intervention in human metabolic disorders, like atherosclerosis and diabetes type 2, as well as for the modulation of inflammation and immune responses. LXR agonists have already yielded promising results as treatment for murine models of atherosclerosis, diabetes and Alzheimer [34]. However, synthetic ligands of LXRs increased hepatic lipogenesis and plasma triglyceride levels, mainly by inducing SREBP-1c expression in the liver. This resulted in a dramatic increase in fatty acid biosynthesis, which led to hepatic steatosis and hypertriglyceridemia [28]. Nevertheless, this hypertriglyceridemic effect of LXR agonists is usually transient and limited to the first few days of treatment, likely due to enhanced VLDL triglyceride hydrolysis resulting from an

increased expression of hepatic lipoprotein lipase (LPL) [39]. Several strategies may be applied to generate LXR agonists that do not exert undesirable side effects. First, most data suggests that the lipogenic effect of LXR agonists in the liver is mediated predominantly by LXRα. Therefore, specific LXRβ agonists may be a possible solution to resolve the side effects of LXR agonist therapy. N-Acylthiadiazolines are a new class of LXR agonists that show increased selectivity for LXRβ [40]. The second possibility to generate LXR agonists without lipogenic properties is the use of steroid LXR activators, like oxysterols. These LXR agonists are less potent in stimulating lipogenesis, as they also inactivate SREBP. A third potential approach to obtain non-lipogenic LXR agonists is to generate tissue selective compounds which will act on macrophages, but not on hepatocytes [41]. Another possible solution is the use of gene-selective LXR modulators that mediate potent transcriptional activation of ABCA1 gene expression while exhibiting minimal effects on SREBP-1c. The synthetic LXR agonist, N,N-dimethyl-3β-hydrocycholenamide (DMCA), represents such a gene-selective LXR modulator [42]. Additionally, GW9365 has been described to be a LXR agonist with only a limited ability to increase expression of lipogenic genes [39].

1.4 Peroxisome proliferating activated receptor

The peroxisome proliferating activated receptor gamma (PPARγ) and LXR are both nuclear receptors and their activity is most likely related due to their common role in macrophage cholesterol metabolism. Moreover, they share other common features, since both receptors form heterodimers with the RXR, bind to a PPAR response element (PPRE) in the nucleus [29] and are able to induce the expression of LXRα, ABCA1, ABCG1 and apoE [43]. Natural ligands for the PPARs include native and modified polyunsaturated fatty acids and eicosanoids. Three different PPAR subtypes have been identified: PPARα, PPARβ and PPARγ. PPARα is expressed in liver, skeletal muscle, kidney, heart and vascular wall. PPARβ is ubiquitously expressed with highest levels in skin and skeletal muscle and PPARγ is predominantly expressed in adipose tissue, intestine and macrophages [44]. PPARs have a role in lipid metabolism, since they are expressed in macrophage foam cells where they enhance the uptake of oxidized LDL by inducing the transcription of scavenger receptor CD36. Activation of PPARγ not only results in cholesterol uptake but it can also increase cholesterol efflux from macrophages. For instance, it was proven that PPARγ could directly induce the expression of LXRs. These results indicate that a coordinated lipid transport exists in macrophages, orchestrated by basically two nuclear receptors: PPARγ and LXR [29]. Besides the role of PPARγ in lipid metabolism, it also plays a role in glucose homeostasis, cell proliferation, cell differentiation and apoptosis. In addition to these metabolic functions, PPARs have also been described to modulate inflammatory responses. PPARs repress the upregulation of inflammatory genes in macrophages in response to various stimuli by a mechanism called transrepression. PPARγ ligands were shown to inhibit TNFα, IL-6 and IL-1β expression in monocytes. Furthermore, PPARγ ligands were shown to inhibit iNOS, MMP-9 and scavenger receptor A expression in macrophages. These effects can be attributed to an inhibition of NFκB, nuclear factor of activated T cells (NFAT), signal transducers and activators of transcription protein (STAT) and activator protein 1 (AP-1) transcriptional activities [44]. PPARγ can inhibit inflammatory gene expression by several mechanisms, including direct interactions with AP1 and NFκB, nucleocytoplasmic redistribution of the p65 subunit of NFκB, modulation of p38 mitogen-activated protein kinase activity, competition

for limiting pools of co-activators and interactions with transcriptional co-repressors [45]. PPARγ is expressed in cells of the monocyt/macrophage lineage and can influence the phenotype of these cells. These receptors are considered to antagonize inflammatory M1 activation, while promoting M2 activation. PPARγ mRNA and protein is induced in peritoneal macrophages and peripheral blood monocytes by IL-4, a typical M2 inducer, suggesting a possible role in alternative activation. M1 stimuli, in contrast, have no effect on PPARγ expression. Additionally, there was a reduced M2 macrophage differentiation reported in the absence of PPARγ and an enhanced M2 macrophage differentiation in response to PPARγ activation. By regulating the expression of the arginase I gene, a marker of the M2 phenotype, PPARγ promotes the M2 phenotype [37, 44, 46]. Based on these studies it might be suggested that LXR and PPARγ are both involved in cholesterol metabolism and perhaps even in inducing the anti-inflammatory phenotype in macrophages. Moreover, as major components of myelin are cholesterol and fatty acids, myelin phagocytosis could lead to an activation of both the LXR and the PPARγ present in macrophages.

1.5 Goal of the project

Recent evidence suggests that myelin-laden macrophages could be neuroprotective in MS. However, underlying mechanisms inducing this protective phenotype still remain to be clarified. One such possible mechanism is the activation of LXRs present in macrophages. LXRs are present in macrophages and are activated by oxidized cholesterol derivatives. Interestingly, since myelin contains cholesterol, LXRs could be involved in the macrophage response after myelin phagocytosis. We hypothesize that LXRs are activated after myelin phagocytosis and induce a protective, anti-inflammatory phenotype in macrophages. **The main objective of this study is to determine the role of LXRs in the macrophage response after myelin phagocytosis.**

Aim I

First, we will investigate 'in vitro' whether LXRs are activated after myelin phagocytosis. Real-time quantitative polymerase chain reaction (*RT-PCR*) will be used to study LXR response gene activation in macrophages. The level of expression of LXRα, LXRβ, apoE, ABCA1, ABCG1 and LDL-R will be determined after myelin phagocytosis. This expression-level will be compared to the expression of the LXR response genes in control macrophages.

Aim II

Next, we will investigate 'in vitro' whether LXR activation by myelin influences the macrophage mediated release of inflammatory mediators. The release of NO, ROS and TNFα will be investigated in macrophages treated with myelin or the LXR agonist TO901317. With these experiments we will discover whether LXRs may be involved in the induction of a protective, anti-inflammatory phenotype in macrophages.

The results of this study will increase the knowledge about a possible protective role of macrophages after myelin phagocytosis. Furthermore, we will elucidate the mechanism by which myelin modulates the phenotype of macrophages. This study may eventually lead to a novel therapeutic approach for MS.

2 Materials and methods

2.1 Cell culture

Peritoneal macrophages were obtained from adult Wistar rats (Harlan, The Netherlands) by peritoneal lavage. After decapitation, the rats were intraperitoneally injected with 10 ml cold phosphate buffered saline (1X PBS, Lonza, Belgium) supplemented with 5 mM ethylenediamine tetraacetic acid (EDTA) (Lonza, Belgium). The peritoneal fluid was withdrawn and centrifuged (Sorvall® RT 600D meyvis analytical systems, USA) at 4°C for 10 minutes at 1400 rpm. The cell pellet was resuspended in RPMI-1640 (Gibko, UK) medium supplemented with 10 % fetal calf serum (FCS, Hyclone, Belgium) and 0,5 % penicilline/streptomycin (P/S) (Gibco, Belgium). Macrophages were counted in a hemocytometer (Marienfeld, Germany) and cell viability was determined by the tryphan blue (VWR, Belgium) exclusion method. Cells were divided over 6-, 24- and 96 well plates (Greiner bio-one cellstar®, Germany) at a density of 1.10^6 cells per ml. Cells were incubated for 2 hours at 37°C in an atmosphere of 5 % CO_2. Non-adherent cells were removed by washing with RPMI. Adherent monolayers of macrophages were maintained in RPMI medium supplemented with 10 % FCS and 0,5 % P/S at 37°C and 5 % CO_2. Macrophages were treated with either the LXR agonist T0901317 (T09, provided by Cayman Chemical Company, USA) or myelin, isolated out of rat brain according to Norton and Poduslo [47]. In addition macrophages were treated with the PPARγ antagonist GW9662 or the LXR antagonist 22(S)-hydroxycholesterol (Sigma, Belgium).

2.2 RNA isolation

Peritoneal rat macrophages were cultured in 6 well plates, containing $2{,}5.10^6$ cells per well in 2,5 ml RPMI supplemented with 10 % FCS and 0,5 % P/S. After 48 hours, total RNA was isolated, with the RNeasy mini kit (Qiagen, The Netherlands), after treatment of macrophages (24 and 48 hours) with either 50 µg myelin or 10 µM T09 in each well. In brief, cells were lysed with RLT lysisbuffer supplemented with 1 % β-mercaptoethanol (β-ME, Sigma, Belgium) and homogenized by using a Qiashredder column. After adding an equal volume of 70 % ethanol, RNA was extracted using the RNeasy column. Contaminants were removed by washing with RW1- and RPE buffer. Finally, RNA was eluted with 25 µl RNase free water and stored at -80°C. The purity of the isolated RNA was determined by running the RNA samples on a 1 % agarose gel. The presence of 2 bands, representing the 18S and 28S RNA, confirmed the purity of the isolated RNA. The RNA concentration was determined with a NanoDrop spectrophotometer (Isogen, The Netherlands).

2.3 cDNA synthesis

RNA was converted to cDNA by reverse transcriptase PCR, with the reverse transcription system (Promega, The Netherlands). The cDNA synthesis mix contained 4 µl $MgCl_2$ (25 mM), 2 µl RTase buffer (10x), 2 µl DNTP mixture (10 mM); 0,5 µl RNasin (20-40 U/µl; 2500U); 0,75 µl AMV RTase (20 U/µl) and 1 µl Oligo(dt) 15 primer. The RNA samples (750 ng) and nuclease free water were added to the cDNA synthesis mix to a final volume of 20 µl. Prior to reverse transcription, RNA was

denaturated for 10 minutes to prevent secondary structures. The reverse transcription reaction was performed on 42°C for 60 minutes, 95°C for 5 minutes and 4°C for 5 minutes, using the iCYCLER (biorad, USA).

2.4 Real-time PCR

RT-PCR analysis was performed using the primers shown in the supplemental information section. Real time quantitative PCR was conducted on a 7500 fast detection system (Applied biosystems). The PCR reaction consisted of fast SYBR green master mix (Applied biosystems, USA), 10 µM of forward and reverse primers, RNase free water and 12,5 ng template cDNA in a total reaction volume of 10 µl. Normalization of real-time quantitative RT-PCR data was accomplished by determination of the most stable reference genes, as previously described [48]. Normfinder and Genorm software were used to compare multiple internal control genes as a prerequisite for accurate RT-PCR expression profiling. The average expression stability of Glyceraldehyde 3-phosphate dehydrogenase (GAPDH), phosphoglycerate kinase (PGK1), hydroxymethylbilane synthase (HMBS), ribosomal protein S9, tata box binding protein (TBP), beta-actin, cyclophilin D, Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein zeta polypeptide (YWHAZ) and 18S ribosomal RNA was determined. Both 18S and PGK1 are the most stable reference genes for the determination of LXR response gene expression in macrophages after T09 and myelin treatment.

Relative quantitation of gene expression was accomplished by using the comparative C_t method ($\Delta\Delta C_t$ method). In this method, the amount of target, normalized to an endogenous reference and relative to a calibrator is given by: $2^{-\Delta\Delta Ct}$. First, the mean and standard deviation values of the replicate sample results were calculated. Secondly, the ΔC_T values were determined by using the following formula: $\Delta C_T = C_{T\ target} - C_{T\ reference}$. The reference samples are the untreated control macrophages. Thirdly, the standard deviation of the ΔC_T was calculated from the standard deviations of the target and reference values using the formula: $s = (s_1^{\ 2} + s_2^{\ 2})^{1/2}$. Fourthly, the $\Delta\Delta\ C_T$ value was calculated by: $\Delta\Delta\ C_T = \Delta\ C_T$ test sample - $\Delta\ C_T$ calibrator sample. The calibrator samples are the reference genes. Next, the standard deviation of the $\Delta\Delta\ C_T$ value was determined. The calculation of $\Delta\Delta\ C_T$ involves subtraction of the $\Delta\ C_T$ calibrator value. This is subtraction of an arbitrary constant, so the standard deviation of the $\Delta\Delta\ C_T$ value is the same as the standard deviation of the $\Delta\ C_T$ value. Finally, the standard deviations of the $\Delta\Delta\ C_T$ values were incorporated into fold-differences. Fold-differences are expressed as a range, which is a result of incorporating the standard deviation of the $\Delta\Delta\ C_T$ value into the fold-difference calculation. The range for target, relative to a calibrator sample, is calculated by: $2^{-\ \Delta\Delta\ CT}$ with $\Delta\Delta\ C_T + s$ and $\Delta\Delta\ C_T - s$, where s stands for the standard deviation of the $\Delta\Delta\ C_T$ value.

2.5 Dihydrorhodamine assay

Peritoneal rat macrophages were incubated with either 50 µg myelin or 10 µM T09 for 3, 24, 48 and 72 hours. Furthermore, cells were treated with 10 µM 22(S)-hydroxycholesterol or 10 µM GW9662, 2 hours prior to myelin treatment (48 hours). Intracellular ROS production was determined using the dihydrorhodamine (DHR) assay. In short, macrophages were stimulated with

phorbol 12-myristate 13-acetate (PMA) (100 ng/ml) for 15 minutes at 37°C and 5 % CO_2. PMA was washed away and 10 µM DHR (Sigma, USA) was added to the cell cultures and incubated for 15 minutes at 37°C and 5 % CO_2. The cells were washed with PBS and captured with PBS + 5 mM EDTA. The captured cells were placed on ice, centrifuged (2000 rpm, 3 minutes) and resuspended in fluorescent activated cell sorting (FACS) buffer, containing 1x PBS, 2 % FCS and sodium Azide. The amount of fluorescent rhodamine, formed out of DHR after contact with oxygen radicals, is measured, in the FL2 channel, by FACS analysis using the FACSCalibur (Becton Dickinson, Belgium) and is used as an indicator of intracellular ROS production.

2.6 Nitric Oxide assay

Macrophages, cultured in a 96 well plate, were incubated with either myelin (25, 50 µg or 100 µg) or T09 (1, 10 or 20 µM) for 24 hours. Furthermore, macrophages were treated with 10 µM 22(S)-hydroxycholesterol or 10 µM GW9662, 2 hours prior to myelin (100 µg) treatment for 24 hours. In addition, cells were treated with 10 µM 22(S)-hydroxycholesterol, 10 µM chloroform, 10 µM GW9662 or 10 µM DMSO for 24 hours. Next, cells were stimulated with 50 ng/ml LPS (Calbiochem®, USA) for 18 hours. Levels of the NO derivative nitrite were determined in the culture medium, using the Griess reagent system (Promega, USA). Briefly, 50 µl of macrophage supernatant was incubated with an equal volume of sulphanilamide solution for 10 minutes at room temperature protected from light. Next, samples were incubated with 50 µl N-1-napthylethylenediaminedihydrochloride (NED) under the same conditions. Optical densities were determined by using a microplate reader at 550 nm (Biorad Benchmark, Japan) and nitrite concentrations were determined by means of a nitrite standard reference curve.

2.7 MTT assay

Cell viability was determined by the Thiazolyl Blue Tetrazolium Bromide (MTT) reduction assay. Peritoneal macrophages, cultured in a 96 well plate, were incubated with 12,5 µl MTT (Sigma-Aldrich, Belgium) per 100 µl at 37°C and 5 % CO_2. After 4 hours, medium was removed and 150 µl DMSO (VWR, Belgium) and 25 µl glycine (Sigma-Aldrich, Belgium) were added to each well. Optical densities were determined using a microplate reader at 550 nm (Biorad Benchmark, Japan).

2.8 ELISA

Macrophages, cultured in a 96 well plate, were incubated with either myelin (25, 50 or 100 µg) or T09 (1, 10 or 20 µM) for 24 hours. Furthermore, macrophages were treated with 10 µM 22(S)-hydroxycholesterol or 10 µM GW9662, 2 hours prior to myelin (100 µg) treatment for 24 hours. In addition, cells were treated with 10 µM 22(S)-hydroxycholesterol, 10 µM chloroform, 10 µM GW9662 or 10 µM DMSO for 24 hours. After stimulation with 50 ng/ml LPS for 18 hours, TNFα levels in cell culture supernatants were determined by enzyme linked immunosorbent assay (ELISA) using the eBiosience kit (Belgium). ELISA plates (96 wells) were coated overnight with 100 µl/well of capture antibody in coating buffer (1/250). After washing with wash buffer (1X PBS/0,05% tween-20), plates were blocked with 200 µl/well 1 X assay diluent at room temperature for 1 hour. Next, 100 µl/ well supernatant (1/30) was added and incubated for 1 hour at room temperature. After adding 100 µl/well detection antibody biotin-conjugate anti-rat TNFα diluted in 1 X assay

diluent (1/250), plates were incubated for 1 hour at room temperature. Next, 100 µl/well of avidine/HRP diluted in 1 X assay diluent (1/250) was added and incubated at room temperature for 30 minutes. Incubation with 100 µl/well substrate solution (1 X tetramethylbenzidine solution) for 15 minutes was followed by adding 50 µl/well stop solution (2N H_2SO_4). Optical densities were determined using a microplate reader at 450nm (Biorad Benchmark, Japan). Cytokine concentrations were determined from standards containing known concentrations of the cytokine.

2.9 Myelin phagocytosis assay

Cells were incubated with 10 µM T09 for 3, 24, 48 and 72 hours. Myelin uptake after T09 treatment was determined using the myelin phagocytosis assay. In short, peritoneal macrophages were incubated with 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate (DiI) labelled rat myelin (25 µg) for 90 minutes at 37°C and 5 % CO_2. Next, cells were washed with PBS and captured with PBS + 5 mM EDTA. The captured cells were placed on ice, centrifuged (2000 rpm, 3 minutes) and resuspended in FACS buffer, containing 1x PBS, 2 % FCS and sodium Azide. Myelin phagocytosis was determined by measuring the intracellular fluorescent intensity, in the FL2 channel, by means of FACS analysis using the FACSCalibur (Becton Dickinson, Belgium).

2.10 Statistics

Results were statistically analyzed with Graph Pad Instant and are expressed as mean values +/- standard error (SEM) for N experiments. When two conditions were compared on significant differences, the unpaired student T test was applied. When tree or more conditions were compared, an analysis of variances (ANOVA) was used. P- values < 0,05 were considered significant. *P<0,05, **P<0,01 and ***P<0,001.

3 Results

The goal of this study was to determine the role of LXRs in the macrophage response after myelin phagocytosis. It was hypothesized that LXRs are activated after myelin phagocytosis and induce a protective, anti-inflammatory phenotype in macrophages. To determine whether LXRs are activated after myelin phagocytosis, RT-PCR was used to study the expression of several LXR response genes after myelin phagocytosis in rat peritoneal macrophages. To determine whether LXR activation induces a protective phenotype in macrophages, inflammatory mediator release after LXR activation was investigated. For this, ROS, NO and cytokine release was determined in macrophages after treatment with either the LXR agonist T09 or myelin.

3.1 LXRs are activated after myelin phagocytosis

To determine whether LXRs are activated after myelin phagocytosis, RT-PCT was used to investigate the effect of myelin treatment on LXR response gene expression in macrophages. First, we determined the stability of different reference genes in macrophages after T09 and myelin treatment. It was demonstrated that both 18S and pgk1 were the most stable reference genes (figure 3). Subsequently, treatment of macrophages with 10 µM T09 for 24 hours significantly increased the expression levels of ABCA1 (figure 4C) and LDL-R (figure 4D), compared to untreated control macrophages. Additionally, incubation of macrophages for 48 hours with 10 µM T09 significantly increased the expression levels of LXRα, apoE, ABCA1, LDL-R and ABCG1 (figure 4A-E) compared to untreated control macrophages. Treatment of macrophages with 50 µg myelin significantly increased the expression of LXRα, apoE, ABCA1 and LDL-R after both 24 and 48 hours. These results indicate that LXRs are most likely activated after myelin phagocytosis and could therefore play a role in the macrophage response after myelin phagocytosis.

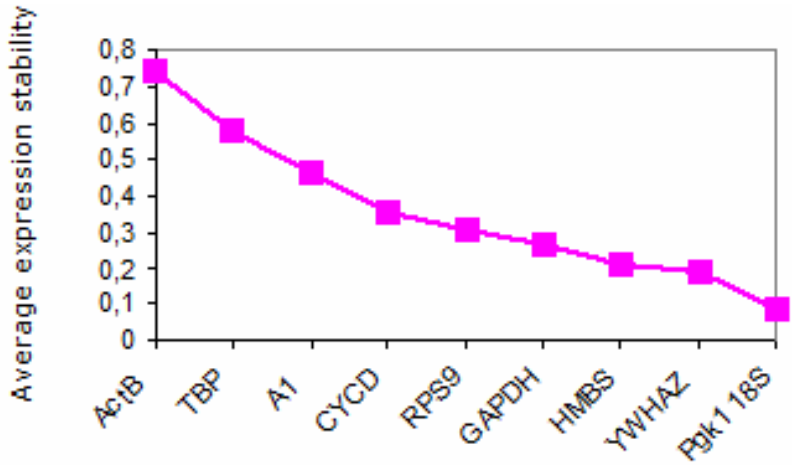

Figure 3: Normalization of RT-PCR data. Genorm software was used to compare multiple internal control genes as a prerequisite for accurate RT-PCR expression profiling. Left: least stable genes, right: most stable genes. ActB: beta actin, TBP: tata box binding protein, A1: ABCA1, CYCD: cyclophilin D, RPS9: ribosomal protein S9, GAPDH: glyceraldehype 3 phosphate dehydrogenase, HMBS: hydroxymethylbiliane synthase, Pgk1: phosphoglycerate kinase.

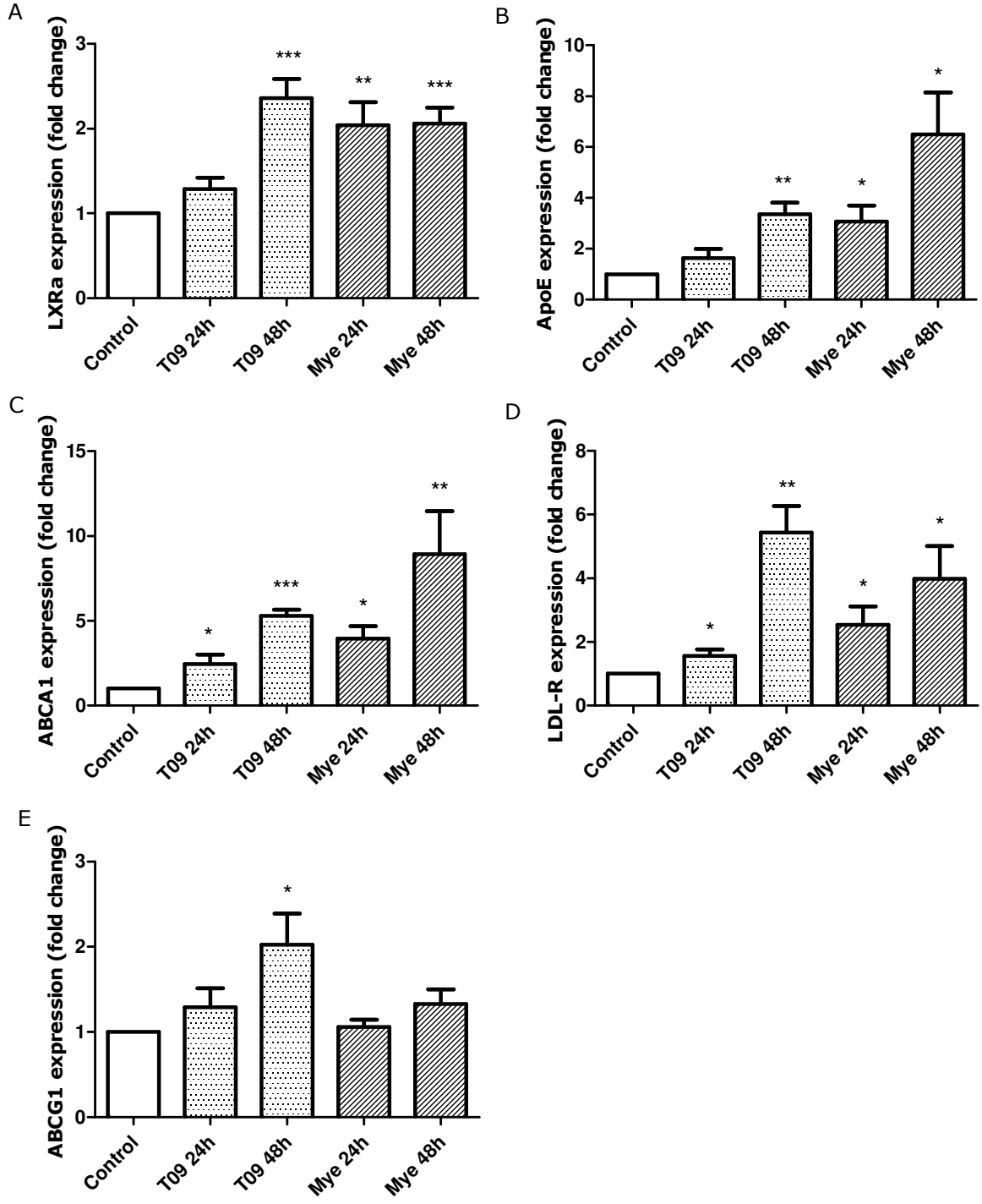

Figure 4: LXR response gene expression in macrophages. Peritoneal rat macrophages were incubated with 10 µM T09 or 50 µg myelin for 24 and 48 hours. The expression of LXRα (A), apoE (B), ABCA1 (C), LDL-R (D) and ABCG1 (E) was determined with RT-PCR and compared with their expression in untreated control macrophages. Values represent the fold change compared to untreated control macrophages and values were normalized to the internal control genes pgk1 and 18S. Experiments were performed in duplicate and data are representative for 4 separate experiments (N=4). Error bars represent standard error of the mean (SEM). Data were statistically analyzed by means of the student t test. *p<0,05; **p<0,01 and ***p<0,001 versus untreated control macrophages. T09: T0901317, mye: myelin.

3.2 Myelin phagocytosis and LXR activation increase ROS production

To determine the effect of myelin phagocytosis and LXR activation on ROS production, macrophages were incubated with myelin or T09 for various periods of time. Results demonstrated that both 50 µg myelin and 10 µM T09 induced a time-dependent increase in ROS production by macrophages, compared to PMA-stimulated control macrophages. Moreover, this increase in ROS production became significant after a minimal incubation-period of 48 hours (figure 5A-B).

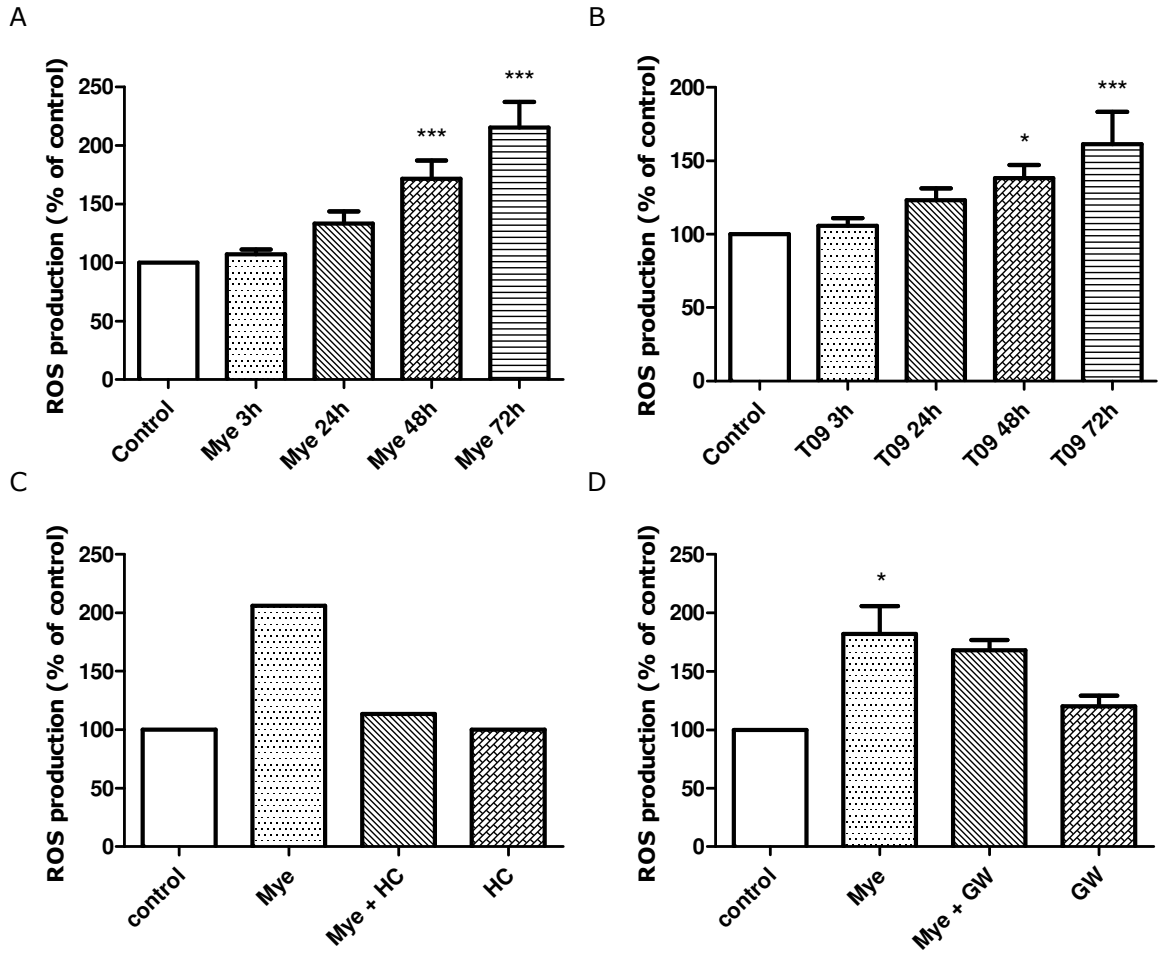

Figure 5: Effect of myelin phagocytosis, LXR activation and PPARγ activation on ROS production by macrophages after PMA stimulation. Peritoneal rat macrophages were incubated with 50 µg myelin (N=4) (A) or 10 µM T09 (N=4) (B) for 3, 24, 48 and 72 hours. Furthermore, macrophages were treated, for 48 hours, with 50 µg myelin, 10 µM 22(S)-hydroxycholesterol (HC) or a combination of both (N=1) (C). Finally, macrophages were treated, for 48 hours, with 50 µg myelin, 10 µM GW9662 or a combination of both (N=2) (D). Intracellular ROS production was determined and results are expressed as percentages of ROS production by PMA-stimulated control macrophages. Experiments were performed in duplicate. Error bars represent SEM. Data were statistically analyzed by one way ANOVA (Dunnet test). *P<0,05; **P<0,01 and ***p<0,001 versus untreated control macrophages. ROS: reactive oxygen species, mye: myelin, T09: T0901317.

To further investigate the effects of LXR activation on ROS production, macrophages were incubated with myelin, the LXR antagonist 22(S)-hydroxycholesterol or a combination of both. Interestingly, a 2 hour pre-incubation with 10 µM 22(S)-hydroxycholesterol reversed the myelin-

induced increase in ROS production. Incubation with 10 µM 22(S)-hydroxycholesterol alone had no effect on ROS production by macrophages (figure 5C).

The PPARγ is another nuclear receptor, present in macrophages, activated by oxidized fatty acids. Since myelin also consists of fatty acids besides cholesterol, the PPARγ receptor could also play a role in the macrophage response after myelin phagocytosis. To determine whether PPARγ activation is involved in the myelin-mediated increase in ROS production by macrophages, ROS production was determined after inhibition of PPARγ signalling. Macrophages were treated with myelin, the PPARγ antagonist GW9662 or a combination of both. As mentioned earlier, incubation of macrophages with 50 µg myelin, for 48 hours, significantly increased ROS production, which could be abolished by the addition of 10 µM 22(S)-hydroxycholesterol. Nevertheless, incubation of macrophages with GW9662, 2 hours prior to myelin incubation, had no significant effect on the myelin-induced increase in ROS production. Treatment of macrophages with 10 µM GW9662 alone had no significant effect on ROS production (figure 5D). The results presented above give strong evidence for the fact that the myelin-induced increase in ROS production is independent on PPARγ signalling and dependent on the activation of the LXR-pathway. However, other pathways may be involved in the myelin-mediated increase in ROS production by macrophages.

3.3 Myelin phagocytosis and LXR activation decrease NO production

To determine the effect of myelin phagocytosis and LXR activation on macrophage-mediated NO production, nitrite concentrations were determined in supernatants of macrophage cultures supplemented with various concentrations of myelin or T09. LPS induced a significant increase in nitrite production in macrophages (data not shown). Interestingly, myelin significantly decreased this LPS-induced NO production at concentrations of 25, 50 and 100 µg (figure 6A). Similarly, incubation of macrophages with the LXR agonist T09, for 24 hours, decreased the LPS-induced NO production in a concentration-dependent manner (figure 6C). To evaluate whether cell viability influenced these observed changes in NO production after the different treatment conditions, macrophage viability was determined. T09 treatment had no significant effect on macrophage viability (figure 6D). In contrast, incubation of macrophages with 50 µg or 100 µg myelin significantly increased macrophage viability (figure 6B).

Next, we investigated whether LXR inhibition had a neutralizing effect on the myelin-reduced NO production. For this, macrophages were incubated with either myelin, 22(S)-hydroxycholesterol or a combination of both. Here, we demonstrated that a 2 hour pre-incubation with 10 µM 22(S)-hydroxycholesterol slightly reversed the myelin-induced decrease in NO production. However, this effect was not significant. Incubation with 10 µM 22(S)-hydroxycholesterol or its solvent chloroform, had no significant effect on NO production by these macrophages (figure 6E). None of the above described treatment conditions had a significant effect on macrophage viability (figure 6F).

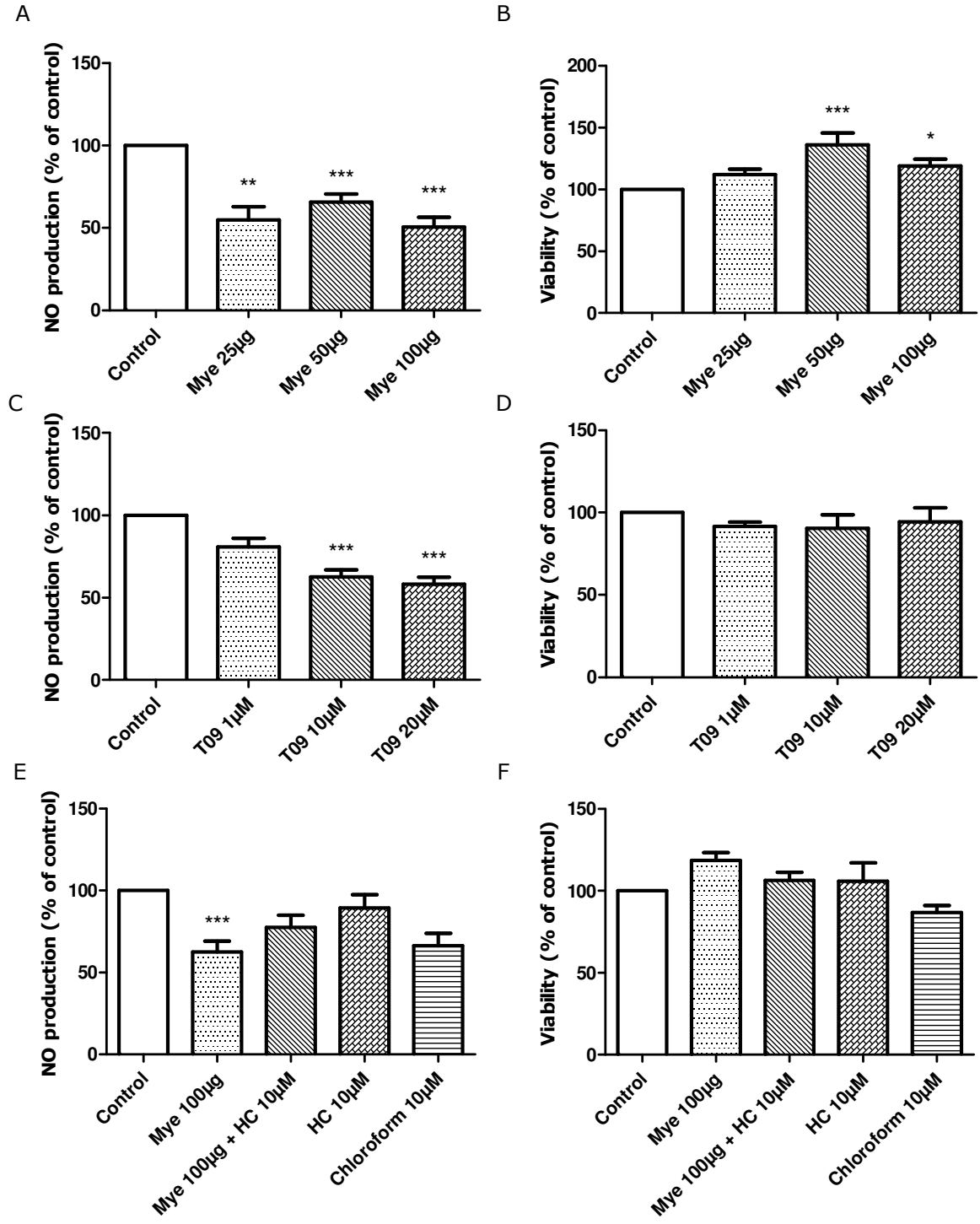

Figure 6: The effect of myelin phagocytosis and LXR activation on NO production and viability of macrophages. Macrophages were incubated with 25, 50 or 100 µg myelin (N=10) (A) or with 1, 10 or 20 µM T09 (N=10) (C). In addition, macrophages were incubated with 100 µg myelin, myelin + 22(S)-hydroxycholesterol, 10 µM 22(S)-hydroxycholesterol or its solvent chloroform (N=8) (E). After 18 hours of LPS stimulation, NO production was determined by the Griess reagent assay. Macrophage viability was determined by using a MTT assay (B,D,F). Results are expressed as percentages of NO production by LPS-stimulated control macrophages and error bars represent SEM. Experiments were performed in triplo and data were statistically analyzed by means of one way ANOVA (Dunnett and Tukey test). *p<0,05; **p<0,01 and ***p<0,001 compared to LPS-stimulated control macrophages. NO: nitric oxide, T09: T0901317, mye: myelin, HC: 22(S)-hydroxycholesterol.

Next, we investigated whether PPARγ activation plays a role in the myelin-dependent decrease in NO production by macrophages. Pre-incubation (2 hours) with 10 µM GW9662 did not significantly affect the myelin-induced decrease in NO production. Treatment of macrophages with 10 µM GW9662 or its solvent DMSO alone had no significant effect on NO production compared to LPS-treated control macrophages (figure 7A). Moreover, cell viability was not affected by the divergent treatment conditions (figure 7B).

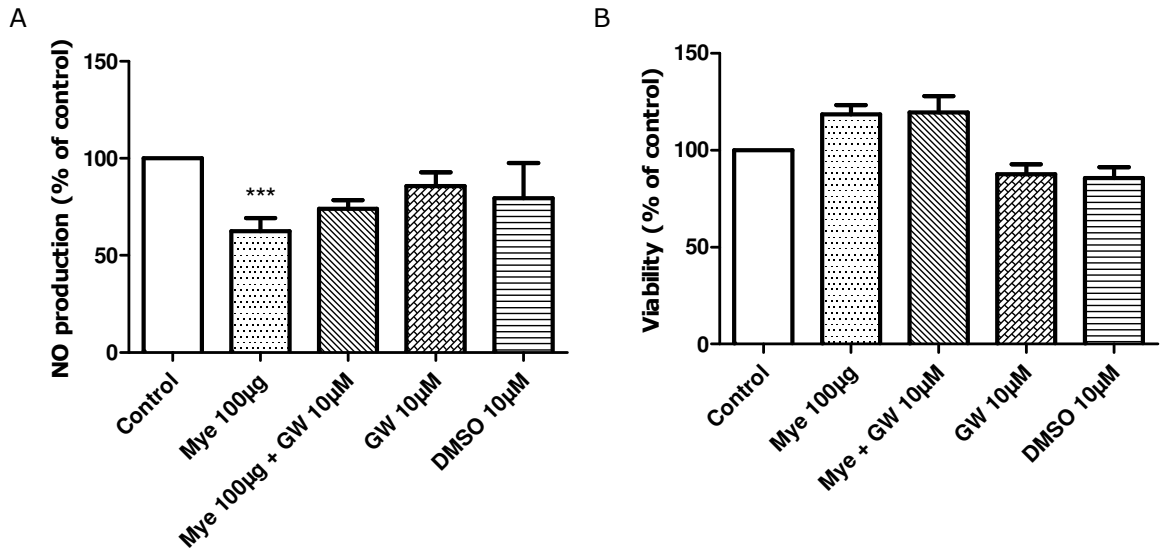

Figure 7: The effect of myelin phagocytosis and PPARγ inhibition on NO production and viability of macrophages. Peritoneal rat macrophages were incubated with 100 µg myelin, 10 µM GW9662 or a combination of both. In addition, macrophages were incubated with 10 µM DMSO, the solvent of GW9662 (A). After 18 hours of LPS stimulation, NO production was determined by the Griess reagent assay and cell viability was assessed by using the MTT assay (B). Results are expressed as percentages of NO production by LPS-stimulated control macrophages. Error bars represent SEM. Results are derived from 8 different experiments (N=8) performed in triplo. Data were statistically analyzed by means of one way ANOVA (Tukey test and Dunnet test). ***p<0,001 compared to LPS-stimulated control macrophages. NO: nitric oxide, mye: myelin, GW: GW9662, DMSO: dimethylsulfoxide.

To determine whether the myelin-induced decrease in NO production also persists at later time periods, macrophages were treated with 50 µg myelin or 10 µM T09 for various periods of time. Myelin demonstrated an inhibitory effect on NO production during the first 24 hours, compared to LPS-stimulated control macrophages. Nevertheless, at later time-points, myelin stimulated NO production. Interestingly, similar to myelin-treated macrophages, T09 demonstrated an inhibitory effect on NO production during the first 24 hours. At later time points, T09 had no significant effect on NO production (figure 8A-B). As demonstrated, T09 had no significant effect on macrophage viability (figure 8D). In contrast, incubation of macrophages with myelin for 48 hours increased macrophage cell viability (figure 8C). The findings reported above suggest that, like the increased ROS production, the myelin-mediated decrease in NO production seem to depend on LXR activation and not on PPARγ signalling. However, other mechanisms may be involved in the myelin-mediated decrease in NO production by macrophages. Furthermore, the myelin induced decrease in NO production disappears at later time periods.

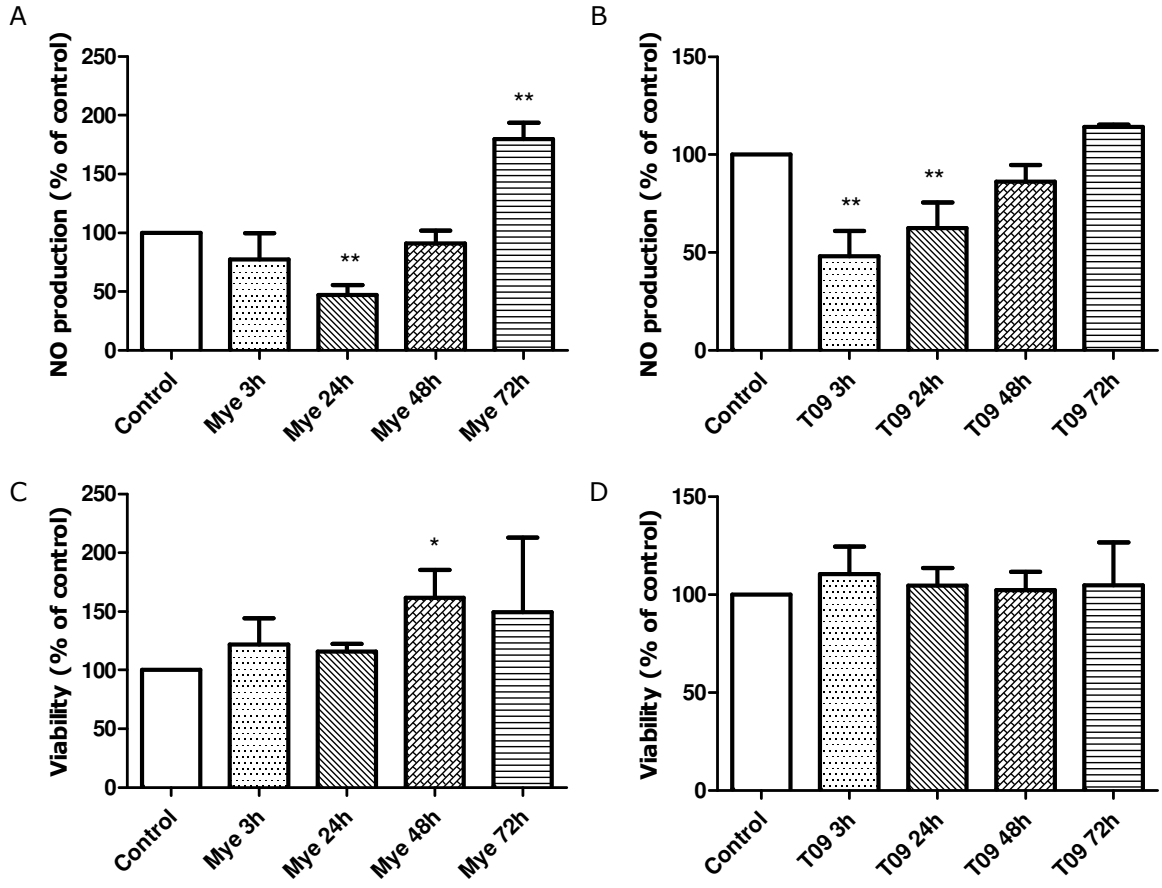

Figure 8: The effect of myelin phagocytosis and LXR activation on NO production by macrophages. Peritoneal macrophages were incubated with 50 µg myelin (A) or 10 µM T09 (B) for 3, 24, 48 and 72 hours. After 18 hours of LPS stimulation, NO production was determined by the Griess reagent assay and compared to the NO production of LPS-stimulated control macrophages. Cell viability was determined by using a MTT assay (C + D). Results are expressed as percentage of NO production by LPS-stimulated control macrophages. Errors bars represent SEM. Results are derived from 5 experiments (N=5) and performed in triplo. Data were statistically analyzed by means of one way ANOVA (Dunnett test). *p<0,05 and **p<0,01 compared to LPS-stimulated control macrophages.

3.4 Myelin phagocytosis decreases TNFα production

In the previous sections, it was demonstrated that differences in NO and ROS production by macrophages following myelin phagocytosis might be mediated by activation of the LXR-signalling pathway. To further address myelin-induced secretional alterations in macrophages, TNFα production was investigated. Macrophages were treated with various concentrations of myelin or T09, after which they were activated with LPS. Stimulation of macrophages with LPS significantly induced TNFα production (data not shown). Incubation of macrophages with myelin for 24 hours significantly decreased this LPS–induced TNFα production in a dose-dependent manner (figure 9A). In contrast, incubation of macrophages with T09 had a dual effect on TNFα production. T09 (1 µM) significantly decreased TNFα production compared to LPS-treated controls. 10 µM T09 had no significant effect on TNFα production and 20 µM T09 significantly increased TNFα production (figure

9B). Both myelin and T09 were not toxic to cells at the concentrations examined as demonstrated by a MTT assay (figure 9C+D).

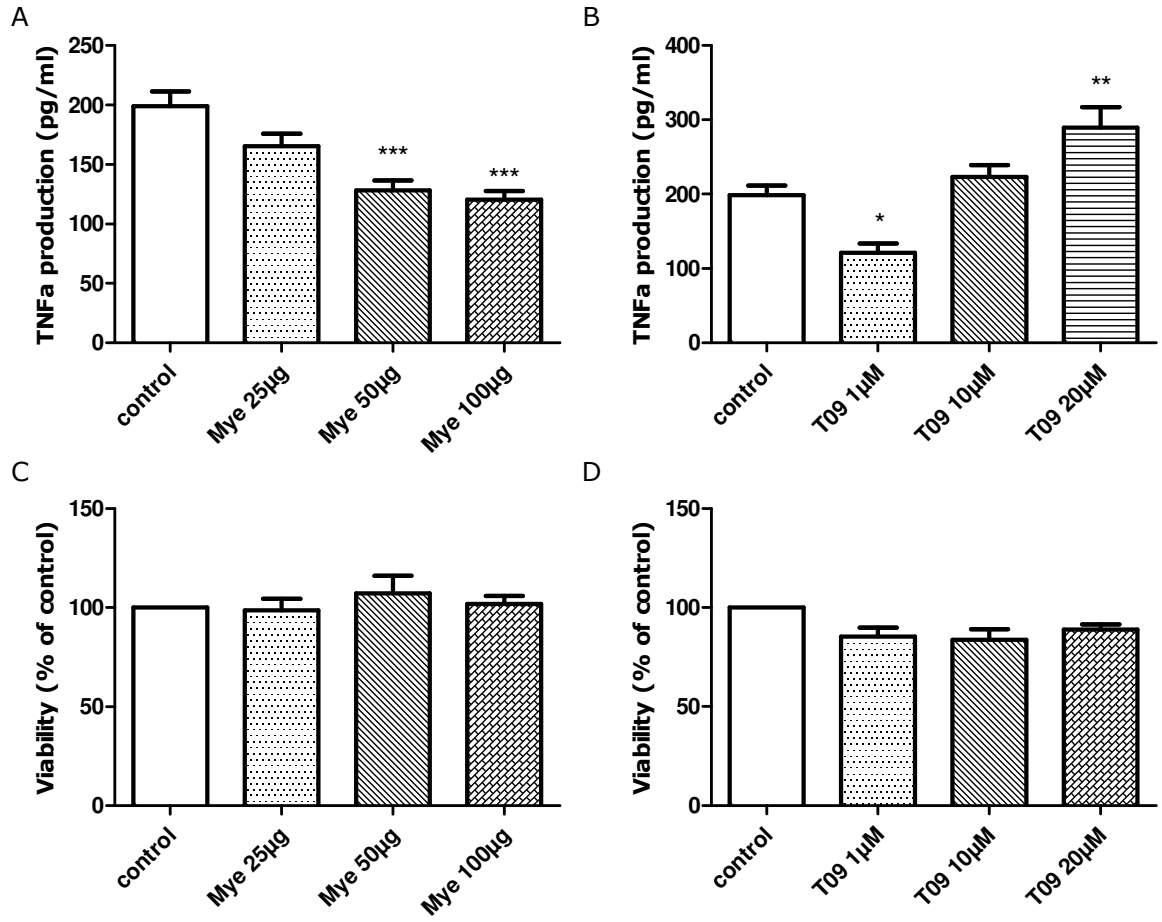

Figure 9: The effect of myelin phagocytosis and LXR activation on TNFα release by macrophages. Peritoneal rat macrophages were incubated with 25, 50 or 100 µg myelin for 24 hours (A). Furthermore, macrophages were treated with 1, 10 or 20 T09 for 24 hours (B). After 18 hours LPS stimulation, TNFα production was determined by ELISA and compared to the TNFα production of LPS-stimulated control macrophages. Results are expressed as mean TNFα production +/- SEM. Cell viability of the same peritoneal macrophages was determined by using a MTT assay (C + D). Results are expressed as percentage of viability of LPS-stimulated control macrophages. Results are derived from 6 different experiments (N=6) performed in triplo. Data were statistically analyzed by means of one way ANOVA (Dunnett test). *p<0,05; p<0,01 and ***p<0,001 compared to LPS-stimulated control macrophages.

To confirm that the observed myelin-mediated decrease in TNFα production was indeed not mediated via LXR signalling, macrophages were treated with the LXR antagonist 22(S)-hydroxycholesterol 2 hours prior to myelin treatment. Pre-incubation with 10 µM 22(S)-hydroxycholesterol did not significantly reverse the myelin-mediated decrease in TNFα production (figure 10A). Additionally, none of the above described treatment conditions had a significant effect on macrophage viability (figure 10B).

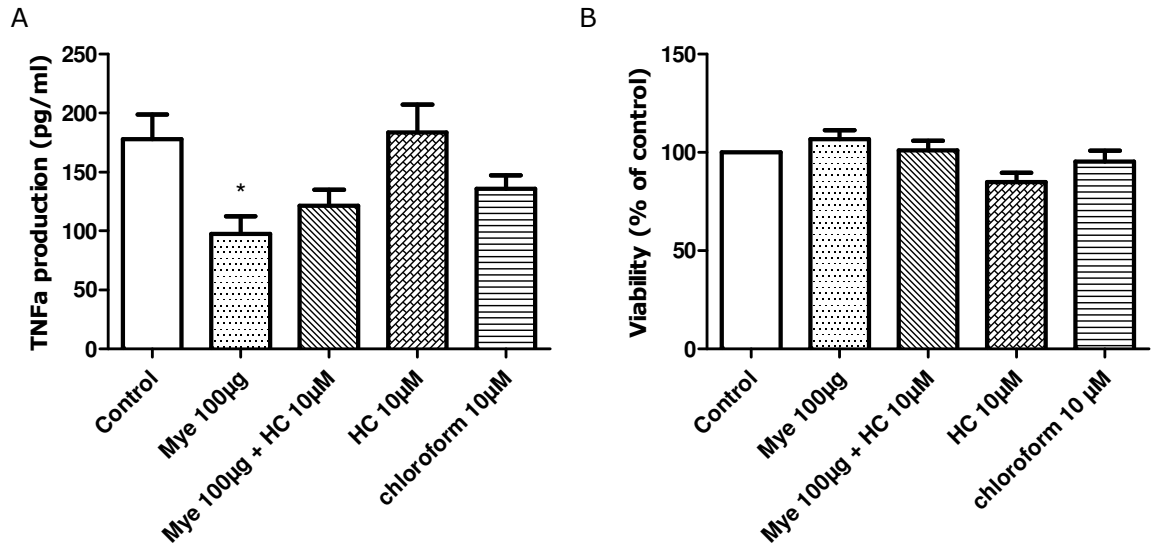

Figure 10: The effect of myelin phagocytosis and LXR inhibition on TNFα production and viability of peritoneal macrophages. Macrophages were incubated with 100 µg myelin, myelin + 22(S)-hydroxycholesterol, 10 µM 22(S)-hydroxycholesterol or its solvent chloroform. After 18 hours LPS stimulation, TNFα production was determined by ELISA. Results are expressed as mean TNFα production and error barrs represent SEM (A). Cell viability was determined by a MTT assay. Results are expressed as percentages of viability of LPS-stimulated control macrophages. Results were derived from 7 different experiments (N=7) performed in triplo. Data were statistically analyzed by means of one way ANOVA (Dunnet and Tukey test). *p<0,05 compared to LPS-stimulated control macrophages. Mye: myelin, HC: 22(S)-hydroxycholesterol.

To determine whether PPARγ plays a role in the myelin-mediated decrease in TNFα production, macrophages were treated with the PPARγ antagonist GW9662, 2 hours prior to myelin treatment. Pre-treatment with 10 µM GW9662 had no effect on the myelin-induced decrease in TNFα production (figure 11A). Additionally, myelin, GW9662 and DMSO had no significant effects on macrophage viability (Figure 11B).

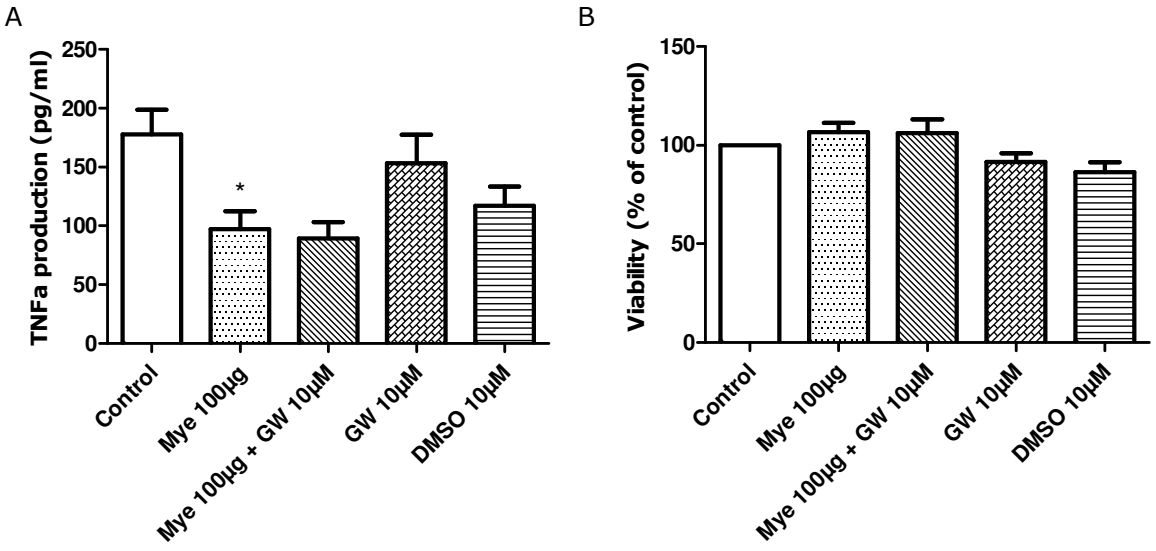

Figure 11: The effect of myelin phagocytosis and PPARγ activation on TNFα production and viability of peritoneal macrophages. Macrophages were incubated with 100 µg myelin, 100 µg myelin + 10 µM GW9662, 10

μM GW9662 or 10 μM DMSO. After 18 hours LPS stimulation, TNFα production was determined by ELISA. Results are expressed as mean TNFα production and error bars represent SEM (A). Cell viability was determined by using a MTT assay. Results are expressed as percentages of viability of LPS-stimulated control macrophages. Results are derived from 7 different experiments (N=7) performed in triplo. Data were statistically analyzed by means of one way ANOVA (Dunnet and Tukey test). *p<0,05 compared to LPS-stimulated control macrophages. Mye: myelin, GW: GW9662.

To determine whether the myelin-induced decrease in TNFα production also persists at later time periods, macrophages were treated with 50 μg myelin or 10 μM T09 for various periods of time. Myelin demonstrated an inhibitory effect on TNFα production during the first 24 hours. Interestingly, similar to myelin-treated macrophages, T09 demonstrated an inhibitory effect on NO production during the first 3 hours. At later time points, T09 increased TNFα production, which is significant after 48 hours (figure 12A). Both T09 and myelin have no significant effects on macrophage cell viability (figure 12B). The results described above, indicate that myelin phagocytosis induces a short-term protective phenotype in macrophages by decreasing TNFα production. However, this effect seems to be independent of LXR and PPARγ signalling.

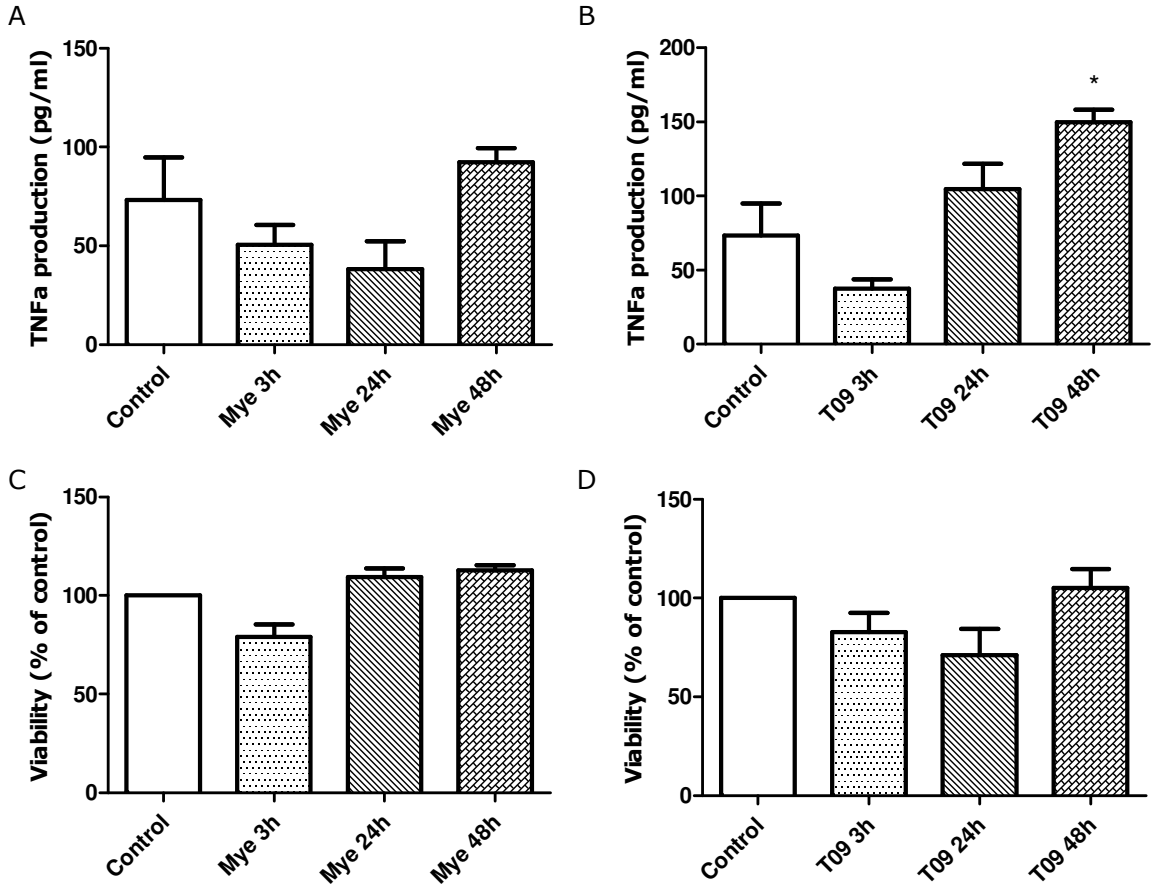

Figure 12: The effect of myelin phagocytosis and LXR activation on TNFα production. Macrophages were incubated with 50 μg myelin (A) or 10 μM T09 (B) for 3, 24 and 48 hours. After 18 hours LPS stimulation, TNFα production was determined by ELISA. Results are expressed as mean TNFα production +/- SEM. Cell viability was determined by a MTT assay (C-D). Results are expressed as percentages of control. Results were derived from 1 experiment (N=1) performed in triplo. Data were statistically analyzed by means of one way ANOVA (Dunnett test). *p<0,05 compared to LPS-stimulated control macrophages.

3.5 LXR activation increases myelin phagocytosis by macrophages

To find out whether there is an influence of LXR stimulation on the phagocytic capacity of macrophages, a myelin phagocytosis assay was performed. Macrophages were treated with 10 µm T09 for divergent periods of time. T09 treatment, for 72 hours, significantly increased myelin phagocytosis by macrophages compared to untreated control macrophages (figure 13). This result indicates that there is a positive feedback loop between myelin phagocytosis and LXR activation.

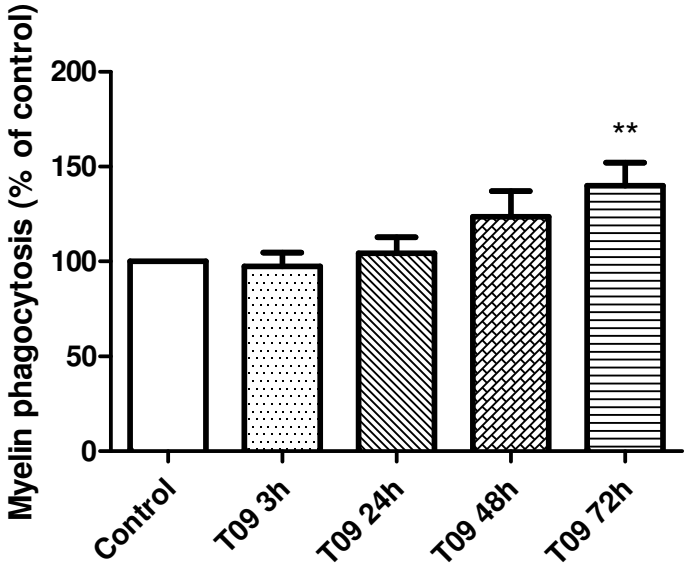

Figure 13: myelin phagocytosis by macrophages after LXR activation. Peritoneal rat macrophages were treated with 10 µM T09 for 3, 24, 48 and 72 hours. After 90 minutes incubation with DiI labelled myelin, myelin phagocytosis was determined by FACS analysis. Results are expressed as percentages of myelin phagocytosis by untreated control macrophages. Error bars represent SEM. Results are derived from 4 different experiments (N=4) performed in duplo. Data were statistically analyzed by means of one way ANOVA (Dunnett test). *p<0,05 compared to untreated control macrophages.

4 Discussion

In this study, we hypothesized that LXRs are activated after myelin phagocytosis and induce a protective, anti-inflammatory phenotype in macrophages. Previous research demonstrated that LXRs are present in macrophages. The goal of this study was to determine the role of these LXRs in the macrophage response after myelin phagocytosis.

First, it was investigated whether LXRs are activated after myelin phagocytosis by macrophages. Several studies have already demonstrated the presence of LXRs in macrophages [35]. In these studies, the LXR agonist T0901317 was able to induce the expression of several genes, like LXRα, apoE, ABCA1, ABCG1 and LDL-R [32, 33]. Surprisingly, it is still not known whether LXRs are also activated after myelin phagocytosis, despite the fact that myelin contains large amounts of cholesterol and cholesterol metabolites are known to be potent LXR ligands. We demonstrated that both the LXR agonist T09 and myelin increased the expression of LXRα, apoE, ABCA1 and LDL-R in a time-dependent manner. These results indicate that LXRs are most likely activated after myelin phagocytosis, suggesting that LXRs could be involved in the macrophage response after myelin phagocytosis. Myelin phagocytosis induces the expression of several LXR response genes involved in cholesterol efflux, like ABCA1 and apoE. After efflux from the macrophage interior to the periphery, cholesterol could be transported to other cells, like neurons and oligodendroglia. Both cell types need cholesterol for their functioning. For example, oligodendroglia need it to structure the myelin in their membranes [49]. In this way, the upregulation of cholesterol transporters and acceptors in macrophages by myelin phagocytosis may thus be beneficial. The PPARγ is another nuclear receptor, present in macrophages, activated by oxidized fatty acids. Moreover, it has been described to be involved in anti-inflammatory responses, as it represses the upregulation of inflammatory genes in macrophages. For example, PPARγ ligands were shown to inhibit TNFα, IL-6, iNOS, MMP-9 and IL-1β expression in macrophages [44] . Since myelin also consists of fatty acids besides cholesterol, the PPARγ receptor may also play a role in the macrophage response after myelin phagocytosis.

Next, we investigated whether LXR activation by myelin induces a protective, anti-inflammatory phenotype in macrophages. In MS and EAE, macrophages were shown to infiltrate the CNS. These infiltrated macrophages may contribute to axonal damage by their production of pro-inflammatory mediators. Macrophages in EAE lesions were shown to produce ROS, NO and pro-inflammatory cytokines, like TNFα [13]. First, we determined the effect of myelin phagocytosis and LXR activation on ROS production by macrophages. Both myelin and the LXR agonist T09 significantly increased ROS production by macrophages over time. These results indicate that the increase in ROS production after myelin phagocytosis is most likely mediated via LXR signalling. However, other pathways may be involved in the myelin-induced increase in ROS production. Furthermore, the LXR antagonist 22(S)-hydroxycholesterol was able to completely reverse the myelin-induced increase in ROS production. This confirmed that the increase in ROS production after myelin phagocytosis is most likely mediated via LXR signalling. However, since results are derived from a single experiment, no statistical analysis could be performed. More experiments are needed to

confirm the involvement of LXR signalling in the myelin-induced increase in ROS production. On the other hand, the PPARγ antagonist GW9662 was not able to reverse the myelin-induced increase in ROS production, indicating that the myelin-induced increase in ROS production is probably not mediated via PPARγ signalling.

Both myelin phagocytosis and LXR activation increased ROS production by macrophages. However, the significance of ROS on the regulation of inflammation is still unclear. ROS produced by the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (NOX2) complex were for a long time considered as detrimental mediators of inflammation. During MS, ROS are released in high concentrations by the respiratory burst of macrophages. ROS can cause oxidative damage to proteins, lipids and nucleic acids resulting in cell death by apoptosis or necrosis. Furthermore, ROS are able to induce DNA damage leading to neurodegeneration and ROS have been implicated as mediators of demyelination and axonal damage [13]. In both MS and EAE, myelin phagocytosis has been shown to induce the generation of ROS [17]. In contrast, recent evidence suggests a possible anti-inflammatory role for ROS. The important role of ROS in inflammatory responses has led to the dogma that ROS promote inflammation and destroy the surrounding tissue and cells. However, recent evident suggests that ROS produced by the NOX2 complex are anti-inflammatory and prevent auto-immune responses. For example, mice with EAE suffer a more severe disease course when they carried a mutation in the neutrophil cytosolic factor 1 (Ncf1). A mutation in Ncf1 leads to a decreased ROS production, since Ncf1 encodes the p47-phagocyte oxidase (PHOX) subunit of the NOX2 complex. These findings suggest an immunoregulatory role for ROS [50]. This view correlates with previous observations that a genetic defect of p47-PHOX enhanced autoimmune encephalomyelitis and arthritis in animal models. Additional support for this hypothesis comes from a previous study showing that p47-PHOX-mediated ROS negatively regulates the expression of the pro-inflammatory cytokines IL-8 and IL-1β in human neutrophils. Furthermore, it was demonstrated that the generation of ROS via p47-PHOX after myelin phagocytosis induced a marked downregulation of microglial inflammatory responses [17]. Finally, inflammatory responses to antioxidants in vivo are not consistent and in some studies, antioxidants have even been proven to have pro-inflammatory effects [50]. Previous research demonstrated another protective role for ROS by suppressing T-cell function. Peroxynitrite is a product of a chemical reaction between NO and the superoxide anion. Peroxynitrite production could result in the nitration of the T-cell receptor and CD8 molecules. This process alters the specific peptide binding of T-cells and renders them unresponsive to antigen-specific stimulation [51]. The role of ROS is very complex and can be associated with both pro- and anti-inflammatory responses. This means that the myelin-mediate increase in ROS production via LXR signalling could have both pro- and anti-inflammatory effects.

Besides their important role in inflammation, ROS have been demonstrated to play a crucial role in the process of myelin phagocytosis. Blocking ROS production with NADPH oxidase inhibitors prevented the phagocytosis of myelin. Furthermore, scavenging ROS with catalase or mannitol decreased the phagocytosis of myelin by macrophages. In addition, Lipoic acid (LA), a non-specific scavenger of ROS, also decreased the phagocytosis of myelin by macrophages [52]. This indicates that ROS play a regulatory role in the phagocytosis of myelin and are required for the phagocytosis of myelin by macrophages.

In addition to ROS, activated macrophages secrete large amounts NO, which can aggravate the disease course of MS. NO has multiple detrimental effects like enhancing demyelination, axonal degeneration and neuronal apoptosis [13]. LXR ligands have been shown to inhibit the expression of iNOS in macrophages in response to bacterial infection or LPS stimulation [53]. We demonstrated that both myelin and T09 decreased NO production by macrophages. These results suggest that the myelin-induced decrease in NO production by macrophages is most likely mediated via LXR signalling. However, other pathways may be involved in the myelin-induced decrease in NO production by macrophages. The decrease in NO production detected in macrophages, mediated by LXR activation and myelin phagocytosis, probably occurs via down-regulation of the iNOS gene. To confirm the underlying mechanism, additional experiments like RT-PCR are indicated. The observed decrease in macrophage-mediated NO production, after treatment with T09 and myelin, could be a consequence of decreased macrophage viability after the different treatment conditions. To rule this out, a MTT assay was used to determine macrophage viability after the different treatment conditions. Both myelin and T09 did not decrease macrophage cell viability, indicating that the observed decrease in NO production is most likely mediated by T09 and myelin and not due to differences in macrophage viability. To confirm the involvement of LXR signalling in the myelin-mediated decrease of NO production, macrophages were treated with the LXR antagonist 22(S)-hydroxycholesterol prior to myelin treatment. The myelin mediated decrease in NO production could not completely be reversed by pre-incubating macrophages with 22(S)-hydroxycholesterol. This indicates that the observed effects after myelin treatment are not only mediated by LXRs and perhaps multiple signalling pathways lead to a reduced NO production following myelin phagocytosis. However, doubt arises in respect to the antagonistic capacity of 22(S)-hydroxycholesterol in cell culture experiments. For instance, it was shown that 22(S)-hydroxycholesterol binds both LXR subtypes with high affinity (150 nM for LXRα and 160 nM for LXRβ), but does not function as an antagonist in cells [54]. Additionally, 22(S)-hydroxycholesterol may never even reach its nuclear receptor target. In our experiments, this could be explained by intracellular myelin hindrance. It may even bind cellular proteins that prevent its localization in the nucleus, may be pumped out of the cells by means of a multidrug resistance related mechanism or may be rapidly metabolized in cell culture. In support of this latter hypothesis, it is shown that the unnatural stereoisomers of several oxysterols, like 22(S)-hydroxycholesterol, are much better substrates for esterification by acyl-coA than their LXR active counterparts. An alternative explanation could be a possible difference in binding potential of the LXR in vivo. In vivo, the LXR exists as part of a multimeric complex bound to the RXR, co-activators or corepressors and DNA [54]. In the future, it would be better to use LXR knock out animals in further experiments.

Next, we determined whether PPARγ plays a role in the myelin-induced decrease in NO production. The PPARγ antagonist GW9662 was not able to reverse the myelin-induced decrease in ROS production, indicating that the myelin-induced decrease in NO production is probably not mediated via PPARγ signalling.

The mechanism underlying the repression of inflammatory genes by LXRs is poorly understood. Transrepression is thought to be the primary mechanism by which LXRs inhibit the expression of pro-inflammatory genes in macrophages. This process is called transrepression because LXREs

have not been identified in the proximal promoters of the repressed genes, which indicates that inhibition does not depend on the binding of heterodimers to LXREs in target gene promoters [43]. In addition to possible competition for transcriptional co-activators, evidence suggests that inhibition of the NFkB pathway is involved. Inhibition of this pathway does not entail inhibition of NFkB translocation to the nucleus, binding to the DNA or degradation of the NFkB inhibitor ikB. Most likely transrepression of NFkB by LXRs involves a nuclear event. Sumoylation of the LXR is a possible mechanism, but still has to be investigated [28]. Ligand binding to the LXR result in a conformational change, such that small ubiquitin-related modifier (SUMO) can bind. SUMOylated LXR then binds to the corepressor complex of NFkB, preventing its degradation by the 19S proteasome and thereby maintaining inflammatory genes in a repressed state [45].

To determine whether the myelin-induced decrease in NO production also persists at later time periods, macrophages were incubated with myelin or T09 for the indicated time periods. Both myelin and T09 demonstrated an initial decrease in macrophage-mediated NO production during the first 24 hours. At later time-points myelin phagocytosis significantly increased NO production. These results indicate that myelin ingestion results in a short-term modulation of macrophage function by decreasing their NO production. However, this effect disappears at later time periods.

In addition to ROS and NO, activated macrophages produce pro-inflammatory cytokines, like TNFα. TNFα is neurotoxic in vitro, increases axonal vulnerability and induces NO production [13]. We demonstrated that myelin phagocytosis decreased TNFa release by macrophages in a concentration dependent manner. This indicates that myelin phagocytosis can induce a protective, anti-inflammatory phenotype in macrophages by decreasing TNFα production. On the other hand, T09 decreased TNFα production at low concentrations and increased it at higher concentrations. These results demonstrate that the myelin-induced decrease in TNFα production is not only mediated via LXR signalling. However, a possible explanation for the observed increase in TNFα production after treatment with high concentrations of T09 is the activation of toll-like receptor 4 (TLR-4) by LXRs. For instance, in human macrophages it was shown that LXR activation leads to an increased TLR-4 expression. This regulation occurs at the transcriptional level via a DR-4 type LXRE in the promoter region of the human TLR-4 gene. Induction of TLR-4 enhances signalling responses in response to LPS, which leads to an increased TNFα secretion [55].

To further investigate the involvement of LXRs in the myelin-induced decrease in TNFα production, macrophages were treated with the LXR antagonist 22(S)-hydroxycholesterol prior to myelin incubation. The LXR antagonist was not able to significantly reverse the myelin-induced decrease in TNFα production, indicating that the observed effects after myelin treatment are not LXR specific. Next, we investigated whether the PPARγ is involved in the myelin-mediated decrease in TNFa production by macrophages. However, the PPARγ antagonist could not reverse the myelin-induced decrease in TNFα production, indicating that the PPARγ was not involved in the decrease in TNFa production after myelin phagocytosis.

To determine whether the myelin-induced decrease in TNFα production also persists at later time periods, macrophages were incubated with myelin or T09 for various periods of time. Myelin

phagocytosis induced an initial decrease in TNFα production by macrophages during the first 24 hours. Similar, LXR activation by T09 induced an initial decrease in TNFα production during the first 3 hours. However, these effects were not significant and experiments need to be repeated. Interestingly, T09 significantly increased TNFα production after 48 hours incubation. Again this could be explained by the activation of TLR-4 by LXRs, since it was demonstrated that short-term pre-treatment with LXR agonists before LPS stimulation, reduced the inflammatory response induced by LPS. On the other hand, pre-treatment of macrophages for 48 hours with LXR agonists resulted in an enhanced LPS response, thus an increase in TNFα production [55]. Together with the observed time-dependent effects of myelin phagocytosis on NO production, these results indicate that myelin ingestion does not modulate NO and TNFα production by macrophages at later periods of time.

Phagocytosis of myelin by macrophages is well documented in MS and EAE [11]. Peritoneal macrophages, isolated from the peritoneal cavity of adult Wistar rats, phagocytose myelin in vitro and this myelin uptake is time and dose dependent (data not shown). To determine whether LXR activation has an influence on the phagocytic capacity of macrophages, a myelin phagocytosis assay was performed. Treatment of macrophages with T09 increased myelin phagocytosis by macrophages over time. Phagocytosis, a major function of macrophages, has important roles in CNS maturation, the clearance of infectious agents and extracellular matrix breakdown and reformation. Macrophage-mediated phagocytosis of intact myelin, surrounding the axons, could be harmfull. This will lead to a reduced conduction capacity and axons are more vulnerable to damaging mediators because their protective sheaths are lost [56]. On the other hand, ingestion of myelin is a prerequisite for repair attempts in MS, since myelin debris impairs remyelination and axonal outgrowth. Uptake of potential auto-immunogenic material could also influence the risk for further immune attacks. The persistence of myelin debris in the lesions may inhibit oligodendrocyte precursor differentiation, by influencing the growth factor environment, and thereby inhibiting remyelination [24, 25]. Thus, the T09-induced increase in myelin phagocytosis could have both pro- and anti-inflammatory effects. More studies have to be performed to investigate whether T09 stimulates phagocytosis of intact myelin or phagocytosis of myelin debris. Interestingly, by stimulating myelin phagocytosis, LXR activation establishes a positive feedback loop.

The results described above indicate that LXRs are indeed activated after myelin phagocytosis. Moreover, we can conclude that multiple mechanisms, probably including LXR signalling, are involved in the myelin-induced protective phenotype in macrophages.

5 Conclusion and synthesis

The relapsing-remitting nature of MS strongly suggests the presence of potent counter-regulatory mechanisms that keep the disease under control. One such mechanism may be the control of inflammation in the CNS by myelin-laden macrophages. Macrophages are the most important effector cells in MS and they are abundantly present in the centre of MS lesions. Classically, these immune cells are hypothesized to be solely detrimental in the disease process, as they contribute to axonal loss by the production of ROS, NO and pro-inflammatory cytokines. However, recent evidence suggests that macrophages could also be neuroprotective in MS. For example, myelin-containing foam cells in the centre of MS lesions have been demonstrated to express a series of anti-inflammatory molecules, while lacking pro-inflammatory cytokines. Underlying mechanisms, inducing this protective phenotype in myelin-laden macrophages, still remain to be clarified. One such possible mechanism is the activation of LXRs after myelin phagocytosis. LXRs are activated by oxidized cholesterol derivatives. Interestingly, since myelin contains cholesterol, LXRs could be involved in the macrophage response after myelin phagocytosis. In this study, it was hypothesized that LXRs are activated after myelin phagocytosis and induce a protective, anti-inflammatory phenotype in macrophages. First, we demonstrated that LXRs are activated by myelin phagocytosis, suggesting that LXRs could be involved in the macrophage response after myelin phagocytosis. Next, the influence of myelin phagocytosis and LXR activation on the release of inflammatory mediators by macrophages was investigated. Both myelin and T09 increased ROS production by macrophages over time, indicating that the myelin-induced increase in ROS production is mediated via LXR signalling. However, other pathways may be involved in the observed increase in ROS production by macrophages after myelin phagocytosis. The significance of ROS on the regulation of inflammation is still unclear. Classically, ROS were considered as detrimental mediators of inflammation. However, there are an increasing number of findings suggesting that ROS could also be anti-inflammatory and have a more immunoregulatory role in inflammation. In addition, both myelin and T09 decreased NO production by macrophages. However, the observed decrease in macrophage-mediated NO production disappeared at later periods of time. These results indicate that the myelin-induced decrease in NO production is mediated via LXR signalling, however other pathways may be involved. Finally, we demonstrated that myelin, in contrast to T09, decreased macrophage-mediated TNFα release in a concentration dependent manner. These results suggest that the myelin-induced decrease in TNFα production is not mediated via LXR signalling. The results described above indicate that multiple mechanisms, probably including LXR signalling, are responsible for the induction of a protective phenotype in macrophages after myelin phagocytosis. For future experiments, it would be interesting to use LXR knock-out animals to confirm the involvement of LXR signalling in the myelin-induced protective phenotype in macrophages. Furthermore, additional mechanisms by which myelin modulates the phenotype of macrophages could be investigated. Also, the effect of myelin phagocytosis on other effector functions of macrophages could be investigated. In addition, it would be interesting to use immunohistochemical stainings to confirm LXR activation in EAE lesions. Future in vivo studies are required to elucidate the role of myelin-phagocytosing macrophages in EAE/MS and the effect of LXR agonist treatment in EAE/MS. The results of this study increased the knowledge about a

possible protective role of macrophages in MS and the underlying mechanism. In the future, it may hopefully lead to a novel therapeutic approach for MS. The ability of LXRs to integrate metabolic and inflammatory signalling makes them attractive targets for the intervention in human metabolic disorders, like atherosclerosis and diabetes type 2, as well as for the modulation of inflammation and immune responses. LXR agonists have already yielded promising results as treatment for murine models of atherosclerosis, diabetes and Alzheimer. Hence, the pharmacological manipulation of the LXRs, present in macrophages, may induce a protective, anti-inflammatory phenotype in these immune cells.

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Supplemental information

Table 1: Primers real time PCR

Gene	Bp length	Sequence 5′-3′	Sequence 5′-3′
LXRα	99	CTT GCT CAT TGC TAT CAG CAT CTT	ACA TAT GTG TGC TGC AGC CTC T
LXRβ	80	AAG GAC TTC ACC TAC AGC AAG GA	GAA CTC GAA GAT GGG ATT GAT GA
ApoE	65	ACT GGG TCG CTT TTG GGA TT	CTC CTC CTG CAC CTG CTC A
ABCA1	103	CCC AGA GCA AAA AGC GAC TC	GGT CAT CAT CAC TTT GGT CCT TG
ABCG1	105	CAA GAC CCT TTT GAA AGG GAT CTC	GCC AGA ATA TTC ATG AGT GTG GAC
LDL-R	114	GCA TCA GCT TGG ACA AGG TGT	GGG AAC AGC CAC CAT TGT TG