The role of differently activated macrophages in axonal damage during multiple sclerosis

Vereyken, E.J.F.

2010

document version
Publisher's PDF, also known as Version of record

CHAPTER

4

Activation of Liver X Receptors by myelin induces the anti-inflammatory phenotype of foamy macrophages

Vereyken E.J.F., Voerman J., Glim J.E., Meurs M., Teunissen C.E., Laman J.D., Dijkstra C.D., Boven L.A.

ABSTRACT

Foamy macrophages are abundantly present in active MS lesions and posses an anti-inflammatory phenotype. This phenotype could be mimicked *in vitro* by ingestion of myelin, after which the response to lipopolysaccharide (LPS) was inhibited. Liver X receptors (LXR) are involved in both lipid metabolism and immune modulation. Our hypothesis is that ingestion of myelin leads to an increase in intracellular cholesterol which in turn could activate LXR, leading to inhibition of the LPS response.

In MS lesions the downstream effects of LXR activity were significantly increased, as revealed by an increased ATP-binding cassette transporter (ABCA1) mRNA expression. This increase in target gene mRNA expression correlated with increased LXR mRNA expression. *In vitro*, myelin ingestion increased LXR mRNA expression and activity. Furthermore, in these macrophages the LPS response, as assesed by TNFα expression, was abrogated. Importantly, when LXR expression was knocked down using LXR small interfering RNA (siRNA), the inhibition of LPS response by myelin ingestion was partly alleviated. This indicates that LXR activation by myelin is, at least partly, responsible for the lack of pro-inflammatory responses.

These results showed that myelin ingestion can induce LXR activity and that the inhibition of the LPS response by myelin ingestion was at least in part due to the activation of LXR. In MS lesions this process of activation of LXR due to myelin ingestion could lead to reduction in the inflammation and thereby to inactive lesions. This process could therefore be partially responsible for the self-limiting nature of MS lesions. LXR agonists could thus possibly be interesting targets for MS therapy by the reduction of inflammation.

INTRODUCTION:

Multiple sclerosis (MS) is a chronic inflammatory autoimmune disease of the central nervous system (CNS), affecting approximately 1 in a 1000 people in Europe and Northern America [1]. The age of onset is between 20 and 40 years of age, making it the most common cause of neurological disability among young adults. MS is characterized by the presence of multiple demyelinated lesions. These lesions are associated with perivascular infiltrates, oligodendrocyte death, axonal damage and loss and scar formation due to astrogliosis [2;3].

It is widely accepted that macrophages play an important role in MS pathology. Macrophages have been implicated in the mechanisms that lead to demyelination and axonal damage [4-11]. However, beneficial roles have also been ascribed to macrophages in relation to remyelination, for example removal of myelin debris, and axonal repair [12-17]. These different roles ascribed to macrophages could be due to polarization in macrophage activation [18-20]. M1, or classically activated macrophages induced by exposure to interferon-gamma (IFN-γ) and LPS, are thought to be cytotoxic. They express high levels of pro-inflammatory molecules. In contrast, the M2, or alternatively activated macrophages generated by exposure to IL-4 or IL-13, are suggested to be involved in immune modulation, tissue remodeling and repair. They produce anti-inflammatory mediators and scavenge debris [19]. In MS lesions foamy macrophages, that have ingested and accumulated myelin derived lipids, are a common feature and have been suggested to be “alternatively activated”. Boven et al. [21] have shown that myelin-laden foamy macrophages in MS lesions have an anti-inflammatory phenotype, since they secrete anti-inflammatory cytokines and display markers of alternatively activated M2 macrophages. Furthermore, *in vitro*, macrophages that have ingested myelin have an immunosuppressive function since they do not respond to pro-inflammatory stimuli [21]. In another study it was found that in isolated mouse sciatic nerve macrophages that have ingested myelin display an anti-inflammatory phenotype [22].

In atherosclerosis the immune regulatory functions of lipids, lipid mediators and lipid-laden macrophages has been extensively studied. It was found that lipid mediators and lipid accumulation can reduce inflammation [23-25]. Macrophages are involved in both inflammation and lipid homeostasis, since they express liver X receptors (LXRs). LXRs are nuclear receptors that play a role in lipid homeostasis and can modulate inflammatory responses [26-28]. LXRs dimerize with the retinoid X receptor and upon ligand binding, bind to LXR-responsive elements (LXREs) in DNA, regulating the expression of target genes. Using trans-repression, LXRs can inhibit the transcription of promoters that do not contain LXREs. Metabolites of cholesterol, oxysterols such as 24(S)-hydroxycholesterol, the major oxysterol in the CNS, are endogenous ligands for LXR [29-31]. Since LXR mediates lipid and cholesterol homeostasis, LXR target genes are genes that participate in lipid metabolism and cholesterol transport, such as ATP-binding cassette transporter A1 (ABCA1) [32] and ATP-binding cassette transporter G1 (ABCG1) [33].

LXRs are not only involved in cholesterol homeostasis, but can modulate immune responses. *In vivo*, LXR agonists reduce the inflammatory response in a model for contact dermatitis and atherosclerosis [34]. Macrophage pro-inflammatory

gene expression of for example iNOS, COX2 and MMP9, is negatively regulated by ligand activation of LXRs *in vitro* [34;35]. LXRs can interfere with NF-κB signaling, via inhibition of DNA binding and IκB-α protein degradation [34;36]. LXR activation inhibits apoptosis: in LXRα/β-/- mice macrophages underwent accelerated apoptosis after infection with the intracellular bacteria Listeria monocytogenes [37]. Furthermore, LXR activation rescues macrophages from apoptotic signaling pathways stimulated by bacterial pathogens and withdrawal of macrophage-colony stimulating factor [28]. Finally, LXR can upregulate the arginase II gene, thereby shifting the arginine metabolism toward polyamine synthesis exerting positive anti-inflammatory effects [38].

Since LXR can activate an anti-inflammatory gene set and negatively influence the pro-inflammatory repertoire of macrophages, our hypothesis is that activation of LXR via the ingestion of myelin leads to the anti-inflammatory phenotype observed in foamy macrophages. Cholesterol is a major constituent of myelin and therefore ingestion of myelin by macrophages could raise intracellular levels of cholesterol and oxysterols which in turn would activate LXR.

Our results show that myelin ingestion leads to LXR activation and the induction of LXR target gene expression. Crucially, we show that by silencing LXR expression via LXR small interfering RNA (siRNA) we can partly restore the inflammatory response in foamy macrophages. Together, these data suggest that in demyelinating MS lesions the inflammatory process probably reduces once macrophages start ingesting myelin through an LXR-dependent mechanism, possibly leading to limitation of lesion expansion.

MATERIALS AND METHODS

RNA extraction from MS lesions

Human autopsy brain of 11 MS patients and 6 non-neurological age-matched controls was provided by the Netherlands Brain Bank in Amsterdam (Coordinator dr. I. Huitinga). The sections used for RNA isolation were selected on basis of the occurrence of preactive and actively demyelinating lesions. The RNA isolation was used to determine the amount of LXR and ABCA1 mRNA expression in lesion versus normal appearing white matter (NAWM) and non-neurological controls. Per brain sample 5 sections of 40 µm of the selected lesions were used for RNA extraction.

Myelin

Human myelin was isolated as previously described by Norton and Poduslo [39], by using density gradient centrifugation. Briefly, brain sections obtained for the Dutch brain bank were homogenized in 0.32 M sucrose (Sigma-Aldrich, Zwijndrecht, the Netherlands). The homogenate was layered on 0.85 M sucrose and centrifuged at 75,000 g for 30 min. The interface containing the myelin was obtained and taken up in de-ionized water. After washing twice (75,000g for 15 min), the myelin concentration was determined by bicinchoninic acid (BCA) assay (Pierce, Ettenleur, the Netherlands) using a bovine serum albumin (BSA) (Sigma-

Aldrich) standard curve [40].

Human macrophages

Peripheral blood human mononuclear cells were isolated as described previously. Heparinized blood from healthy donors was separated by using a Ficoll density gradient (GE healthcare lifesciences AB, Diegum, Belgium). Afterwards, the monocytes were further purified using a Percoll density gradient (GE healthcare lifesciences AB) which resulted in >80% pure monocytes. The purified monocytes were cultured in suspension in Teflon flasks at a concentration of $1x10^6$ cells/ml in RPMI (Invitrogen, Breda, the Netherlands) with 5% penicillin-streptomycin-glutamine (Invitrogen) and 5% human AB serum (Sanquin, Amsterdam, the Netherlands). After 5-7 days of culturing in teflon flasks (Nalgene, Roskilde, Denmark) the monocytes had developed into macrophages and the macrophages were seeded in 96-wells plates, at a concentration of $5x10^5$ cells/ml. Before each experiment macrophages were washed to remove non-adherent cells, resulting in >95% pure macrophages cultures.

To induce the foamy phenotype in human macrophages, macrophages were exposed to 25 µg/ml myelin for 48 h, as described previously by Boven et al [21].

cDNA synthesis

RNA was isolated using the mRNA capture kit (Roche, Almere the Netherlands) as described by the manufacturer. Macrophages were washed with phosphate buffered saline (PBS) and lysis buffer was added. 50 µl of lysis buffer and 4 µl of biotin-labelled oligo-dT was incubated for 5 min at 37°C. Subsequently the mix was added in a streptavidin coated tube and incubated for 5 min at 37°C. The tube was washed twice with washing buffer. The cDNA synthesis was performed using reverse transcriptase system (Promega, Leiden, The Netherlands) as described by the manufacturer. In the streptavidin coated tubes per sample 6 µl 25 mM $MgCl_2$, 3 µl reverse transcriptase buffer, 2 µl deoxyribonucleotides, 0.4 µl RNAsin, 0.4 µl AMV reverse transcriptase, 0.6 µl random hexamer primers and 17.6 µl water was added. The samples were incubated for 10 min at room temperature (RT) and a further 15 min at 42°C. The final step is 5 min at 99°C. The Eppendorf tubes were opened and the sample was placed in a new, non-streptavidin coated tube.

Real-time quantitative PCR

To verify target gene mRNA expression real-time quantitative-transcription-polymerase chain reaction (qPCR) was performed using a LightCycler. SYBR GREEN (Applied biosystems; Nieuwekerk a/d IJsel, the Netherlands) was used as detection system as indicated by the manufacturer. Target gene expression levels were corrected for glyceraldehyde 3-phosphage dehydrogenase (GAPDH) mRNA levels. The primer pairs used are listed in table 1.

LXR silencing experiment

LXR expression in macrophages was silenced to determine the effect of LXR on the foamy phenotype of myelin ingested macrophages.

Gene	sequence	
LXR	forward	AGTGTCGGCTTCGCAAAT
	reverse	GGCGGATCTGTTCTTCTGAC
ABCA	forward	GCCTGCTAGTGGTCATCCTG
	reverse	GCCTGCTAGTGGTCATCCTG
TNF-α	forward	TGTTGTAGCAAACCCTCAAGC
	reverse	GCTGGTTATCTGTCAGCTCCA
GAPDH	forward	TCCACTGGCGTCTTCAC
	reverse	GGCAGAGATGATGACCCTTTT

Table 1: Primer sequences

Macrophages were seeded in 96-wells plate. The cells were washed and exposed to either nothing, LXR siRNA or control siRNA (siCONTROL Non-targeting siRNA Pool, D-001206-13-05) using the DharmaFECT transfection protocol (Thermoscientific, Epsom, UK). After 48 h of exposure to siRNA the cells were washed. Subsequently the macrophages were exposed to myelin at 25 µg/ml for 24 h. The cells were washed and incubated with LPS for 6 h. Afterwards RNA was isolated.

Statistical analysis

The data are presented as mean ± SEM. Statistical analysis was performed in SPSS (15.0.0, Chicago, USA). Data were analysed using a one-way ANOVA with Bonferroni correction and p-values <0.05 were considered significant. For the correlation studies Spearman analysis was used.

RESULTS

Expression of LXR mRNA is higher in lesions compared to NAWM and correlates with ABCA

Autopsy brain of 16 MS patients was used for RNA isolation to determine the amount of LXR and LXR target gene mRNA expression in lesion versus NAWM and non-neurological controls. The sections used for RNA isolation were investigated for the presence of preactive and actively demyelinating lesions [41] using immunohistochemistry. Per brain sample 5 sections of 40 µm were used for RNA analysis.

In active and pre-active MS lesions, LXR mRNA expression was not significantly increased, although there was a trend that tissue containing more lesions had increased LXR expression (figure 1A). The relative expression of LXR mRNA in MS lesion was 2.6 ± 1.04, in NAWM the relative expression was 0.8 ± 0.19 and in control brain the relative expression was 1.1 ± 0.19. Determining protein expression by either staining and western blot is not possible, due to the lack of a specific antibody for LXR. To determine the extent of LXR activation in lesions the mRNA expression of ABCA1 was determined. In MS lesions the relative expression

of both ABCA1 mRNA was significantly increased compared to NAWM and non-demented controls (see figure 1). The relative expression of ABCA1 mRNA was 1.5 ± 0.48 in MS lesion, 0.4 ± 0.09 in NAWM and 0.4 ± 0.08 in control brain. Although LXR mRNA expression was not significantly increased, a strong correlation existed between ABCA1 and LXRα mRNA levels (figure 1 b).

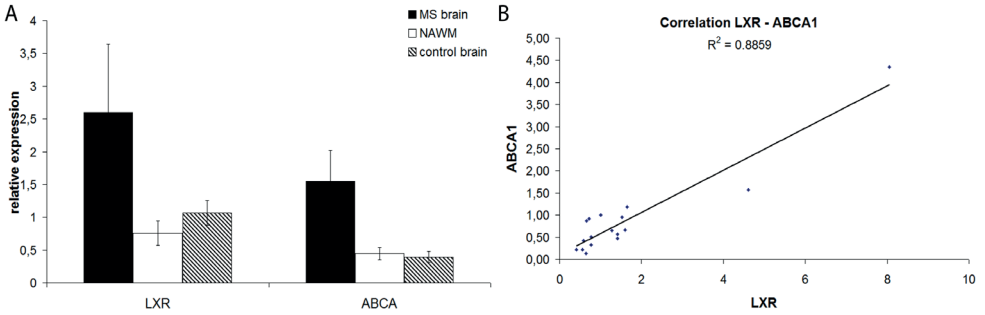

Figure 1: LXR and LXR target gene expression in lesion versus NAWM versus control brain. Human autopsy brain was used to determine the amount of LXR and LXR target gene mRNA expression in normal brain versus NAWM and MS lesions using qPCR. A) In MS brain LXR gene mRNA expression is not significantly increased compared to NAWM or control brain. ABCA was significantly increased in MS lesion compared to NAWM and control brain. B) Correlation between LXR and ABCA. A significant correlation was found between LXR an ABCA expression $p<0.001$.

Ingestion of myelin leads to LXR activation in macrophages

Since in active MS lesions high numbers of foamy macrophages are present, we determined whether foamy macrophages in MS lesions could be the source of the upregulated LXR and LXR target gene expression. An *in vitro* model for foamy macrophages was used as described by Boven et al [21]. Human macrophages were incubated with 25 µg/ml myelin for 48 h, after which they had a foamy appearance and an abrogated inflammatory LPS response (see figure 5B) as described previously [21].

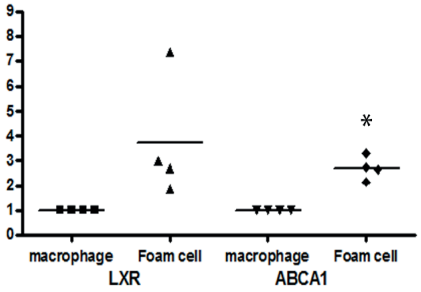

Figure 2: In vitro foamy macrophages expression of LXR and ABCA1 mRNA. After 2 days of exposure to 25 µg/ml myelin macrophages were considered foamy. Using qPCR the level of LXR and ABCA1 mRNA expression was determined relative to control macrophages. Data presented are the mean of four separate experiments. *= $p<0.05$. LXR mRNA expression in foam cells showed a trend towards higher expression, but this was not significant. For ABCA1 the mRNA expression was significantly higher in foam cells compared to normal macrophages.

Using qPCR the relative level of LXR and target gene mRNA expression was determined. Macrophages that have ingested myelin displayed a trend towards a higher level of LXR mRNA expression (figure 2). The relative mRNA expression of LXR in foamy macrophages was 3.7 (± 2.48). Similar as in MS lesions, ABCA1

mRNA expression was significantly enhanced. The relative expression of ABCA1 in foamy macrophages was 2.7 (± 0.24). These findings indicate that LXR activity was increased in the foamy macrophages and that myelin is or contains a ligand for LXR.

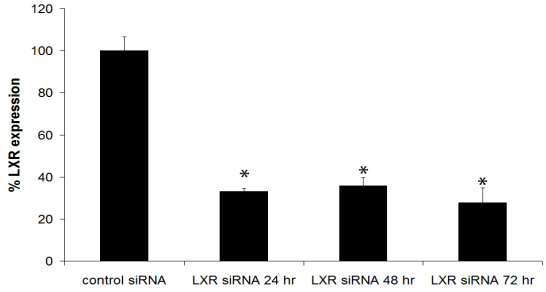

Figure 3: LXR knockdown after siRNA is visible after 24 h. Macrophages were exposed to LXR siRNA for 24, 48 or 72 h. Data presented are the mean of three separate experiments. *= p<0.05. Expression of LXR mRNA was reduced to 33% after 24 h and was still visible after 72 h.

LXR is downregulated after siRNA

To determine whether it is the myelin induced LXR activation that ultimately leads to the abrogated inflammatory response in foamy macrophages, we used siRNA techniques to silence LXR expression in macrophages and subsequently determined whether the LPS response was restored after exposure to myelin. First, we determined whether adding LXR siRNA actually led to a knock down of LXR expression. Macrophages were exposed to LXR siRNA, control siRNA or vehicle alone and left to incubate for 24, 48 or 72 h. Subsequently qPCR was performed to determine LXR expression (figure 3). At all time points tested the macrophages exposed to LXR siRNA showed a significantly lower amount of LXR mRNA expression compared to control macrophages. The level of LXR mRNA expression was reduced by about 70%.

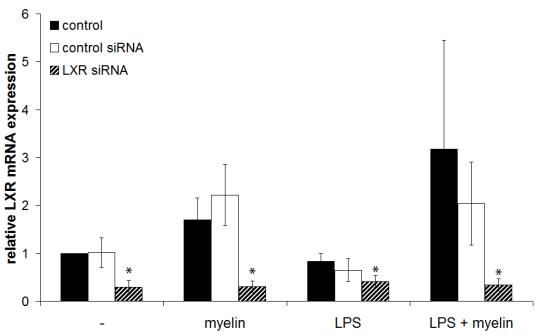

Figure 4: LXR mRNA expression. Macrophages, treated with nothing, control or LXR siRNA for 48 h, were exposed to 25 µg/ml myelin for 24 h and to LPS for 6 h afterwards. Data presented are the mean of five separate experiments. *= p<0.05. In all conditions tested LXR siRNA led to a reduction in LXR mRNA expression by approximately 70%. Control siRNA did not significantly change the LXR mRNA expression compared to control macrophages.

LXR siRNA alleviates inhibition of LPS response by myelin

Next, we determined whether the myelin-induced abrogation of the LPS response was mediated by LXR. Macrophages were exposed to LXR siRNA for 48 h and subsequently washed. The cells were left to recover for a day and exposed to myelin. After 24 h the cells were washed and LPS was added for 6 h. Tumor

necrosis factor alpha (TNFα) mRNA expression was measured as read-out for the inflammatory response and LXR mRNA as a measure for the LXR response.

Measuring LXR mRNA, we observed again that after exposure to myelin the induction of LXR mRNA expression was increased as compared to control macrophages (1.7 ± 0.46 compared to 1), although not significantly (see figure 4). Addition of LPS did not alter LXR mRNA significantly. Addition of LPS after myelin ingestion increased LXR mRNA expression (3.2 ± 2.31) not significantly. Control siRNA treated macrophages showed similar LXR mRNA expression compared to control macrophages. After siRNA treatment again the mRNA expression of LXR was significantly decreased, to approximately 30% of control LXR mRNA expression.

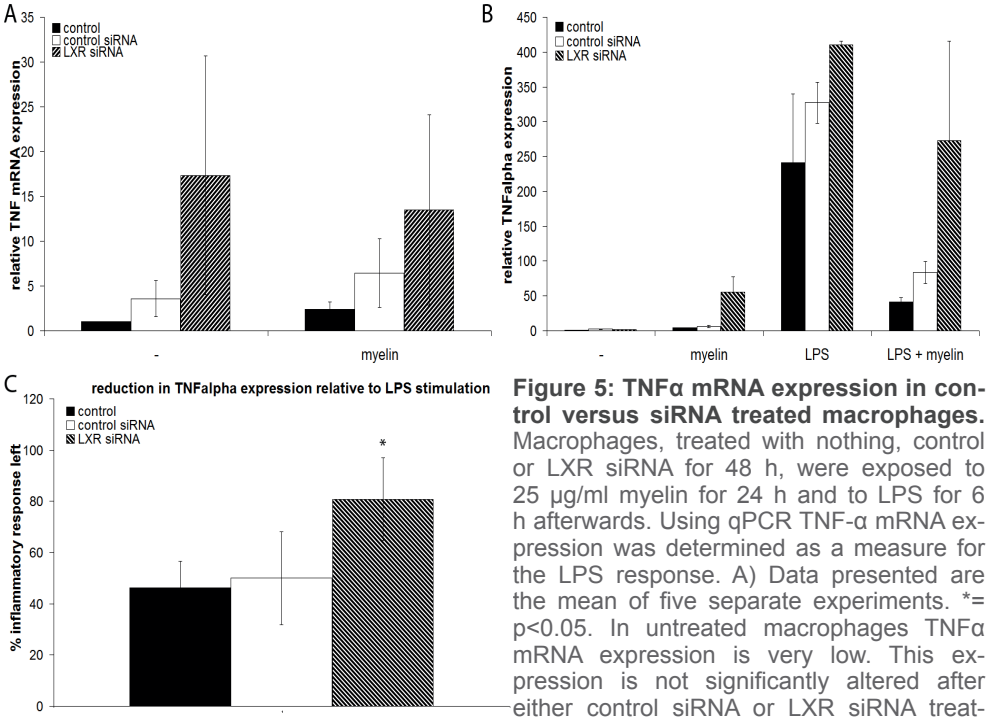

Figure 5: TNFα mRNA expression in control versus siRNA treated macrophages. Macrophages, treated with nothing, control or LXR siRNA for 48 h, were exposed to 25 µg/ml myelin for 24 h and to LPS for 6 h afterwards. Using qPCR TNF-α mRNA expression was determined as a measure for the LPS response. A) Data presented are the mean of five separate experiments. *= $p<0.05$. In untreated macrophages TNFα mRNA expression is very low. This expression is not significantly altered after either control siRNA or LXR siRNA treatment. Myelin ingestion also does not alter the TNFα mRNA expression. B) Representative siRNA experiment. In the control situation, TNFα mRNA expression is very low. This does not change after control or LXR siRNA treatment. After myelin ingestion, TNFα mRNA expression does not alter significantly. An increase is seen with LXR siRNA, but this is negligible. LPS stimulation of macrophages did increase TNFα mRNA expression in control macrophages. In control siRNA treated macrophages this increase in TNFα mRNA expression was also visible, as was it in LXR siRNA treated macrophages. Myelin ingestion prior to LPS stimulation decreased TNFα mRNA expression in control macrophages. Treatment with control siRNA did not affect the consequence of myelin on TNFα mRNA expression after LPS stimulation. The inhibitory effect of myelin on LPS induced TNFα mRNA expression was reduced by LXR siRNA. C) Myelin ingestion by macrophages reduced the LPS response in both control and control siRNA treated macrophages. In LXR siRNA treated macrophages, the LPS response after myelin ingestion was significantly higher compared to control macrophages. Data presented are the mean of four separate experiments. *= $p<0.05$.

In control macrophages TNFα expression was low. Myelin ingestion did not significantly alter the expression of TNFα mRNA expression in control macrophages (2.3 ± 0.8). Treatment with LXR siRNA did not change the TNFα mRNA expression significantly in either of these two conditions; control (17 ±13.3) or after myelin ingestion (14 ±10.6). A significant increase of TNFα mRNA expression was found after LPS stimulation; however the amount of upregulation was subject to large variation between donors. We therefore show a representative siRNA experiment (figure 5b). LXR siRNA treated macrophages also showed a significant increase in TNFα mRNA expression after LPS stimulation. As has been shown previously by Boven et al [21], myelin ingestion by macrophages prior to LPS stimulation led to a significant decrease in TNFα mRNA expression. After LXR siRNA the inflammatory response to LPS was partly restored, since TNFα mRNA expression was higher compared to control macrophages treated with myelin and LPS (figure 5b).

To determine the effect of LXR siRNA on the inflammatory response in foamy macrophages, TNFα mRNA expression was assessed in control and foamy macrophages stimulated with LPS. In foamy macrophages that had ingested myelin the inflammatory response after LPS stimulation was reduced to 46% (± 10.3) of values for LPS stimulated control macrophages. In control siRNA treated macrophages after myelin ingestion the LPS stimulated TNFα mRNA expression was 50% (± 18.2), similar to control foamy macrophages. LXR siRNA treatment of foamy macrophages partly rescued the inflammatory response, since the TNFα mRNA expression was 81 % (±16.1) of the control condition where macrophages were exposed to LPS only. The inflammatory response in LXR siRNA treated foamy macrophages was significantly higher compared to control foamy macrophages.

DISCUSSION

The aim of this study was to determine whether LXR activation by myelin ingestion could be crucial in the development of the AA phenotype of foamy macrophages. We show that in MS lesions LXR target gene mRNA expression of ABCA1 is significantly increased and, importantly, correlates with the expression of LXR mRNA and the presence of foamy macrophages. *In vitro*, we show that myelin induces LXR target gene mRNA expression. This expression of LXR target genes by the ingestion of myelin suggests that myelin is or contains a ligand for LXR. Furthermore, this shows that the *in vitro* model for foamy macrophage generation very nicely mimicked the *in vivo* situation, as both situations gave similar results.

Previously myelin ingestion by macrophages has been shown to abrogate the LPS response [21]. Since LXR activity is known to downregulate the LPS response [23-25], we determined whether LXR activation by myelin is responsible for the dampened LPS response in foamy macrophages, by silencing the LXR mRNA gene expression. LXR knockdown did result in partial rescue of the LPS response, as visualized by TNFα mRNA expression. After myelin ingestion in control macrophages, the LPS response was reduced to 46% of the LPS response in control macrophages. In control siRNA treated macrophages after myelin ingestion the LPS response was reduced to 50% of control macropahges. After LXR siRNA the LPS response was recovered to 80%. The fact that the LPS response was not totally rescued could be

due to the approximately 30% LXR expression that was still present in the siRNA treated macrophages. Our data show that LXR activity is indeed involved in the down modulation of a pro-inflammatory phenotype by myelin ingestion or LPS.

Our experiments were in line with several studies indicating that LXR activation could reduce the inflammatory response to LPS. First, cytokine release was reduced in response to LPS after LXR activation in human monocytes [42]. Activation of LXR by exposure to a synthetic agonist (18 hr) suppressed LPS induced tissue factor expression in human monocytes, partly due to blocking NF-κB signaling pathway [43]. Finally, anti-inflammatory effects of LXR activation in human T-cells, since expression of Th1 cytokines in T-cells was reduced by 30 minutes pretreatment or direct combined stimulation of LXR agonist with pro-inflammatory stimuli [44].

We observed that silencing of LXR leads to an increased LPS response. In contrast, pre-treatment of human macrophages with an LXR agonist led to an increased LPS response, as shown by increased TLR4 expression and TNF-α production after LPS stimulation. Shorter pre-treatment times than 48 h did not show this increase in LPS response. A bimodal effect was shown as 0 and 6 h pretreatment significantly decreased TNF-α production, while 12 and 24 h did not induce significant differences in TNF-α production [45]. In our study we used 24 h pre-treatment with myelin, however we did not use a direct LXR agonist. Myelin might need to be processed before it contains LXR agonists, which might explain why our longer pre-treatment time did not give an increase in LPS response. The role of LXR activation in the inflammatory response in the human settings is not as clear cut or as well described as in mice.

Our results showed that in human macrophages LXR knockdown via siRNA led to a reduction in the inhibition of the LPS response by macrophages. These data are confirmed in several mouse studies, showing that LXR activation led to a decrease in inflammation both *in vivo* and *in vitro* [36;46-48]. In these studies a link has been observed between LXR activation and a decrease in neurodegeneration, suggesting that LXR activation could be beneficial in MS. Treatment with T0901317, an LXR agonist, reduced peak and cumulative disease scores in EAE mice, and inflammation as visualized by T-cell and macrophage infiltration and cytokine production [49]. Since both antigen-specific T-cell proliferation and cytokine production was not reduced in the LXR agonist treated mice, it could well be that macrophages are responsible for the beneficial effects of LXR agonists. In a model for Niemann-Pick type C disease activation of LXR by the agonist T0901317 induced increased cholesterol efflux from the brain and a slowed neurodegeneration [46]. In an APP/PS1 mouse model for Alzheimer's disease loss of LXRα or –β expression exacerbates Alzheimer related pathology and that LXR activation potently inhibited the glial cell response to inflammatory stimuli, for example to fibrilar Aβ [47]. In a model for experimental stroke in rats LXR activation by the synthetic agonists, GW3965 and T0901317, mediates a potent neuroprotective effect accompanied again by a significant decrease in the production of inflammatory mediators like iNOS [48]. Finally, it has been shown *in vitro* that activating LXR blocks the production of NO and pro-inflammatory cytokines by LPS-stimulated primary mouse microglia and astrocytes [36]. An explanation for the effect of the LXR siRNA on the recovery of the

LPS response could be explained by the fact that LXR is constitutively expressed in macrophages and always reduces the LPS response slightly. Under homeostatic conditions LXR would function as a brake on the LPS response. After LXR siRNA, the reduction of the LPS response would no longer be present. LXR siRNA could therefore lead to disinhibition of the macrophages and rescue of the LPS response after tolerization towards LPS with contaminated myelin. An indication that the macrophages were disinhibited was that when LXR siRNA treated macrophages were stimulated with only LPS the TNFα expression was higher compared to control macrophages stimulated with LPS.

In conclusion, it is likely that myelin is, or contains, a ligand for LXR. In MS lesions the ingestion of myelin by macrophages could lead to activation of LXR. The activation of LXR was shown to down regulate the LPS response, which in turn might induce the self-limiting nature of MS lesions. LXR activation could thus be beneficial in MS.

REFERENCE LIST

1. Pugliatti,M., S.Sotgiu, and G.Rosati. 2002. The worldwide prevalence of multiple sclerosis. Clin. Neurol. Neurosurg. 104:182-191.
2. Lassmann H. 1998. Pathology of Multiple Sclerosis. In McAlpine's Multiple Sclerosis. Livingstone C., editor. 323-358.
3. Charcot,M. 1868. Histolgie de le sclerose en plaques. 141:554-558.
4. Bitsch,A., J.Schuchardt, S.Bunkowski, T.Kuhlmann, and W.Bruck. 2000. Acute axonal injury in multiple sclerosis. Correlation with demyelination and inflammation. Brain 123 (Pt 6):1174-1183.
5. Kuhlmann,T., G.Lingfeld, A.Bitsch, J.Schuchardt, and W.Bruck. 2002. Acute axonal damage in multiple sclerosis is most extensive in early disease stages and decreases over time. Brain 125:2202-2212.
6. Trapp,B.D., J.Peterson, R.M.Ransohoff, R.Rudick, S.Mork, and L.Bo. 1998. Axonal transection in the lesions of multiple sclerosis. N. Engl. J. Med. 338:278-285.
7. Koning,N., L.Bo, R.M.Hoek, and I.Huitinga. 2007. Downregulation of macrophage inhibitory molecules in multiple sclerosis lesions. Ann. Neurol. 62:504-514.
8. Huitinga,I., van Rooijen N., C.J.De Groot, B.M.Uitdehaag, and C.D.Dijkstra. 1990. Suppression of experimental allergic encephalomyelitis in Lewis rats after elimination of macrophages. J. Exp. Med. 172:1025-1033.
9. Heppner,F.L., M.Greter, D.Marino, J.Falsig, G.Raivich, N.Hovelmeyer, A.Waisman, T.Rulicke, M.Prinz, J.Priller, B.Becher, and A.Aguzzi. 2005. Experimental autoimmune encephalomyelitis repressed by microglial paralysis. Nat. Med. 11:146-152.
10. Hendriks,J.J., C.E.Teunissen, H.E.de Vries, and C.D.Dijkstra. 2005. Macrophages and neurodegeneration. Brain Res. Brain Res. Rev. 48:185-195.
11. Newman,T.A., S.T.Woolley, P.M.Hughes, N.R.Sibson, D.C.Anthony, and V.H.Perry. 2001. T-cell- and macrophage-mediated axon damage in the absence of a CNS-specific immune response: involvement of metalloproteinases. Brain 124:2203-2214.
12. Baer,A.S., Y.A.Syed, S.U.Kang, D.Mitteregger, R.Vig, C.Ffrench-Constant, R.J.Franklin, F.Altmann, G.Lubec, and M.R.Kotter. 2009. Myelin-mediated inhibition of oligodendrocyte precursor differentiation can be overcome by pharmacological modulation of Fyn-RhoA and protein kinase C signalling. Brain 132:465-481.
13. Kotter,M.R., W.W.Li, C.Zhao, and R.J.Franklin. 2006. Myelin impairs CNS remyelination by inhibiting oligodendrocyte precursor cell differentiation. J. Neurosci. 26:328-332.

14. Teunissen,C.E., C.D.Dijkstra, B.Jasperse, F.Barkhof, H.Vanderstichele, E.Vanmechelen, C.H.Polman, and L.Bo. 2006. Growth-associated protein 43 in lesions and cerebrospinal fluid in multiple sclerosis. Neuropathol. Appl. Neurobiol. 32:318-331.
15. Larsen,P.H., J.E.Wells, W.B.Stallcup, G.Opdenakker, and V.W.Yong. 2003. Matrix metalloproteinase-9 facilitates remyelination in part by processing the inhibitory NG2 proteoglycan. J. Neurosci. 23:11127-11135.
16. Kerschensteiner,M., E.Gallmeier, L.Behrens, V.V.Leal, T.Misgeld, W.E.Klinkert, R.Kolbeck, E.Hoppe, R.L.Oropeza-Wekerle, I.Bartke, C.Stadelmann, H.Lassmann, H.Wekerle, and R.Hohlfeld. 1999. Activated human T cells, B cells, and monocytes produce brain-derived neurotrophic factor in vitro and in inflammatory brain lesions: a neuroprotective role of inflammation? J. Exp. Med. 189:865-870.
17. Kerschensteiner,M., C.Stadelmann, G.Dechant, H.Wekerle, and R.Hohlfeld. 2003. Neurotrophic cross-talk between the nervous and immune systems: implications for neurological diseases. Ann. Neurol. 53:292-304.
18. Edwards,J.P., X.Zhang, K.A.Frauwirth, and D.M.Mosser. 2006. Biochemical and functional characterization of three activated macrophage populations. J. Leukoc. Biol. 80:1298-1307.
19. Mosser,D.M. and J.P.Edwards. 2008. Exploring the full spectrum of macrophage activation. Nat. Rev. Immunol. 8:958-969.
20. Mantovani,A., A.Sica, S.Sozzani, P.Allavena, A.Vecchi, and M.Locati. 2004. The chemokine system in diverse forms of macrophage activation and polarization. Trends Immunol. 25:677-686.
21. Boven,L.A., Van Meurs M., Van Zwam M., A.Wierenga-Wolf, R.Q.Hintzen, R.G.Boot, J.M.Aerts, S.Amor, E.E.Nieuwenhuis, and J.D.Laman. 2006. Myelin-laden macrophages are anti-inflammatory, consistent with foam cells in multiple sclerosis. Brain 129:517-526.
22. van Rossum D., S.Hilbert, S.Strassenburg, U.K.Hanisch, and W.Bruck. 2008. Myelin-phagocytosing macrophages in isolated sciatic and optic nerves reveal a unique reactive phenotype. Glia 56:271-283.
23. Ares,M.P., M.Stollenwerk, A.Olsson, B.Kallin, S.Jovinge, and J.Nilsson. 2002. Decreased inducibility of TNF expression in lipid-loaded macrophages. BMC. Immunol. 3:13.
24. Greaves,D.R. and S.Gordon. 2005. Thematic review series: the immune system and atherogenesis. Recent insights into the biology of macrophage scavenger receptors. J. Lipid Res 46:11-20.
25. Serhan,C.N., N.Chiang, and T.E.Van Dyke. 2008. Resolving inflammation: dual anti-inflammatory and pro-resolution lipid mediators. Nat. Rev. Immunol. 8:349-361.
26. Rigamonti,E., G.Chinetti-Gbaguidi, and B.Staels. 2008. Regulation of macrophage functions by PPAR-alpha, PPAR-gamma, and LXRs in mice and men. Arterioscler. Thromb. Vasc. Biol. 28:1050-1059.
27. Zelcer,N. and P.Tontonoz. 2006. Liver X receptors as integrators of metabolic and inflammatory signaling. J. Clin. Invest 116:607-614.
28. Valledor,A.F., L.C.Hsu, S.Ogawa, D.Sawka-Verhelle, M.Karin, and C.K.Glass. 2004. Activation of liver X receptors and retinoid X receptors prevents bacterial-induced macrophage apoptosis. Proc. Natl. Acad. Sci. U. S. A 101:17813-17818.
29. Lehmann,J.M., S.A.Kliewer, L.B.Moore, T.A.Smith-Oliver, B.B.Oliver, J.L.Su, S.S.Sundseth, D.A.Winegar, D.E.Blanchard, T.A.Spencer, and T.M.Willson. 1997. Activation of the nuclear receptor LXR by oxysterols defines a new hormone response pathway. J. Biol. Chem. 272:3137-3140.
30. Janowski,B.A., P.J.Willy, T.R.Devi, J.R.Falck, and D.J.Mangelsdorf. 1996. An oxysterol signalling pathway mediated by the nuclear receptor LXR alpha. Nature 383:728-731.

31. Janowski,B.A., M.J.Grogan, S.A.Jones, G.B.Wisely, S.A.Kliewer, E.J.Corey, and D.J.Mangelsdorf. 1999. Structural requirements of ligands for the oxysterol liver X receptors LXRalpha and LXRbeta. Proc. Natl. Acad. Sci. U. S. A 96:266-271.
32. Repa,J.J., S.D.Turley, J.A.Lobaccaro, J.Medina, L.Li, K.Lustig, B.Shan, R.A.Heyman, J.M.Dietschy, and D.J.Mangelsdorf. 2000. Regulation of absorption and ABC1-mediated efflux of cholesterol by RXR heterodimers. Science 289:1524-1529.
33. Sabol,S.L., H.B.Brewer, Jr., and S.Santamarina-Fojo. 2005. The human ABCG1 gene: identification of LXR response elements that modulate expression in macrophages and liver. J. Lipid Res 46:2151-2167.
34. Joseph,S.B., A.Castrillo, B.A.Laffitte, D.J.Mangelsdorf, and P.Tontonoz. 2003. Reciprocal regulation of inflammation and lipid metabolism by liver X receptors. Nat. Med. 9:213-219.
35. Castrillo,A., S.B.Joseph, C.Marathe, D.J.Mangelsdorf, and P.Tontonoz. 2003. Liver X receptor-dependent repression of matrix metalloproteinase-9 expression in macrophages. J. Biol. Chem. 278:10443-10449.
36. Zhang-Gandhi,C.X. and P.D.Drew. 2007. Liver X receptor and retinoid X receptor agonists inhibit inflammatory responses of microglia and astrocytes. J. Neuroimmunol. 183:50-59.
37. Joseph,S.B., M.N.Bradley, A.Castrillo, K.W.Bruhn, P.A.Mak, L.Pei, J.Hogenesch, R.M.O'connell, G.Cheng, E.Saez, J.F.Miller, and P.Tontonoz. 2004. LXR-dependent gene expression is important for macrophage survival and the innate immune response. Cell 119:299-309.
38. Marathe,C., M.N.Bradley, C.Hong, F.Lopez, C.M.Ruiz de Galarreta, P.Tontonoz, and A.Castrillo. 2006. The arginase II gene is an anti-inflammatory target of liver X receptor in macrophages. J. Biol. Chem. 281:32197-32206.
39. Norton,W.T. and S.E.Poduslo. 1973. Myelination in rat brain: method of myelin isolation. J. Neurochem. 21:749-757.
40. van der Goes A., M.Kortekaas, K.Hoekstra, C.D.Dijkstra, and S.Amor. 1999. The role of anti-myelin (auto)-antibodies in the phagocytosis of myelin by macrophages. J. Neuroimmunol. 101:61-67.
41. De Groot,C.J., E.Bergers, W.Kamphorst, R.Ravid, C.H.Polman, F.Barkhof, and Van der Valk P. 2001. Post-mortem MRI-guided sampling of multiple sclerosis brain lesions: increased yield of active demyelinating and (p)reactive lesions. Brain 124:1635-1645.
42. Myhre,A.E., J.Agren, M.K.Dahle, M.V.Tamburstuen, S.P.Lyngstadaas, A.J.Collins, S.J.Foster, C.Thiemermann, A.O.Aasen, and J.E.Wang. 2008. Liver X receptor is a key regulator of cytokine release in human monocytes. Shock 29:468-474.
43. Terasaka,N., A.Hiroshima, A.Ariga, S.Honzumi, T.Koieyama, T.Inaba, and T.Fujiwara. 2005. Liver X receptor agonists inhibit tissue factor expression in macrophages. FEBS J. 272:1546-1556.
44. Walcher,D., A.Kummel, B.Kehrle, H.Bach, M.Grub, R.Durst, V.Hombach, and N.Marx. 2006. LXR activation reduces proinflammatory cytokine expression in human CD4-positive lymphocytes. Arterioscler. Thromb. Vasc. Biol. 26:1022-1028.
45. Fontaine,C., E.Rigamonti, A.Nohara, P.Gervois, E.Teissier, J.C.Fruchart, B.Staels, and G.Chinetti-Gbaguidi. 2007. Liver X receptor activation potentiates the lipopolysaccharide response in human macrophages. Circ. Res 101:40-49.
46. Repa,J.J., H.Li, T.C.Frank-Cannon, M.A.Valasek, S.D.Turley, M.G.Tansey, and J.M.Dietschy. 2007. Liver X receptor activation enhances cholesterol loss from the brain, decreases neuroinflammation, and increases survival of the NPC1 mouse. J. Neurosci. 27:14470-14480.
47. Zelcer,N., N.Khanlou, R.Clare, Q.Jiang, E.G.Reed-Geaghan, G.E.Landreth, H.V.Vinters, and P.Tontonoz. 2007. Attenuation of neuroinflammation and Alzheimer's

disease pathology by liver x receptors. Proc. Natl. Acad. Sci. U. S. A 104:10601-10606.
48. Morales,J.R., I.Ballesteros, J.M.Deniz, O.Hurtado, J.Vivancos, F.Nombela, I.Lizasoain, A.Castrillo, and M.A.Moro. 2008. Activation of liver X receptors promotes neuroprotection and reduces brain inflammation in experimental stroke. Circulation 118:1450-1459.
49. Hindinger,C., D.R.Hinton, S.J.Kirwin, R.D.Atkinson, M.E.Burnett, C.C.Bergmann, and S.A.Stohlman. 2006. Liver X receptor activation decreases the severity of experimental autoimmune encephalomyelitis. J. Neurosci. Res 84:1225-1234.