Lipids, inflammation and atherosclerosis

Stitzinger, M.

Chapter 4

LIPID ACCUMULATION BY β-VLDL ALTERS LPS INDUCED EXPRESSION OF LIPID RELATED GENES IN MURINE MACROPHAGES

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ABSTRACT

Macrophage-derived foam cells play a prominent role in the initiation and progression of the atherosclerotic plaque. Expression of genes responsible for macrophage lipid homeostasis and their regulation by lipid loading and inflammatory mediators, such as LPS, is essential for preventing foam cell formation. We investigated the effect of foam cell formation on the response of macrophages to LPS with a specific emphasis on the expression of lipid related genes. To induce foam cells, murine macrophage RAW 264.7 cells were incubated with β-VLDL for 24 hours. Subsequently, cells were incubated with LPS for 6 hours and expression of inflammatory and lipid related genes were analysed using quantitative real-time PCR-analysis. β-VLDL loading of the macrophages reduced basal, but not LPS induced TNF-α gene expression and secretion. The effect of LPS on gene expression of LRP-1, SR-BI, ABCG1, PPARγ, LXRβ, SREBP-1 and SREBP-2 was similar for both control cells and cells pre-incubated with β-VLDL. However, the effect of LPS on the expression of LDLr, ABCA1, apoA-I, apoE and PPARα was potentiated, attenuated or even reversed by preloading of the cells with lipids. These data point to an interaction between lipid metabolism and inflammation in macrophages, which is likely to modulate the pathogenesis of atherosclerosis.

INTRODUCTION

One of the early steps in atherosclerosis involves the infiltration of circulating monocytes into the vessel wall in response to the enhanced expression of adhesion molecules and chemoattractants by activated endothelial cells. Subsequently, the infiltrated monocytes differentiate into macrophages and transform into foam cells as a result of accumulation of cholesteryl esters from modified lipoproteins. These foam cells play a prominent role in the initiation and progression of the atherosclerotic plaque[1-3].

Several lipoproteins, such as β-very low-density lipoprotein (β-VLDL), a lipoprotein naturally induced by cholesterol-rich feeding of animals[4,5], or experimentally modified lipoproteins including acetylated low-density lipoprotein (acLDL) and oxidized LDL (oxLDL)[6,7], induce *in vitro* foam cell formation. Cholesterol efflux from the macrophage/foam cell is another important process in the development of foam cell formation, because foam cell formation is the result of an imbalance in cholesterol homeostasis[8].

The transformation of macrophages into foam cells can be affected by a variety of factors, amongst which inflammatory mediators such as lipopolysaccharide (LPS)[9]. LPS is a component of the membrane of Gram-negative bacteria and, when released from bacteria, is one of the most potent inducers of inflammation. LPS activates monocytes and macrophages leading to the production of cytokines such as tumor necrosis factor (TNF)-α, interleukin (IL)-1β and IL-6, which serve as endogenous mediators of inflammation through interactions with various target cells[10]. In addition, LPS is able to alter lipid metabolism in macrophages by affecting expression of lipid metabolism related genes[9,11,12].

Recent studies show the effect of foam cell formation on the inflammatory response of macrophages to LPS[13-16]. However, until now, it is unknown whether foam cell formation also affects the expression of lipid related genes in response to LPS. In the present study, we investigated how β-VLDL mediated foam cell formation affects the response of RAW 264.7 murine macrophage cells to LPS with a specific emphasis on the expression of lipid related genes. This response is highly relevant as these genes in concert with the inflammatory genes will determine the regression capacity of existing foam cells and, thus, of atherosclerotic lesion formation.

MATERIALS AND METHODS

Isolation of β-VLDL

β-VLDL was obtained from rats fed a RMH-B diet containing 2% cholesterol, 5% olive oil, and 0.5% cholic acid for 2 weeks (Abdiets). The rats were fasted overnight and anesthetized after which blood was collected by puncture of the abdominal aorta. Serum was centrifuged at 40,000 rpm in a discontinuous KBr gradient for 18 hours as reported earlier[17]. β-VLDL was collected and dialysed against phosphate buffered saline (PBS) containing 1 mM EDTA. Isolated β-VLDL was characterized as described by Van Eck *et al*[18]. Animal experiments were performed at the Gorlaeus Laboratories of the Leiden/Amsterdam Center for Drug Research in

accordance with the national laws. The Ethics Committee for Animal Experiments of Leiden University approved all experimental protocols.

Cell culture and treatment
RAW 264.7 murine macrophage cells were cultured in complete medium containing DMEM supplemented with 10% fetal bovine serum, 100 IU/ml penicillin and 100 µg/ml streptomycin (P/S) and 4 mM L-glutamine (all from BioWitthaker™) at 37°C in an humidified atmosphere with 5% CO_2. Unless stated otherwise, RAW cells ($0.75x10^5$) were seeded in 24-well plates for 16 hours, after which non-adherent cells were removed and adherent cells were loaded with 50 µg/ml β-VLDL in complete medium for 24 hours. Cells were washed twice with PBS to remove excess β-VLDL and serum-components. Subsequently, cells were incubated for 6 hours with 50 ng/ml LPS from Salmonella minnesota R595 (List Biological Laboratories Inc) in DMEM supplemented with 0.2% bovine serum albumin, P/S and 4 mM L-glutamine.

TNF-α detection
After treatment of the RAW cells with β-VLDL and LPS, supernatant was collected and analysed for TNF-α levels by ELISA (OptEIA kit, PharMingen).

Lipid accumulation
RAW cells were seeded on cover glasses (12-mm from Menzel GmbH & Co KG) in a 24-well plate, after which they were incubated with β-VLDL and LPS as described above. Cells were stained for lipid accumulation with oil red O (Sigma) and photographed using a Leica DMRE microscope (1000× magnification) coupled to a video camera.

Gene expression
After treatment with β-VLDL and LPS, cells were washed with ice-cold PBS and lysed using TriZol® reagent (Invitrogen Life technologies). RNA was isolated from cell lysates according to manufacturer's instruction and reverse transcribed using RevertAid™ reverse transcriptase. Gene expression analysis was performed using real-time SYBR Green technology (Eurogentec) with the primers displayed in **Table 1**. Hypoxanthine-guanine phosphoribosyltransferase (HPRT), β-actin and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were used as standard housekeeping genes. Relative gene expression numbers were calculated by subtracting the threshold cycle number (Ct) of the target gene from the average Ct of housekeeping genes and raising 2 to the power of this difference. The average Ct of three housekeeping genes was used to exclude that changes in the relative expression were caused by variations in the separate housekeeping gene expressions. The average Ct values for housekeeping genes did not differ significantly between the groups.

Table 1: Primers for quantitative real-time PCR analysis

Gene	GenBank Accession	Forward primer	Reverse Primer
β-actin	X15267	GGACCCGAGAAGACCTCCTT	GCACATCACTCAGAATTTCAATGG
ABCA1	NM013454	GGTTTGGAGATGGTTATACAATAGTTGT	TTCCCGGAAACGCAAGTC
ABCG1	NM009593	AGGTCTCAGCCTTCTAAAGTTCCTC	TCTCTCGAAGTGAATGAAATTTATCG
ApoA-I	NM009692	ACTCTGGGTTCAACCGTTAGTCA	TCCCAGAAGTCCCGAGTCA
ApoE	NM009696	AGCCAATAGTGGAAGACATGCA	GCAGGACAGGAGAAGGATACTCAT
GADPH	NM008084	TCCATGACAACTTTGGCATTG	TCACGCCACAGCTTTCCA
HPRT	J00423	TTGCTCGAGATGTCATGAAGGA	AGCAGGTCAGCAAAGAACTTATAG
LDLr	Z19521	CTGTGGGCTCCATAGGCTATCT	GCGGTCCAGGGTCATCTTC
LRP-1	NM008512	TGGGTCTCCCGAAATCTGTT	ACCACCGCATTCTTGAAGGA
LXRβ	NM009473	AAGCTGGTGAGCCTGCGC	CGGCAGCTTCTTGTCCTG
PPARα	NM011144	TGAACAAAGACGGGATG	TCAAACTTGGGTTCCATGAT
PPARγ	NM011146	CATGCTTGTGAAGGATGCAAG	TTCTGAAACCGACAGTACTGACAT
SR-BI	NM016741	GGCTGCTGTTTGCTGCG	GCTGCTTGATGAGGGAGGG
SREBP-1	AB017337	GACCTGGTGGTGGGCACTGA	AAGCGGATGTAGTCGATGGC
SREBP-2	AF374267	TGAAGCTGGCCAATCAGAAAA	ACATCACTGTCCACCAGACTGC
TNF-α	X02611	GCCAGCCGATGGGTTGTA	AGGTTGACTTTCTCCTGGTATGAGA

Immunoblotting

RAW cells ($3.0x10^5$) were seeded in a 6 well-plate, after which they were treated with β-VLDL and LPS as described above. Subsequently, cells were washed with ice-cold PBS and lysed in 50 mM Tris-HCl, 150 mM NaCl, 1% Triton X-100, 0.5% deoxycholate, 1% SDS containing 0.02 µg/ml leupeptin, 0.02 µg/ml aprotinin, and 0.02 µg/ml trypsin inhibitor. Cell debris was removed by centrifugation at 10,000 rpm for 10 min. Equal amounts of protein (25 µg) were separated on 7.5% SDS-PAGE gels and electrophoretically transferred to Protran nitrocellulose membrane (Schleicher & Schnell). Immunolabeling was performed using rabbit polyclonal SR-BI (amino acids 496-509 from Abcam) as primary antibody and goat-anti-rabbit IgG (Jackson ImmunoResearch) as secondary antibody. Finally, immunolabeling was detected by enhanced chemiluminescence (ECL plus, Amersham Biosciences), scanned by a Typhoon 9400 variable mode imager (Amersham Pharmacia Biotech) and analysed using ImageQuant™ software (Amersham Pharmacia Biotech).

RESULTS

Foam cell formation

In order to study the effect of foam cell formation on LPS induced changes in lipid related gene expression, we first established foam cell formation. β-VLDL, a cholesteryl ester (CE)-rich lipoprotein present in the plasma of animals fed a cholesterol rich diet[4], is able to induce foam cell formation[19,20]. RAW 264.7 cells were incubated with 50 µg/ml β-VLDL for 24 hours, after which the cells were stained using oil red O to determine the extent of lipid accumulation. Control cells (**Fig. 1A**) and cells treated with 50 ng/ml LPS for 6 hours (**Fig. 1B**), did not show any lipid accumulation, whereas foam cell formation was clearly visible in the cells treated with 50 µg/ml β-VLDL (**Fig. 1C**). LPS treatment for 6 hours did not affect the lipid accumulation in cells pre-incubated with β-VLDL (**Fig. 1D**).

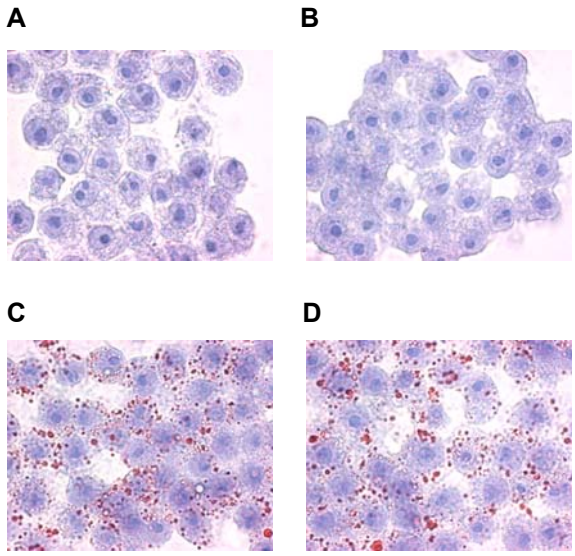

Fig. 1: Effect of LPS and β-VLDL on intracellular lipid accumulation. **A/B** RAW cells were cultured for 24 hours in complete medium, after which they were incubated for 6 hours with **A** serum-free medium or **B** LPS in serum-free medium. **C/D** RAW cells were incubated with β-VLDL in complete medium for 24 hours, after which were incubated for 6 hours with **C** serum-free medium or **D** LPS in serum-free medium. All cells were stained for lipid accumulation using oil red O, after which they were photographed.

TNF-α response

Lipid loading has been shown to influence basal and LPS induced TNF-α secretion[13-16,21], but the effects differed remarkably between the types of lipoprotein used. To investigate the effect of β-VLDL induced lipid loading on TNF-α, we treated RAW cells with 50 µg/ml β-VLDL for 24 hours. Subsequently, cells were incubated with 50 ng/ml LPS in serum-free medium for 6 hours. RNA of the cells was isolated for the detection of TNF-α gene expression by quantitative real-time PCR and supernatant was collected to analyse TNF-α secretion by ELISA. As expected, LPS treatment resulted in a 3.18-fold induction of TNF-α gene expression. Interestingly, the basal gene expression level of TNF-α was significantly 1.29-fold decreased by incubation with β-VLDL. However, pre-incubation with β-VLDL did not prevent or inhibit the TNF-α inducing effect of LPS (**Fig. 2A**). These effects of β-VLDL and LPS were even more profound on TNF-α secretion. β-VLDL induced lipid loading resulted in a 1.82-fold decrease in basal TNF-α secretion, whereas LPS treatment led to a 15.3-fold higher TNF-α secretion. Again, pre-incubation with β-VLDL did not affect LPS induced TNF-α secretion (**Fig. 2B**).

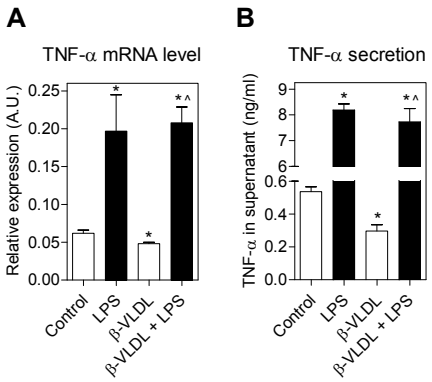

Fig. 2: Effect of LPS and β-VLDL on TNF-α. RAW cells were incubated with β-VLDL in complete medium for 24 hours, after which they were incubated for 6 hours with LPS in serum-free medium. **A** Quantitative real-time PCR was used to determine the gene expression of TNF-α. Values are expressed as relative gene expression compared to housekeeping gene expression (arbitrary units). **B** Supernatant was analysed for TNF-α levels by ELISA. All values are expressed as mean±SEM. Significant differences compared to control: *, significant differences compared to β-VLDL: ^

Receptors for β-VLDL

Several studies show that LPS and/or lipid loading influences the expression of low-density lipoprotein receptor (LDLr), LDLr related protein (LRP)-1 and scavenger receptor class B, type I (SR-BI)[11,12,22-26], which are all involved in β-VLDL uptake. To investigate whether β-VLDL loading modulates the effects of LPS on these genes, RAW cells were treated with β-VLDL and/or LPS as described above, after which RNA was isolated for quantitative real-time PCR-analysis. Treatment of cells with LPS resulted in a significant 1.49-fold decrease in LDLr gene expression and treatment with β-VLDL led to a strong 7.01-fold decrease. Strikingly, pre-incubation with β-VLDL did not only prevent further downregulation of LDLr gene expression by LPS, but in cells pre-incubated with β-VLDL, LDLr gene expression was even significantly 1.46-fold upregulated after LPS treatment (**Fig. 3A**). LRP-1 gene expression was significantly 2.05-fold downregulated by LPS, but was not affected by β-VLDL. Also pre-incubation of cells with β-VLDL, did not affect the ability of LPS to reduce LRP-1 gene expression (**Fig. 3B**). Both LPS and β-VLDL significantly decreased SR-BI gene expression 2.33-fold and 2.63-fold, respectively. After pre-incubation with β-VLDL and treatment with LPS, SR-BI gene expression was 9.09-fold lower than in control cells (**Fig. 3C**). However, we did not observe an effect of LPS on SR-BI protein levels compared to control cells. On the other hand, β-VLDL was able to reduce SR-BI protein levels 2.65-fold ($p<0.01$) compared to control and, strikingly, LPS was able to induce a 2.0-fold decrease of SR-BI protein levels in β-VLDL pre-treated cells ($p<0.05$, **Fig. 3D**). In addition, we analysed gene expression of the VLDL receptor (VLDLr) and CD36, which are also involved in lipoprotein uptake. VLDLr gene expression was not detectable in our system and the expression of CD36 was not affected by β-VLDL and/or LPS (data not shown).

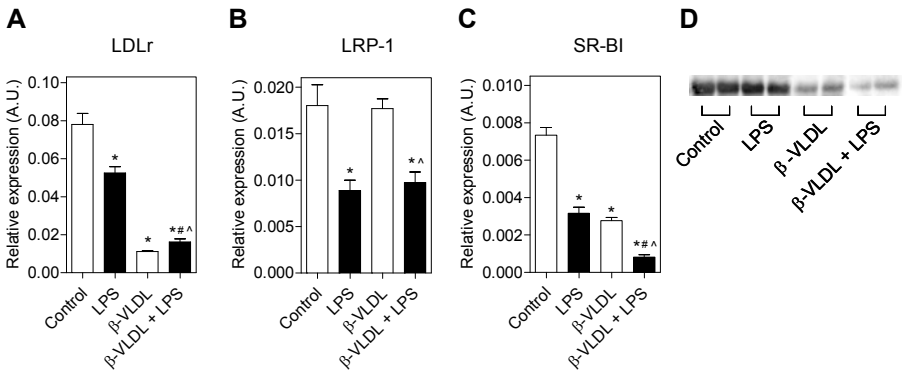

Fig. 3: Effect of LPS and β-VLDL on LDLr, LRP-1 and SR-BI. RAW cells were incubated with β-VLDL in complete medium for 24 hours, after which they were incubated for 6 hours with LPS in serum-free medium. Quantitative real-time PCR was used to determine the gene expression of **A** LDLr, **B** LRP-1 and **C** SR-BI. Values are expressed as relative gene expression compared to housekeeping gene expression (arbitrary units) and expressed as mean±SEM. Significant differences compared to control: *, significant differences compared to LPS: #, significant differences compared to β-VLDL: ^. **D** SR-BI protein levels were determined by western blot.

Cholesterol efflux mediators

LPS and lipid loading are also known to influence cholesterol efflux by affecting the expression of ATP-binding cassette (ABC) transporters ABCA1 and ABCG1[12,24,26,27]. The gene expression of ABCA1 in control cells showed a tendency (p=0.1) towards a 1.41-fold downregulation by LPS treatment, whereas incubation with β-VLDL significantly increased the gene expression 1.76-fold. Strikingly, after β-VLDL pre-incubation, LPS significantly increased the ABCA1 gene expression 2.77-fold compared to the expression in control cells and this expression was significantly higher (1.57-fold) than the expression in cells treated with β-VLDL alone (**Fig. 4A**). LPS significantly reduced ABCG1 gene expression 2.27-fold and β-VLDL significantly induced the expression 1.5-fold. ABCG1 gene expression upon LPS treatment after pre-incubation with β-VLDL was significantly higher than after LPS alone and significantly lower than after β-VLDL alone (**Fig. 4B**), indicating that LPS and β-VLDL affect ABCG1 gene expression in a contrasting manner. Apolipoprotein (apo)A-I and apoE play an important role in cholesterol efflux as extracellular cholesterol acceptors. In addition, apolipoproteins, such as apoA-I and apoE, play a crucial role in the protective effects of lipoproteins on biological responses to LPS, as reviewed by Berbee *et al*[28]. Both LPS and β-VLDL alone did not affect apoA-I gene expression. Strikingly, after pre-incubation with β-VLDL, the cells strongly responded to LPS by upregulating apoA-I gene expression 5.96-fold (**Fig. 4C**). In contrast, LPS significantly reduced apoE gene expression 1.64-fold and β-VLDL alone had no effect, but after β-VLDL pre-incubation, LPS was no longer able to affect apoE gene expression (**Fig. 4D**).

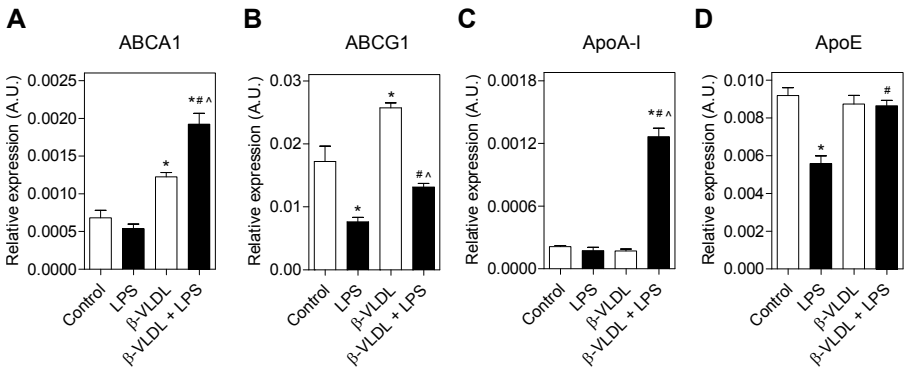

Fig. 4: Effect of LPS and β-VLDL on ABCA1, ABCG1, ApoA-I and ApoE. RAW cells were incubated with β-VLDL in complete medium for 24 hours, after which they were incubated for 6 hours with LPS in serum-free medium. Quantitative real-time PCR was used to determine the gene expression of **A** ABCA1, **B** ABCG1, **C** ApoA-I and **D** ApoE. Values are expressed as relative gene expression compared to housekeeping gene expression (arbitrary units) and expressed as mean±SEM. Significant differences compared to control: *, significant differences compared to LPS: #, significant differences compared to β-VLDL: ^.

Nuclear receptors and transcription factors

Peroxisome proliferator-activated receptors (PPAR), liver X receptors (LXR) and sterol-regulatory element binding proteins (SREBP) are involved in the transcriptional regulation of many lipid related genes, such as the genes discussed above. The gene expression of these nuclear receptors are affected by LPS and/or lipid loading[24,27,29]. Interestingly, PPARα gene expression was not affected by LPS and β-VLDL alone, but was almost 10-fold upregulated by LPS after pre-incubation with β-VLDL (**Fig. 5A**). Gene expression of PPARγ was significantly 4.0-fold downregulated by LPS, but was not affected by β-VLDL. Pre-incubation of cells with β-VLDL did not affect the ability of LPS to reduce PPARγ gene expression (**Fig. 5B**). In our experimental set-up, LPS and/or β-VLDL did not affect gene expression of PPARδ and LXRα (data not shown). LXRβ gene expression was significantly decreased 1.47-fold and 1.43-fold by LPS and β-VLDL, respectively. LPS was able to further decrease LXRβ gene expression to a 1.96-fold lower level than in control cells (**Fig. 5C**). SREBP-1 gene expression was also significantly decreased to similar levels by LPS and β-VLDL, 1.92-fold and 2.01-fold respectively. After pre-incubation with β-VLDL, LPS treatment resulted in a 3.17-fold decrease in gene expression of SREBP-1 compared to the expression in control cells (**Fig. 5D**). LPS significantly 1.73-fold downregulated SREBP-2 gene expression and β-VLDL decreased the expression 4.0-fold. LPS was able to further decrease the gene expression of SREBP-2 to a 6.67-fold lower level than in control cells (**Fig. 5E**).

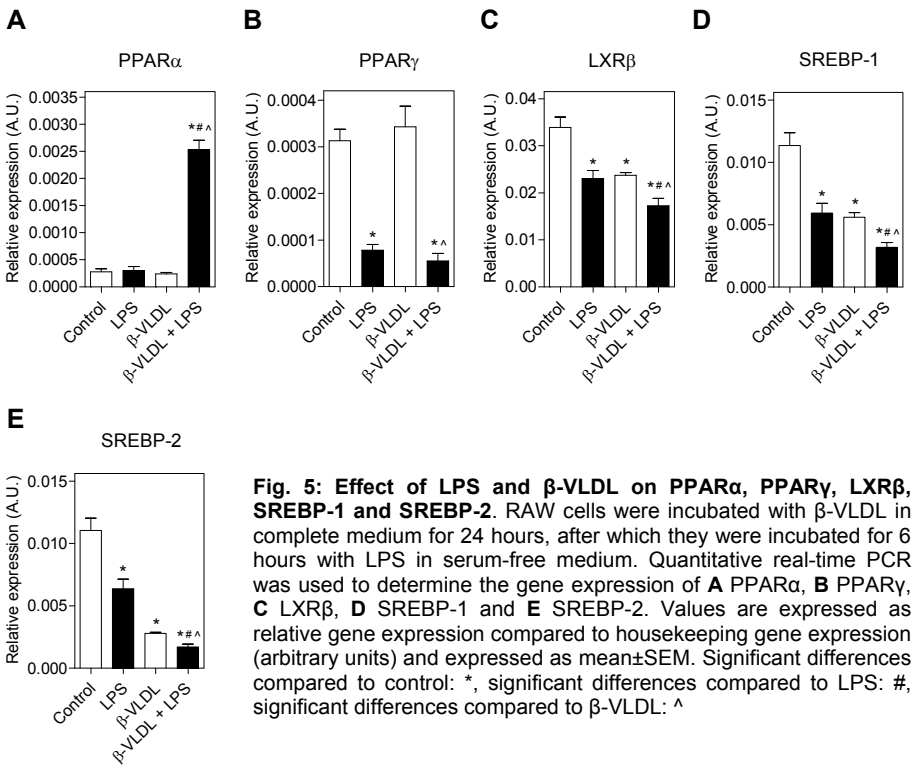

Fig. 5: Effect of LPS and β-VLDL on PPARα, PPARγ, LXRβ, SREBP-1 and SREBP-2. RAW cells were incubated with β-VLDL in complete medium for 24 hours, after which they were incubated for 6 hours with LPS in serum-free medium. Quantitative real-time PCR was used to determine the gene expression of **A** PPARα, **B** PPARγ, **C** LXRβ, **D** SREBP-1 and **E** SREBP-2. Values are expressed as relative gene expression compared to housekeeping gene expression (arbitrary units) and expressed as mean±SEM. Significant differences compared to control: *, significant differences compared to LPS: #, significant differences compared to β-VLDL: ^

DISCUSSION

Recent studies have shown that lipid loading of macrophages influences their inflammatory status and their response to inflammatory stimuli, such as LPS[13-16]. LPS not only induces a pro-inflammatory response in macrophages, but it also affects the expression of many lipid related genes[9,11,12], which play an important role in foam cell formation and, thus, in atherosclerotic lesion development. In the current study we investigated whether foam cell formation using β-VLDL alters the response of macrophages to LPS with a specific emphasis on the expression of lipid related genes.

In human monocyte-derived macrophages, VLDL treatment induced basal TNF-α secretion and potentiated LPS induced TNF-α secretion[15]. In contrast, we now show that β-VLDL reduced basal TNF-α secretion and did not affect LPS induced TNF-α secretion. The discrepancy between the effects of VLDL and β-VLDL on TNF-α can probably be explained by the composition of these lipoproteins: VLDL is rich in triglycerides, whereas β-VLDL is a CE-rich lipoprotein. In line with this hypothesis, acLDL, another CE-rich lipoprotein, has been found to reduce the inducibility of TNF-α in human monocyte-derived macrophages[16]. In addition, oxLDL affects the responsiveness of macrophages to LPS[13,30,31], however, this lipoprotein has characteristics, which are greatly different from β-VLDL, such as inducing apoptosis.

We showed that LPS affected the expression of various lipid related genes in control and β-VLDL pre-incubated macrophages. However, we did not observe any effect of LPS on the intracellular lipid accumulation because of our experimental set-up. Control cells showed no lipid accumulation, indicating that complete medium did not contain foam cell inducers. Cells (pre-) incubated with β-VLDL did accumulate lipids, after which they were carefully washed to remove excess β-VLDL and serum-components. At the time of LPS exposure, no cholesterol donors or acceptors were present, explaining why LPS did not cause a difference in lipid accumulation despite the many effects on the expression of lipid related genes.

We find that LRP-1 is one of the lipid related genes affected by LPS in RAW cells. In fact, LPS was able to reduce LRP-1 expression level in both control cells and cells pre-incubated with β-VLDL. In agreement, LaMarre *et al* demonstrated that LRP-1 expression was markedly decreased by LPS and interferon-γ in RAW cells[11]. In adipocytes, PPARγ has been shown to regulate LRP-1 gene expression and function[32]. We suggest that LRP-1 gene expression in macrophages is also regulated by PPARγ, because the gene expression of PPARγ, like LRP-1, was downregulated by LPS in control cells as well as in cells pre-incubated with β-VLDL. The downregulation of PPARγ by LPS was observed earlier in brown adipocytes[33], but we are the first to report this effect of LPS in macrophages.

In mouse heart and liver, LPS reduced PPARα expression[34,35], but no effect of LPS on macrophage PPARα was reported before. Hoekstra *et al* showed that feeding rats an atherogenic diet increased β-VLDL levels and led to a large increase in PPARα expression in Kupffer cells[24]. We noticed that β-VLDL alone does not alter PPARα expression in RAW cells, but pre-

incubation with β-VLDL made the cells sensitive for LPS induced upregulation of PPARα. The effect on PPARα in Kupffer cells observed by Hoekstra *et al* might be an indirect effect of the increased β-VLDL levels making the Kupffer cells more sensitive for PPARα induction by inflammatory mediators.

Strikingly, the same pattern in gene expression of PPARα was observed for apoA-I in our experiments, suggesting a direct link between expression of PPARα and apoA-I. In agreement, hepatic mRNA levels and plasma levels of apoA-I are regulated by specific PPARα agonists[36,37]. However, until now, it is believed that macrophages do not express or synthesize apoA-I. We are the first to report mRNA expression of apoA-I in macrophages and an increase in apoA-I expression by LPS in cells pre-incubated with β-VLDL, possibly mediated by PPARα. Further research is needed to find out whether the rise in apoA-I mRNA levels leads to actual synthesis of apoA-I in macrophages and to unravel the implications for cholesterol efflux.

The gene expression of apoE, another apolipoprotein, was found to be downregulated by LPS in mouse kidney[27] and upregulated by VLDL in mouse peritoneal macrophages[38]. In agreement, we did show a downregulation of apoE gene expression in RAW cells caused by LPS, but, in contrast, loading of these cells with β-VLDL did not affect apoE gene expression. However, pre-incubation of RAW cells with β-VLDL abolished the inhibiting effect of LPS on apoE expression, indicating that treatment with β-VLDL removed the responsiveness of these cells to LPS induced downregulation of apoE expression.

ABCA1 and ABCG1 prevent accumulation of excess cholesterol and the expression of these cholesterol transporters is regulated by LPS and lipid loading[12,24,26,27]. As expected from literature, downregulation of ABCG1 and, at least a trend towards, downregulation of ABCA1 by LPS was observed in our experiments. In addition, β-VLDL loading of the cells resulted in an increase of ABCA1 and ABCG1 gene expression in an attempt to remove excess cholesterol from RAW cells. Pre-incubation with β-VLDL did not affect the ability of LPS to downregulate ABCG1 expression, but it completely reversed the effect of LPS on ABCA1 expression in RAW cells from reduction into induction.

The responsiveness of RAW cells to LPS effects on LDLr expression was also changed by pre-incubation with β-VLDL. In line with literature[22,39], LDLr expression in RAW cells was decreased by LPS treatment and strongly decreased by β-VLDL loading. After pre-incubation with β-VLDL, LPS was not longer able to further reduce LDLr expression, but even slightly increased LDLr expression compared to the expression in cells loaded with β-VLDL.

The changes in SREBP-1, SREBP-2 and LXRβ gene expression induced by LPS and/or β-VLDL shared the same pattern. Both LPS and β-VLDL downregulated the expression of these genes and LPS was also able to further reduce the expression in β-VLDL loaded cells. In contrast with our data, Wang *et al* showed that LXRα gene expression was suppressed and LXRβ was unaffected by LPS in mouse kidney[27], while we show that LXRβ was downregulated and LXRα was unaffected by LPS in RAW cells. In agreement with our data, they observed a decrease in the expression of diverse LXR target genes, such as apoE, ABCA1, ABCG1 and SREBP-1.

SREBP-2 is suggested to be involved in the regulation of the expression of LRP-1, because LDL induced LRP-1 upregulation is reduced by an inhibitor of SREBP-2 in human vascular smooth muscle cells[40]. However, treatment of RAW cells with β-VLDL resulted in a dowregulation of SREBP-2, but LRP-1 gene expression was not affected by β-VLDL incubation. Moreover, we show that downregulation of PPARγ did coincide with LRP-1 downregulation, indicating that not SREBP-2 but PPARγ is more likely to be responsible for the regulation of LRP-1 expression in macrophages.
In line with studies of Baravova *et al* and Han *et al*[12,41], both LPS and β-VLDL downregulated SR-BI gene expression in our experiments. However, Khovidhunkit *et al*[23] showed that, in contrast to their results in liver, the effect of LPS on SR-BI gene expression is not always representative for the effect on protein level in RAW cells. Therefore, we analysed SR-BI protein level and, in agreement with Khovidhunkit *et al*, LPS was not able to decrease SR-BI protein levels in control RAW cells. In contrast, the reduction in SR-BI gene expression induced by β-VLDL was also observed on protein level and in cells pre-incubated with β-VLDL, LPS was able to further decrease both mRNA and protein levels of SR-BI. In the liver, but not all tissues, regulation of SR-BI protein expression is dependent on the adaptor protein PDZK1 and not solely on SR-BI mRNA levels[42,43]. Until now it is unknown whether PDZK1 plays a role in the regulation of SR-BI protein expression in macrophages, but it might explain the different effect of β-VLDL and LPS on SR-BI protein level, while they both reduce mRNA levels of SR-BI.
In short, the effect of LPS on gene expression of LRP-1, SR-BI, ABCG1, PPARγ, LXRβ, SREBP-1 and SREBP-2 was similar for both control cells and cells pre-incubated with β-VLDL. However, the effect of LPS on LDLr, ABCA1, apoA-I, apoE and PPARα gene expression and SR-BI protein level was potentiated, attenuated or even reversed by pre-incubation of the cells with β-VLDL, as illustrated in **Fig. 6**. SR-BI protein level in macrophages is unaffected by LPS, but in foam cells, LPS treatment results in a decrease of SR-BI protein level, thereby inhibiting cholesterol efflux to HDL and cholesterol uptake. On the other hand, in macrophages under normal conditions, LPS inhibits gene expression of mediators of cholesterol efflux to apoA-I and apoE, whereas in β-VLDL loaded foam cells, these mediators are upregulated by LPS. The coinciding downregulation of LDLr in macrophages and upregulation in foam cells, suggests that, overall, LPS induces a higher intracellular cholesterol content in macrophages and a lower intracellular cholesterol content in foam cells.
In conclusion, both LPS and β-VLDL did affect many lipid related genes in RAW cells. In addition, our results show that foam cell formation induced by β-VLDL markedly alters the effects of LPS on these lipid related genes. These data provide a better understanding of the complex interaction between lipid metabolism and inflammation in macrophages, which is important to unravel the mechanism underlying atherogenesis.

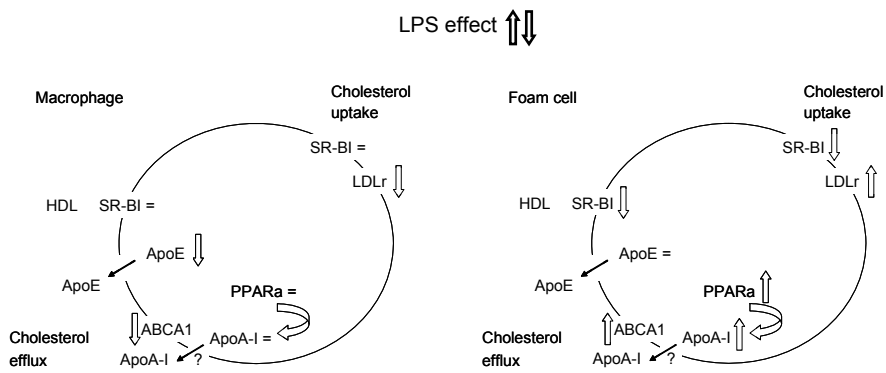

Fig. 6: Schematic overview of different response of macrophages and foam cells to LPS.

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