ABC transporters and scavenger receptor BI : important mediators of lipid metabolism and atherosclerosis

Meurs, I.

Chapter 7

Identification of novel macrophage cholesterol-responsive genes in peritoneal and bone marrow-derived macrophage foam cells

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ABSTRACT

Objective- Excessive accumulation of cholesterol by macrophages leading to their transformation into foam cells is the earliest pathological hallmark of atherosclerosis. Therefore, the mechanisms by which macrophages regulate cholesterol homeostasis are of high interest. The objective of this study was to identify new genes that are differentially expressed during macrophage foam cell formation induced by βVLDL, acetylated LDL (acLDL), and oxidized LDL (oxLDL), lipoproteins commonly used for generating lipid-laden macrophages that mimic macrophage foam cells in atherosclerotic lesions.

Methods and Results- Peritoneal macrophages (PM) or bone marrow-derived macrophages (BMDM) were loaded with βVLDL, acLDL, or oxLDL for 48h and, subsequently, tRNA was isolated for microarray analysis. Interestingly, the use of the 3 types of pro-atherogenic lipoproteins led to different patterns of lipid accumulation in both types of macrophages, visualized using oil red O staining. Furthermore, 3214 genes in PM and 3062 genes in BMDM from the original database of 42851 genes were found to be significantly regulated upon loading with the pro-atherogenic lipoproteins. Four novel genes of high interest were identified, namely MRC1, CLEC4N, SORT1, and SCARF2, which are implicated in receptor-mediated endocytosis.

Conclusion- Four genes involved in receptor-mediated endocytosis have been identified, namely MRC1, CLEC4N, SORT1, and SCARF2, which represent possible new entities which may be relevant for macrophage lipoprotein handling. These entities might serve as targets to modulate macrophage foam cell formation and the initiation of atherosclerotic lesion development.

INTRODUCTION

Epidemiological studies have unequivocally shown that high levels of plasma apolipoprotein B (apoB)-containing lipoproteins are an important risk factor atherosclerosis. One of the earliest events in atherosclerosis is the adherence of monocytes to the endothelium and the transmigration into the arterial intima, where they differentiate into macrophages. Upon differentiation, macrophages start to accumulate large amounts of lipids by the uptake of apoB-containing lipoproteins, leading to the formation of macrophage-derived foam cells.[1-3] Extensive studies have shown that the apoB containing low-density lipoprotein (LDL) has a major role in foam cell formation. Uptake of LDL via the low-density lipoprotein receptor (LDLr) is subject to feedback regulation of the LDLr. Therefore accumulation of excessive amounts of LDL-derived cholesterol by macrophages is thought to require modification of LDL in a way that permits rapid unregulated internalization.[4] It is now well established that oxidized LDL (oxLDL) rather than native LDL is mainly responsible for the build-up of cholesterol in atherosclerotic lesions.[5]

Macrophages take up modified LDL, such as oxLDL and acetylated LDL (acLDL), through the macrophage scavenger receptor pathway, including the scavenger receptors CD36, scavenger receptor A (SR-A), lectin-like oxidized low-density lipoprotein receptor-1 (LOX-1), and scavenger receptor B type I (SR-BI).[6,7] In addition, cholesterol from native unmodified LDL is also taken up, although to a lesser extent, via the LDLr, LDL receptor-related protein 1 (LRP1), very-low-density lipoprotein receptor (VLDLr), and SR-BI.[7] β-Very low-density lipoprotein (βVLDL), a lipoprotein fraction that accumulates in the plasma of patients with the genetic disorder type III hyperlipoproteinemia and in experimental animals fed a high-cholesterol diet, is another type of atherogenic lipoprotein which efficiently transforms macrophages into foam cells.[8] The primary receptors responsible for the uptake of βVLDL by macrophages are the LDLr[9-11] and SR-BI.[11,12] SR-BI promotes the selective uptake of CE from lipoproteins [12,13], while the LDLr, LRP1, and VLDLr take up lipids via the classical receptor-mediated endocytosis pathway. Upon receptor-mediated endocytosis, the pro-atherogenic particles are delivered through endosomes to lysosomes, where at an acidic pH, the protein and lipid components of the lipoprotein are degraded to products that can easily transverse the lysosomal membrane.[4] The proteins are subjected to proteolytic hydrolysis, whereas the cholesterol component, mainly cholesteryl ester (CE), is hydrolyzed by acidic cholesteryl ester hydrolase (ACEH).[14] The resulting excess free cholesterol is then transported across lysosomal membranes to the endoplasmic reticulum, where it is re-esterified to CE by the enzyme acyl-CoA:cholesterol acyltransferase (ACAT).[15,16] This cholesteryl ester is then stored in the cytoplasm as cytosolic lipid droplets where it can be continually hydrolyzed by a neutral cholesterol esterase and re-esterified by the enzyme ACAT.[4,17] Upon lipid droplet formation, lipid esters accumulate between the two leaflets of the endoplasmic reticulum membrane (or other membranes), gradually grow into a globular shape, and are finally pinched off from the ER to become independent cytosolic lipid droplets.[18,19] Different proteins have been identified to be associated with lipid droplets i.e. stabilization of lipid droplets, including cell death inducing DFFA-like effector (Cide), perilipin (Plin), adipocyte differentiation-related protein (ADRP; also called as adipophilin), and tail-interacting protein

of 47 kDa (TIP47).[20-22] Excessive accumulation of CE, stored as cytoplasmic lipid droplets, leads to formation of macrophage foam cells, the pathological hallmark of atherosclerotic lesion development. Since only free cholesterol can be transported from the cells to extracellular cholesterol acceptors, hydrolysis of CE into free cholesterol, catalyzed by neutral cholesteryl ester hydrolase (CEH), is an obligatory step in the removal of excess cholesterol from macrophages.[23, 24] Subsequently, free cholesterol can be transported to ApoA-I or lipid-poor ApoA-I, a process mediated by ABCA1, or can be re-esterified by the enzyme ACAT. In addition to ABCA1, ABCG1 has also been implicated in cholesterol efflux from macrophages. ABCG1 mediates cellular cholesterol and phospholipid efflux from macrophages to mature HDL and other extracellular phospholipid-containing acceptors, but not to lipid-free apolipoproteins.[25]

Overall, macrophage lipid homeostasis involves several processes, such as lipid uptake, lipid storage, intracellular transport, and lipid efflux. Therefore, to prevent macrophage foam cell formation, proteins of different pathways can be modulated to improve lipid homeostasis. To date improved knowledge about the specific pathways involved in macrophage lipid homeostasis might lead to new target identification. Identification of novel cholesterol-responsive genes will increase the knowledge of proteins involved in the maintenance of macrophage cholesterol homeostasis, which may lead to the development of novel therapeutic targets to prevent macrophage foam cell formation.

The aim of this study was to identify new genes that are differentially expressed during macrophage foam cell formation (peritoneal macrophage (PM) or bone marrow-derived macrophage (BMDM)) induced by the commonly used pro-atherogenic lipoproteins βVLDL, oxLDL, and acLDL.

MATERIALS AND METHODS

Animals

Female C57Bl/6 mice, 10 weeks of age, and maintained on sterilized regular chow diet containing 4.3% (w/w) fat and no added cholesterol (RM3, Special Diet Services, Witham, UK) and water *ad libitum* were used. Animal experiments were performed at the Gorlaeus Laboratories of the Leiden/Amsterdam Center for Drug Research in accordance with the National Laws. All experimental protocols were approved by the Ethics Committee for Animal Experiments of Leiden University.

Isolation of lipoproteins

Beta-very-low-density lipoprotein (βVLDL) 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.[26] βVLDL (density <1.019 g/mL) was collected and dialysed against phosphate buffered saline, containing 1 mM EDTA (PBS/1mM EDTA). Isolated βVLDL was characterized as described previously.[27] Furthermore, low-density lipoprotein (LDL) (density 1.063 to 1.019 g/mL) was isolated from plasma of healthy human volunteers by

ultracentrifugation in a KBr discontinuous gradient and dialysed against PBS/1mM EDTA according to Redgrave *et al.*[26]. For generation of oxLDL, LDL was oxidatively modified by incubation of 200 µg/mL of LDL with 10 µM $CuSO_4$ at 37°C for 20 h. Oxidation was terminated by dialysis against PBS containing 0.5 mM EDTA for at least 24 h. For generation of acLDL, LDL was acetylated by repeated additions of acetic anhydride according to Basu *et al.*[28]. Concentrations of βVLDL, oxLDL, and acLDL were based on protein content using the BCA assay. Cholesterol levels of βVLDL, acLDL and oxLDL were quantified using enzymatic colorimetric assays with 0.048 U/mL cholesterol oxidase (Sigma), 0.03 U/mL cholesteryl esterase (Seikagaku, Tokyo, Japan), and 0.065 U/mL peroxidase (Roche Diagnostics, Mannheim, Germany) in reaction buffer (1.0 KPi buffer, pH=7.7 containing 0.01 M phenol, 1 mM 4-amino-antipyrine, 1% polyoxyethylene-9-laurylether, and 7.5% methanol). Precipath (standardized serum; Roche Diagnostics, Mannheim, Germany) was used as internal standard.

Peritoneal macrophage harvesting

Five days after peritoneal injection of 1mL of 3% Brewer thioglycollate medium (Difco, Detroit, MI) in C57/Bl6 mice, peritoneal macrophages (PM) were harvested by lavage of the peritoneal cavity with 10mL of PBS. After three washing steps, the cells were plated in multiwell culture dishes with DMEM containing 10% fetal calf serum (FCS). After 4 hours the non adherent cells were removed by washing and the adherent macrophage were cultured overnight in DMEM containing 10% FCS until start of the experiment.

Generation of bone marrow-derived macrophages

Bone marrow cells, isolated from female C57/Bl6 mice, were cultured for 7 days in complete RPMI medium supplemented with 20% FCS and 30% L929 cell-conditioned medium, as the source of macrophage colony-stimulating factor (M-CSF), to generate bone marrow-derived macrophages (BMDM). After 7 days of culture, the bone marrow-derived macrophages were harvested using 4mM EDTA, washed three times and plated in multiwell culture dishes with DMEM containing 10% FCS until start of the experiment.

Macrophage lipid loading

PM or BMDM were incubated with βVLDL (50 µg/mL), acLDL (50 µg/mL), or oxLDL (20 µg/mL) in DMEM containing 0.2% BSA for 48 hours at 37°C. Subsequently, the cells were washed three times with PBS, fixed in 3.7% neutral-buffered formalin (Formal-fixx; Shandon Scientific Ltd, UK) for 30 minutes, and stained with oil Red O to visualize neutral lipid accumulation or snap frozen in liquid nitrogen and stored at -80°C until tRNA isolation.

Microarray protocol

tRNA was isolated from PM or BMDM loaded with βVLDL, acLDL, or oxLDL (3 samples per condition) using an RNAeasy mini kit (Qiagen, Chatsworth, CA) for microarray analysis. Upon receipt of the tRNA samples, ServiceXS (Leiden, The Netherlands) analyzed the concentration and the integrity of the RNA samples using the NanoDrop ND-100 Spectrophotometer and Agilent 2100 Bioanalyzer, respectively. Amplification and labeling of the RNA samples was performed according to the manufacturer's specifications (Illumina, San Diego, CA). Hereto the Ambion® Illumina TotalPrep RNA Amplification Kit (Ambion,

#IL1791) was used, which generates biotinylated, amplified cRNA. Hybridisation of the labelled RNA samples to the MouseWG-6 v2.0 array, for analysis of 45281 mouse targets per sample, was performed according to manufacturer's specifications (Illumina, San Diego, CA). Signal was developed with streptavidin-Cy3 and the BeadChip is scanned with the Illumina BeadArray Reader (Illumina, San Diego, CA). Microarray data were acquired and imported in Excel (Microsoft,Excel 2010) for further analysis.

Data analyses

Genes were considered reliably expressed when expression was observed in all the arrays with a detection values > 0.05. Consequently, detection values <0.05 were removed from the database. The original database of the microarray consisted of 45281 genes, however, after correction for the low detection values, the database of PM and BMDM consisted of 34512 and 34496 genes, respectively. The means of the triplicates obtained for the individual genes for each loading condition were calculated and expressed as relative to control non-foamy cells. Significant differences between the indicated loading conditions and control non-foamy cells were calculated by using the two-tailed student's T-test. Hierarchical clustering of significant genes was performed using TIBCO Spotfire 3.1 (http://spotfire.tibco.com). Genome wide analysis at molecular function and biological process level was performed using the Panther database. (www.pantherdb.org).[29]

Statistical analyses were performed using a two-tailed student's T-test. P value <0.05 were considered significant.

RESULTS

Different lipoproteins induces distinct loading patterns in PM and BMDM

We designed this experiment to identify new genes that are differentially expressed during macrophage foam cell formation. To induce foam cell formation *in vitro*, macrophages (PM and BMDM) were loaded with βVLDL, acLDL, or oxLDL. Both oxLDL and βVLDL are physiologic atherogenic lipoproteins. OxLDL is generated by oxidation of native LDL in the vessel wall[30, 31], while βVLDL is a lipoprotein fraction that accumulates in the plasma of patients with the genetic disorder type III hyperlipoproteinemia[32] and in experimental animals fed a high-cholesterol diet[33, 34]. AcLDL is chemically modified, but nevertheless a commonly used pro-atherogenic lipoprotein to induce foam cell formation. Evidently, the different lipoproteins exhibited different total cholesterol (TC) levels. βVLDL is highly enriched in cholesterol (10.0 μg TC/μg protein), whereas acLDL and oxLDL contained 1.80 μg TC/μg protein and 0.5 μg TC/μg protein, respectively.

The effects of βVLDL, acLDL, and oxLDL on macrophage foam cell formation were investigated by incubation of PM and BMDM with the respective lipoproteins for a period of 48 hours. Interestingly, the use of the different lipoproteins led to distinct patterns of lipid accumulation in both types of macrophages (Fig. 1). As shown, lipid droplets unite and form large intracellular lipid deposits in macrophages after βVLDL loading. AcLDL loading resulted in small cytosolic lipid droplets, distributed throughout the macrophage,

while loading with oxLDL resulted in a more diffuse lipid distribution. Interestingly, also clear differences were found in lipid loading patterns between PM and BMDM after βVLDL loading. The large intracellular lipid deposits in PM were distributed throughout the cell, whereas βVLDL-induced lipid droplets were primarily located near the plasma membrane in BMDM. Furthermore, acLDL-induced foam cell formation appeared more evident in BMDM compared to PM, while oxLDL-induced foam cell formation was increased in PM compared to BMDM.

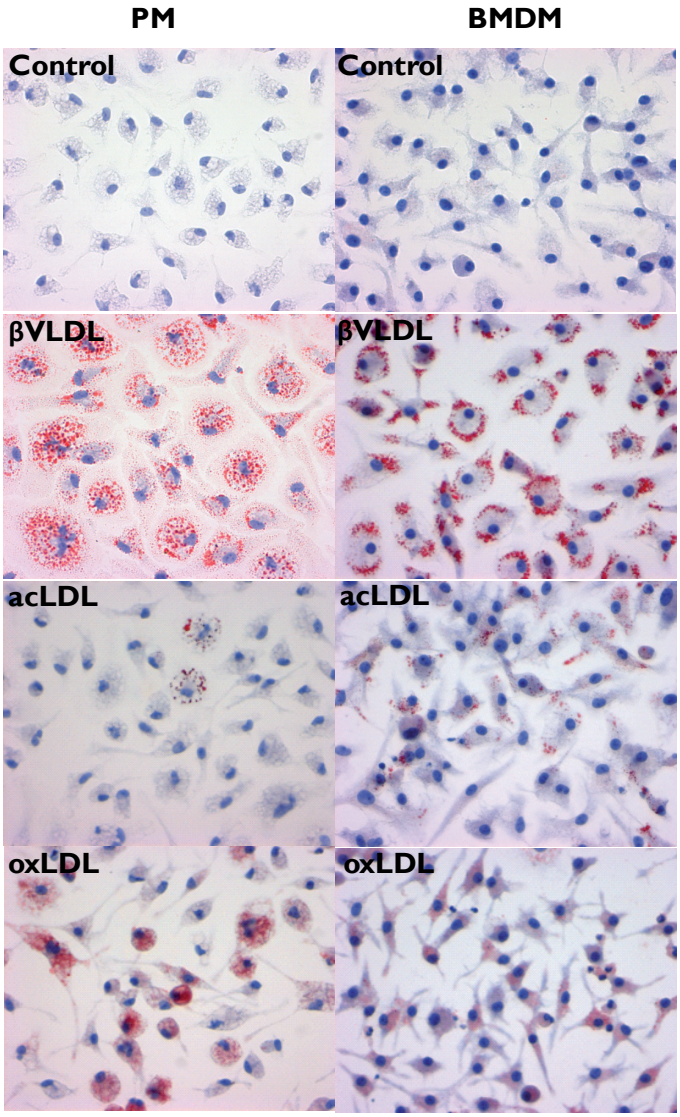

Fig. 1. The effect of different lipoproteins on the lipid loading pattern of PM (A) and BMDM (B)
Peritoneal macrophages (PM) and bone marrow-derived macrophages (BMDM) were incubated with βVLDL (50 μg/ml), acLDL (μg/ml), or oxLDL (20 μg/ml) for 48 hours. Lipid accumulation was visualized with oil red O staining. Original magnification x400

mRNA expression levels of key genes involved in lipid metabolism in PM and BMDM

The effects of the different modified lipoproteins on mRNA expression levels of genes which are anticipated to play role in macrophage lipid homeostasis, including those involved in lipoprotein uptake, storage, and metabolism were determined in PM and BMDM. The genes of interest are all listed in Table 1, of which significantly differentially expressed genes in PM and BMDM compared to control non-foamy cells are indicated in Table 2A and Table 2B, respectively. mRNA expression of LOX-1 was too low to represent reliable expression levels, therefore this gene was excluded from the results.

Table 1. Genes of interest involved in macrophage lipid homeostasis

Genes		
ABCA1	CideC	PPARα
ABCG1	HMGCR	PPARβ
ACAT1	LDLR	PPARγ
ACAT2	LRP1	PPARδ
ACAT3	LXR	SR-AI
CD36	Plin	SR-BI
Cideb	Plin4	VLDLR

ABC= ATP binding-cassette transporter; ACAT= acetyl-Coenzyme A acetyltransferase; Cide= cell death inducing DFFA-like effector; HMGCR= 3-hydroxy-3-methylglutaryl-CoA reductase; LDLr= low-density lipoprotein receptor; LRP1= low-density lipoprotein receptor-related protein 1; LXR= liver x receptor; Plin= perilipin; PPAR= peroxisome proliferator activated receptor; SR-BI= scavenger receptor class BI; SR-A= scavenger receptor A; VLDLr= very low-density lipoprotein receptor.

Table 2A. Genes of interest involved in macrophage lipid homeostasis, which are significantly regulated in PM upon foam cell formation compared to control non-loaded PM

Gene	βVLDL Fold-change	acLDL Fold-change	oxLDL Fold-change	T-test βVLDL	T-test acLDL	T-test oxLDL
ABCG1	1.65	1.28	1.28	<0,05	ns	ns
ACAT2	0,42	0,34	0,42	<0,001	<0,001	<0,001
HMGCR	0,67	0,64	0,67	<0,01	<0,001	<0,01
LDLR	0,38	0,40	0,52	<0,001	<0,001	<0,01
LRP	1,02	0,88	0,75	ns	ns	<0,05

ACAT= acetyl-Coenzyme A acetyltransferase; HMGCR= 3-hydroxy-3-methylglutaryl-CoA reductase; LDLr= low-density lipoprotein receptor; LRP= low-density lipoprotein receptor-related protein; ns= not significant.

Table 2B. Genes of interest involved in macrophage lipid homeostasis, which are significantly regulated in BMDM upon foam cell formation compared to control non-loaded BMDM

Gene	βVLDL Fold-change	acLDL Fold-change	oxLDL Fold-change	T-test βVLDL	T-test acLDL	T-test oxLDL
ACAT2	0,34	0,41	0,49	<0,01	<0,01	<0,05
HMGCR	0,72	0,83	1,07	<0,05	ns	ns
LDLR	0,35	0,50	0,65	<0,01	<0,05	<0,05
$PPAR_{\delta}$	0,61	1,27	2,60	ns	ns	<0,05
VLDLR	1,44	1,18	1,11	<0,01	ns	ns

ACAT= acetyl-Coenzyme A acetyltransferase; HMGCR= 3-hydroxy-3-methylglutaryl-CoA reductase; LDLr= low-density lipoprotein receptor; PPAR= peroxisome proliferator activated receptor; VLDLr= very low-density lipoprotein receptor; ns= not significant.

First, genes involved in lipoprotein uptake i.e. CD36, LRP1, LDLr, SR-A, SR-BI, and VLDLr were evaluated. In BMDM, foam cell formation induced by βVLDL loading led to a significant increase in VLDLr gene expression (1.4-fold, $p<0.01$). Furthermore, LDLr expression was significantly reduced (~0.45-fold) in both PM and BMDM after incubation with βVLDL, acLDL, or oxLDL compared to non-foam cells (Table 2A and 2B). The expression of CD36, SR-A, and SR-BI were not significantly regulated upon loading of PM and BMDM with βVLDL, acLDL, or oxLDL. Gene expression of 3-hydroxy-3-methylglutaryl-CoA reductase (HMGCR) which is the rate-controlling enzyme of endogenous cholesterol synthesis, was significantly decreased in both PM and BMDM upon lipid loading compared to control cells. Reduced LDLr and HMGCR expression levels upon lipid loading were in agreement with previous studies[35, 36], indicating cholesterol accumulation within the macrophages.

Secondly, mRNA expression analysis of genes involved in intracellular lipid storage and trafficking showed that gene expression of ACAT2, a major cholesterol esterification enzyme, was significantly reduced in both types of macrophages compared to control cells. In addition, mRNA expression levels of the nuclear receptors peroxisome proliferator activated receptors (PPAR) and liver x receptor (LXR) in PM and BMDM upon lipid loading were determined. In BMDM, foam cell formation induced by βVLDL loading resulted in increased PPARδ gene expression levels after oxLDL loading (2.6-fold, $p<0.05$). Strikingly, genes involved in lipid droplet formation were determined, including Cide b and c, Plin, and Plin4 showed no differences in mRNA expression levels after loading of PM and BMDM with βVLDL, acLDL, or oxLDL. Unfortunately, gene expression analysis of ADRP (plin2) and TIP47 (plin3) was not included in the mouseWG-6 v2.0 array. Thus, the distinct loading patterns observed in PM and BMDM upon incubation with the different lipoproteins cannot be explained by changes in the expression of genes involved in lipid droplet formation.

Third, mRNA expression analyses of genes involved in cholesterol efflux showed that PM loaded with the three pro-atherogenic lipoproteins exhibited increased expression of ABCG1, which only reached statistical significance in PM loaded with βVLDL (βVLDL: 1.65-fold, $p<0.05$; acLDL: 1.28-fold, $p>0.05$; oxLDL: 1.28-fold, $p>0.05$). A remarkable finding, however, was that ABCA1 was not significantly induced in both types of macrophages upon incubation with the different lipoproteins.

Identification of novel genes regulated upon loading of PM and BMDM with different lipoproteins

Once the genes with a detection value <0.05 were removed from the original database of 42851 genes, the database of PM and BMDM consisted of 34512 and 34496 genes, respectively. For PM, 3214 genes out of the 34512 detectable genes were significantly regulated upon lipid loading, whereas for BMDM 3062 genes out of the 34496 genes were significantly regulated. The number of genes that were specifically regulated upon incubation with the different lipoproteins are illustrated in Fig. 2. Interestingly, in both PM and BMDM, oxLDL loading resulted in a higher amount of significantly changed genes than βVLDL and acLDL loading (oxLDL: 1653 and 1395 genes for PM and BMDM respectively, versus 166 and 779 genes with βVLDL and 561 and 123 genes with acLDL for PM and BMDM, respectively).

First, the genes which were significantly changed upon incubation with all three lipoproteins were identified (PM: 184 genes, BMDM: 186 genes, as these are likely candidates to be

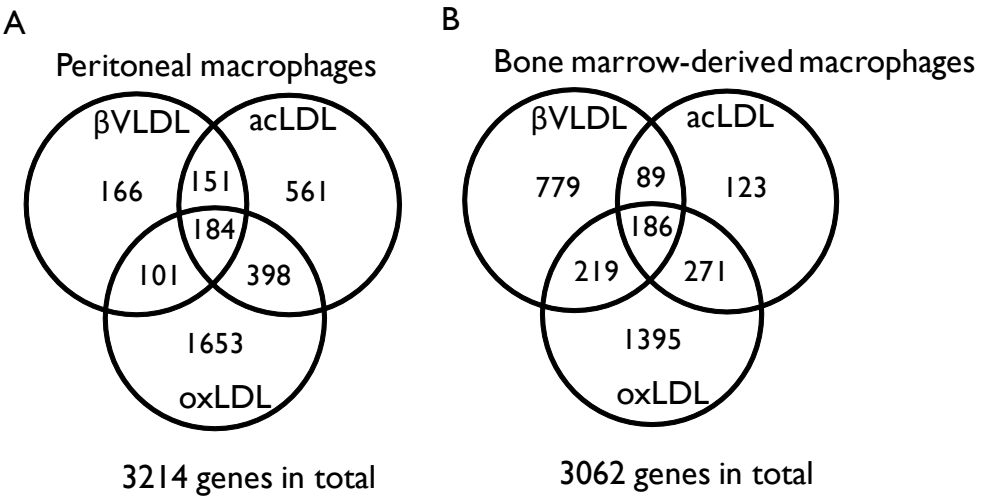

Fig. 2. Distribution of significantly regulated genes over the modified lipoproteins βVLDL, acLDL, and oxLDL in PM (A) and BMDM (B).
Of the 3214 genes found to be regulated during foam cell formation of PM, 184 genes were regulated upon incubation with βVLDL, acLDL, and oxLDL. For BMDM, 186 genes of in total 3062 were regulated by all 3 types of lipoproteins.

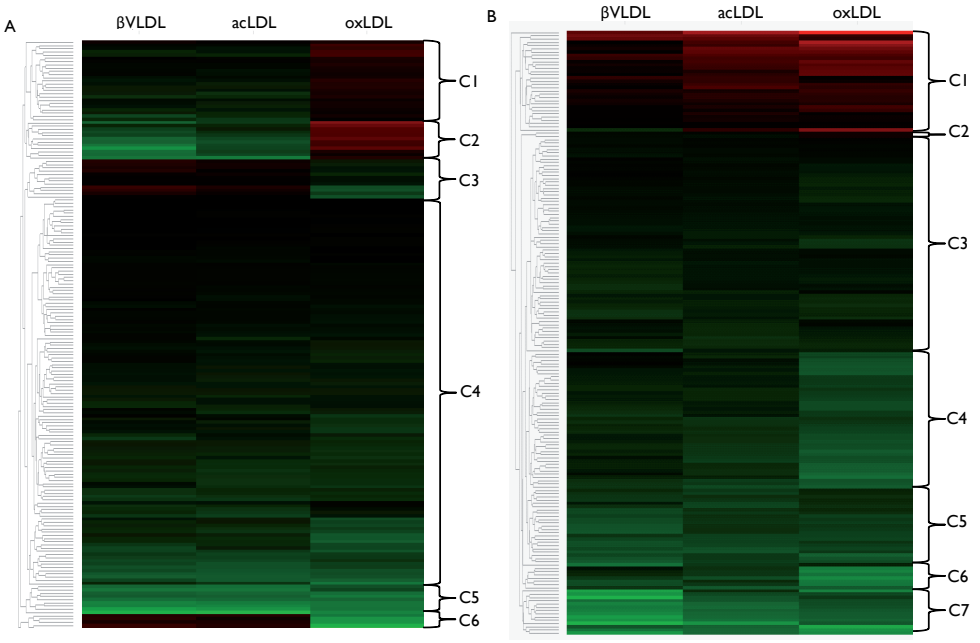

Fig. 3. Hierarchical clustered display of genes which are significantly regulated in PM (A) and BMDM (B) with βVLDL, acLDL, and oxLDL.
The color scale ranges from saturated green to saturated red, indicating low to high expression against the average relative expression in non-foamy control cells. Identification of the genes present in the specific clusters of PM and BMDM are listed in Table 4A and 4B, respectively.

strongly involved in macrophage lipid homeostasis. Hierarchical clustering of this specific group of genes is displayed in Fig. 3A and 3B. Identification of the genes present in the specific clusters of PM and BMDM are listed in Table 3A and 3B. In the hierarchical clustering of genes significantly regulated in PM, 6 clusters were identified of which 4 showed different expression patterns with the three pro-atherogenic lipoproteins, including cluster 1,2,3, and 6 (Fig. 3A). Cluster 1 and 2 represent genes which were significantly downregulated in PM loaded with βVLDL and acLDL and significantly upregulated upon loading with oxLDL compared to non-foamy control cells. Cluster 1 contains the genes dual specificity phosphatase 4 (DUSP4), a member of the superfamily of protein-tyrosine phosphatises, that are involved in the regulation of MAPK signaling in response to oxidative stress [37] and B-cell CLL/lymphoma 2A1C (BCL2A1C), which plays a role in inhibition of apoptosis[38]. DUSP4 and BCL2A1C are significantly increased in PM after loading with oxLDL (2.7-fold, $p<0.001$ and 1.4-fold, $p<0.05$, respectively). Several studies have shown that oxLDL reduces the expression of Bcl-2 [39, 40], thereby promoting the cellular susceptibility to apoptosis. On the contrary, Bcl-2 expressing cells have an enhanced capacity to suppress oxidative stress signals [41]. The increased expression of DUSP4 and BLC2A1C, observed in the present study, might indicate that oxLDL loading induces oxidative stress, which might cause increased susceptibility to apoptosis. As a result expression of BLC2A1C is induced due to a feedback mechanism to suppress these oxidative stress signals. On the contrary, expression of DUSP4 and BCL2A1C were significantly downregulated upon loading of PM with βVLDL and acLDL (βVLDL: 0.6-fold, $p<0.01$ and 0.7-fold, $p<0.05$, respectively; acLDL: 0.7-fold, $p<0.01$ and 0.7-fold, $p<0.05$, respectively).

Cluster 2 contains mainly transport genes which are involved in small molecule transport/ extracellular transport, including solute carrier family 6A12 (SLC6A12), and amino acid transport, including SLC6A1 and SLC7A11. The expression of SLC6A12, SLC6A1, and SLC7A11 was significantly upregulated after oxLDL loading and downregulated upon βVLDL and acLDL loading compared to control cells. Cluster 3 represents genes in PM which are significantly upregulated in PM loaded with βVLDL and acLDL and significantly decreased by oxLDL loading. A gene of particular interest found in cluster 3 is Mrc1 mannose receptor, C type 1 (MRC1). MRC1 is a member of the mannose receptor C-type lectin superfamily, that represent a unique group of multifunctional receptors.[42] A characteristic feature of this family is that they all have the ability to be rapidly internalised from the plasma membrane via clathrin-coated vesicles for delivery into the endosomal system and therefore at least a major part of their function involves ligand delivery into intracellular compartments.[42] The expression of MRC1 is increased 2.4-fold and 1.8-fold by βVLDL ($p<0.01$) and acLDL ($p<0.05$) loading, and decreased 2.5-fold with oxLDL ($p<0.05$).

Cluster 4 represents genes in PM which are all significantly downregulated upon loading with all 3 types of lipoproteins. A gene of interest in this cluster is C-type lectin domain family 4, member n (CLEC4N), which is also a member of the mannose receptor C-type lectin superfamily and, like MRC-1, also plays a role in receptor-mediated endocytosis [42]. CLEC4N showed a highly significant >2-fold decrease in expression for all three lipoproteins compared to control non-loaded cells (βVLDL: 2.4-fold, $p<0.05$; acLDL: 2.2-fold, $p<001$; oxLDL: 3.0-fold, $p<0.001$). Cytochrome P450, family 51 (CYP51) and sterol-C4-methyl oxidase-like (SC4MOL) are genes involved in cholesterol metabolism[29] and their expression

Table 3A. Clusters 1-6 of genes which are significantly regulated in βVLDL, acLDL, and oxLDL-loaded PM compared to non-loaded control cells

Cluster 1					
1190002H23RIK	CBR3	IAP	MGST2	SFXN1	TSHZ1
4933428A15RIK	DUSP4	LAT	PANX1	SLC39A4	UBE2E2
ACPP	ETS2	LOC630729	PTGES	SLC7A11	YBX3
BCL2A1C	HVCN1	MET	RAMP3	TRAF1	

Cluster 2					
CLEC4E	GPR68	INHBA	IRG1	SLC6A12	SLC7A11
CLECSF9	H2-M2	IRG1	ORM1	SLC6A9	

Cluster 3					
CDC42EP3	HEXB	MRC1	PTGDS2	SEPP1	TFRC
CFP	ITM2B	PLXNC1	RNASE4	SLC16A6	

Cluster 4					
0610007P14RIK	ALDH1L1	DHCR7	LBR	NFKBIA	SLC25A1
1810033B17RIK	ALDOC	DPYSL2	LDLR	NSDHL	SLC6A13
2400009B08RIK	ATG9A	FADS1	LOC383368	OLFM1	SLC9A3R2
2810002I04RIK	ATG9B	FDPS	LOC637711	OLFR1	SLFN2
4833426J09RIK	BC003324	FXYD2	LSS	PANK1	SOCS3
4930415G15RIK	BEST1	GALNT9	LUZP1	PCSK9	SPINT1
5830472M02RIK	C3	GNG12	LY6C1	PCYT1A	SPP1
6230425C21RIK	C530042P11RIK	GP38	MAPK8	PCYT2	SQLE
8030402P03RIK	CALD1	GPRK6	MFSD7	PI4K2B	SREBF2
9030216K14RIK	CLEC4B1	GPSN2	MMAB	PKP4	STK38L
9130230L23RIK	CLEC4N	HIVEP3	MMP14	PMVK	TKT
A830026L17RIK	CSNK1G2	HK1	MTAP6	PSEN2	TMEM97
AACS	CXCL16	HMGCR	MT-ND5	PTPRE	TNFRSF1B
ACAT2	CYP2B19	HSD11B1	MVK	RRAS	WIPI1
ACSS2	DBI	ICAM1	MYADM	SCD2	WNT6
ADAM17	DHCR24	INSIG1	NFE2	SEMA4A	ZCCHC6
AKAP4	DHCR24	KYNU	NFKB2	SERINC2	ZFP459

Cluster 5				
CYP51	HSD17B7	LOC666559	SC4MOL	STARD4
FDPS	LOC100040592	MVD	SQLE	

Cluster 6					
AIF1	AIF1	C1QA	C1QB	C1QC	IFI27

Table 3B. Clusters 1-7of genes which are significantly regulated in βVLDL, acLDL, and oxLDL-loaded BMDM compared to non-loaded control cells

Cluster 1					
ACTA2	1110032E23RIK	EGLN3	GADD45G	SGK1	4732458O05RIK
HVCN1	ADSSL1	LONRF3	PANX1	ALOX5AP	AATK
MBC2	TFRC	SORT1	GDPD1	PIRA3	GPR83
RUSC2	ST5	ADSSL1	PPAP2C	LHFPL2	VWF
SORT1	GPR68	NEDD4L	TESK1	PLEKHA8	SOAT1

Cluster 2	
TSPAN33	FCHO1

Cluster 3					
SLCO3A1	ITPR1	ELMO1	SMCHD1	LRMP	MCM5
MSRB2	6430706D22RIK	C79267	ST3GAL6	SEZ6L2	ABI3
MMP25	FCGR2B	PRICKLE1	DHCR24	2610027C15RIK	DHCR24
MAG	FADS2	TMEM110	LBR	USF1	MAD
RGAG4	ZFP281	GMIP	VTI1A	TES	4933401P20RIK
CREB3	NSMAF	ZMYM3	ETV5	A530032D15RIK	DBNL
SP140	NOD1	CCDC28B	LOC100047937	TRIM21	LPXN
NSDHL	DMWD	FMNL3	MBNL1	LOC100047937	SFXN5
HSPA2	BC067047	LOC100046211	GTF2I	E130207H16RIK	LOC100040462
AKNA	RHOBTB1	GLIPR2	6330442E10RIK	PPM1K	ASPH
A530023O14RIK	ADRBK1	TAOK3	ASPH	IL6RA	TES

Cluster 4					
IL6RA	CTSC	OGFRL1	LOC677008	RAPGEF5	RASSF5
DCAKD	DPYSL2	IQGAP2	UPP1	DUSP2	EGR1
SLA	SSBP4	EBI3	SPATA13	LRMP	TMEM2
RAB31	DUSP2	RAPGEF5	1200013B08RIK	5730403B10RIK	PNPLA1
2900019M05RIK	CCR5	STAP1	SLC7A7	TNFRSF11A	CARHSP1
DMWD	SOCS3	IL1RN	ITPKB	CCND3	LMO2
PFTK1	2310004N11RIK	FCGR3	2410025L10RIK	THA1	ETV5

Cluster 5					
ACAT2	CH25H	HDC	STARD4	SLC25A10	CD72
PCYT2	TPST1	SCARF2	PVRL2	CKB	LOC100040592
SQLE	FLOT1	STARD7	USP18	LOC625360	MBOAT5
CD69	MMP13	IL1RN	SREBF2	MMP13	

Cluster 6					
BC013712	ITGAL	CCR5	SLC28A2	PLD4	FGD2

Cluster 7					
CXCL9	CYP51	HSD17B7	LOC100048556	FDPS	SC4MOL
CCL7	CCND3	LOC666559	CXCL10	OTTMUSG	SQLE

was significantly >3-fold decreased by the lipoproteins (βVLDL: 5.3- and 3.4-fold; acLDL: 5.0- and 3.7-fold; oxLDL: 4.4- and 4.2-fold, respectively; cluster 5). Cluster 6 of PM did not contain genes which were highly regulated or possibly involved in lipid homeostasis.

Hierarchical clustering of genes significantly regulated in BMDM by all three lipoproteins (186 genes) resulted in 7 clusters. (Fig. 3B and Table 3B). Cluster 1 of BMDM showed genes which were all significantly increased by βVLDL, acLDL and oxLDL loading. One gene of particular interest in this cluster is sortilin 1 (SORT1), a sorting receptor that direct proteins through secretory and endocytic pathways.[43, 44] BMDM loaded with βVLDL, acLDL and oxLDL showed a significant 4.9-fold ($p<0.01$), 4.7- ($p<0.001$),and 2.4-fold ($p<0.001$) increase in SORT1 expression compared to non-loaded BMDM, respectively. Cluster 2 represent 2 genes, tetraspanin 33 (TSPAN33) and FCH domain only 1 (FCHO1) which were significantly upregulated by acLDL and oxLDL loading, whereas loading with βVLDL resulted in a significant decrease in gene expression. TSPAN33 is a membrane-bound signalling molecule, whereas FCHO1 is a structural constituent of the cytoskeleton.[29] Clusters 3-7 contain genes which were significant downregulated by βVLDL, acLDL, and oxLDL, although the degree of reduction varied with the different loading conditions. From these clusters, only scavenger receptor, class F, member 2 (SCARF2; cluster 5) was of particularly interest in this study. SCARF2 is a member of the scavenger family and several members of this familiy have been shown to play a role in receptor-mediated endocytosis and lipid metabolism.[7, 45, 46]. SCARF2 was significantly reduced in BMDM loaded with βVLDL, acLDL, and oxLDL compared to control cells (βVLDL: 2.1-fold, $p<0.05$; acLDL: 2.4-fold, $p<0.01$; oxLDL: 2.4, $p<0.01$).

In addition to identification of genes which were significantly regulated by all three lipoproteins, also genes which were highly upregulated or downregulated (>2 fold) by one specific lipoprotein were identified in PM and BMDM (Table 4A and 4B). Table 4A shows that fatty acid binding protein 3 (FABP3), involved in fatty acid metabolism, is highly upregulated (6.5-fold, $p<0.05$) in BMDM loaded with oxLDL. PM loaded with acLDL showed a 2.0-fold decrease ($p<0.05$) in the expression of ABC transporter B8 (ABCB8)(Table 4B). In BMDM, foam cell formation induced by βVLDL resulted in decreased expression of genes involved in fatty acid metabolism and fatty acid transport, PAQR& and DBI, respectively and decreased expression of SLC6A13, a protein involved in small molecule transport. In addition, incubation of BMDM with acLDL caused a decrease in expression of ApoC2 (2.6-fold, $p<0.05$), a transporter apolipoprotein.

Furthermore, genes which were highly upregulated or downregulated (>2 fold) by two specific lipoproteins compared to non-foamy control cells are presented in Table 5A and 5B. A gene of interest is arginase 1 (ARG1), a protein suggested to be involved in amino acid catabolism and a classical macrophage M2 marker. ARG1 is highlighted in this study as it shows a 10-fold ($p<0.01$) and a 4-fold increase ($p<0.001$) in PM loaded with βVLDL and acLDL, respectively (Table 5A). Furthermore, loading of BMDM with acLDL or oxLDL caused a 2.5-fold ($p<0.01$) and a 2.3-fold increase ($p<0.01$) in expression of lysosomal acid lipase 1 (LIP1), which hydrolyzes intracellular triglycerides and cholesterol esters derived from plasma lipoproteins.[8]

Identification of genes which were significantly downregulated by two of the three lipoproteins showed a high reduction in gene expression of stearoyl-Coenzyme A desaturase 1 (SCD1), involved in fatty acid metabolism, in PM incubated with βVLDL and acLDL compared to non-

foamy control cells (4.5-fold, $p<0.05$; 5.6-fold, $p<0.05$, respectively)(Table 5B).
Overall, these findings show that several new genes were identified which are possibly involved in lipid uptake, storage and metabolism are significantly regulated during foam cell formation of PM and BMDM by βVLDL, acLDL, and oxLDL.

Table 4A . Genes of interest in cholesterol metabolism upregulated >2 fold by one specific lipoprotein compared to non-foamy control cells

Cell Type	Lipoprotein	Genes of interest	Fold -increase	P-value	Biological process
PM	βVLDL	ADFP	4.1	P<0.001	1
		ABCC3	2.2	P<0.05	2
		SLC25A20	2.0	P<0.01	3
	acLDL	-	-	-	
	oxLDL	0	-	-	
BMDM	βVLDL	0	-	-	
	acLDL	0	-	-	
	oxLDL	FABP3	6.47	P<0.05	4

Biological process: 1)Regulation of lipid , fatty acid, and steroid metabolism; 2)Extracellular transport and import; 3)Transporter/ mitochondrial carrier protein; 4) Lipid and fatty acid /binding transport. PM= peritoneal macrophages; BMDM= bone marrow-derived macrophages

Table 4B . Genes of interest in cholesterol metabolism downregulated >2 fold by one specific lipoprotein compared to non-foamy control cells

Cell Type	Lipoprotein	Genes of interest	Fold -decrease	P-value	Biological Process
PM	βVLDL	-	-	-	
	acLDL	ABCB8	2.0	P<0.05	1
	oxLDL	-	-	-	
BMDM	βVLDL	PAQR&	2.2	P<0.05	2
		DBI	2.1	P<0.05	3
		SLC6A13	2.3	P<0.05	4
	acLDL	ApoC2	2.6	P<0.05	5
	oxLDL	-	-	-	

Biological process: 1) Extracellular transport and import; 2)Lipid and fatty acid metabolism; 3) Lipid and fatty acid transport; 4) Small molecule transport; 5) Transporter apolipoprotein. PM= peritoneal macrophages; BMDM= bone marrow-derived macrophages

Table 5A . Genes of interest in cholesterol metabolism upregulated >2 fold by two specific lipoproteins compared to non-foamy control cells

Cell Type	Lipoprotein	Genes of interest	Fold – increase	Fold-increase	P-value	P-value	Biological Process
PM	βVLDL +acLDL	ARG1	10.2	3.9	P<0.01	P<0.001	1
	βVLDL +oxLDL	-	-	-	-	-	
	acLDL+ oxLDL	-	-	-	-	-	
BMDM	βVLDL +acLDL	ADFP	3.8	2.1	P<0.001	P<0.01	2
	βVLDL +oxLDL	-	-	-	-	-	
	acLDL+ oxLDL	ABCB4	3.6	8.2	P<0.01	P<0.001	3
		CAV1	2.4	2.3	P<0.05	P<0.05	4
		ACSS2	4.4	5.0	P<0.01	P<0.001	5
		EPHX1	3.9	6.0	P<0.01	P<0.001	6
		LIP1	2.5	2.3	P<0.01	P<0.01	6

Biological process: 1) Cell adhesion; 2)Regulation of lipid , fatty acid and steroid metabolism; 3) Extracellular transport and import; 4) Lipid and fatty acid transport; 5) Fatty acid metabolism; 6) Lipid metabolism. PM= peritoneal macrophages; BMDM= bone marrow-derived macrophages

Table 5B. Genes of interest in cholesterol metabolism downregulated >2 fold by two specific lipoproteins compared to non-foamy control cells

Cell Type	Lipoprotein	Genes of interest	Fold – decrease	Fold-decrease	P-value	P-value	Biological Process
PM	βVLDL +acLDL	SCD1	4.5	5.6	P<0.05	P<0.05	1
	βVLDL +oxLDL	-	-	-	-	-	
	acLDL+ oxLDL	-	-	-	-	-	
BMDM	βVLDL +acLDL	ACSL3	2.3	2.0	P<0.05	P<0.05	1
		MVD	3.6	2.8	P<0.05	P<0.05	2
	βVLDL +oxLDL	-	-	-	-	-	
	acLDL+ oxLDL	-	-	-	-	-	

Biological process: 1) Fatty acid metabolism; 2) Cholesterol metabolism. PM= peritoneal macrophages; BMDM= bone marrow-derived macrophages

DISCUSSION

In the present study, the impact of foam cell formation induced by commonly used (modified) lipoproteins to mimic *in vivo* lipid-loading on the genome-wide expression of PM and BMDM was investigated. To induce foam cell formation, PM and BMDM were loaded with βVLDL, acLDL, or oxLDL for 48 hours. Interestingly, the use of the different (modified) lipoproteins

caused distinct cholesterol accumulation patterns in both types of macrophages. Loading of macrophages with acLDL, a non-physiologic lipoprotein, resulted in small cytosolic lipid droplets distributed throughout the macrophage, whereas macrophages loaded with oxLDL exhibited diffusely distributed small cytosolic lipid droplets. Previous studies showed that cholesterol delivered to macrophages by oxLDL does not enter the ACAT substrate pool due to impaired lysosomal degradation of oxLDL.[47-49] This impaired lysosomal degradation causes intralysosomal lipid deposition, analogous to findings in foam cells in atherosclerotic lesions.[50] In contrast to oxLDL, acLDL is efficiently degraded and is a potent stimulator of ACAT, leading to the accumulation of ACAT-derived cholesteryl esters in cytoplasmic lipid droplets.[50] Interestingly, this study showed a remarkable reduction in the expression of ACAT2 by all the three pro-atherogenic lipoproteins in both types of macrophages. These findings are in contrast with a previous study by Batt *et al.*[51], which showed that modified lipoproteins increase the transcription of the ACAT gene in human macrophages. However, our study was performed using PM and BMDM. Downregulation of the ACAT2 expression in the murine macrophages can be considered a protective mechanism to increase the amount of free cholesterol available for cholesterol efflux from the cells to extracellular cholesterol acceptors.

Furthermore, βVLDL induces lipid accumulation in large intracellular lipid deposits. A different loading pattern was seen between loading of PM and BMDM with βVLDL. In PM, the βVLDL loading resulted in the accumulation of lipid vesicles throughout the cell, whereas lipid from βVLDL was stored more near the outer membrane in BMDM. In line with the findings in PM, Tabas *et al.*[52], previously demonstrated also widely-distributed droplet accumulation in murine PM after βVLDL loading. The different loading pattern observed in BMDM compared to PM, might indicate that lipid metabolism is differently regulated in the two types of macrophages from different origin. All macrophages package and store neutral lipids in discrete intracellular storage droplets. However, the processes by which lipoproteins are taken up and the subsequent intracellular handled by macrophages are likely to influence the lipid loading pattern and the amount of lipid which accumulates intracellularly. Strikingly, genes which are known to be involved in lipid droplet formation and catabolism, like Cide b and c, Plin, and Plin4 [21,22], were not affected upon loading of PM and BMDM with the different pro-atherogenic lipoproteins. A possible explanation for this finding might be that these genes are posttranscriptionally regulated.

Furthermore, the expression levels of genes which have been implicated in macrophage foam cell formation, including those involved in lipoprotein uptake and metabolism were determined. In line with previous findings[21], the mRNA expression of the LDLr was downregulated in both types of macrophages upon loading with the different lipoproteins in response to the raised intracellular cholesterol levels. Unlike the LDLr, the relative mRNA transcripts for the scavenger receptors (SR-A, CD36, and SR-BI) were unaffected after incubation of PM and BMDM with the different lipoproteins. Thus, excessive accumulation of cholesterol by macrophages by uptake of modified lipoproteins via scavenger receptors cannot be prevented. Furthermore, BMDM loaded with βVLDL exhibited significantly increased VLDLr gene expression. Suzuki *et al.*[53] have previously reported that the VLDLr, unlike the LDLr, is not downregulated during βVLDL-induced foam cell formation.[53] The VLDLr is thus expected to play a more important role in macrophage foam cell formation

as compared to the LDLr. In agreement, our group previously showed that the macrophage VLDLr indeed facilitates atherosclerotic lesion development.[54] These findings indicate that macrophage VLDLr facilitates atherosclerotic lesion development, probably by mediating the accumulation of atherogenic lipoproteins.

It is important to note that the expression of ABCA1, the key transporter in cholesterol and phospholipid efflux from macrophages [55], was not significantly induced during all conditions *in vitro*. The expression of ABCA1 in macrophages is tightly controlled by intracellular cholesterol levels.[56, 57] Its activity is dramatically increased on cholesterol loading of macrophages and the subsequent transformation into foam cells.[58] However, as in this study the mRNA expression of ABCA1 was not induced, the importance of other genes in macrophage cholesterol homeostasis might also be underestimated.

Importantly, the present study identified genes which are significantly regulated upon foam cell formation, suggesting a role in macrophage cholesterol homeostasis. Of particular interest are MRC1, CLEC4N, SORT1, and SCARF2. MRC1, CLEC4N, and SCARF2 are anticipated to play a role in receptor-mediated endocytosis[29, 42], while SORT1 acts both as a receptor for neuromediators and growth factors at the plasma membrane and is involved in the binding and transport of lysosomal proteins. [43] In the present study, foam cell formation induced the expression of MRC1, SORT1, and SCARF2, while the mRNA expression level of CLEC4N was decreased. Although, to date the exact molecular functions and the regulatory mechanisms of these proteins remains largely unknown, their regulated expression pattern during foam cell formation and their anticipated role in receptor-mediated endocytosis suggest the involvement of these proteins in the uptake of lipoproteins and macrophage cholesterol homeostasis. Previously, several studies reported that the human gene locus of SORT1 at 1p13.3 was associated with plasma LDL levels and with a risk of myocardial infarction in several genome-wide association studies.[44, 59-61]. Overall these findings indicate that SORT1 may also play an important role in foam cell formation and thus atherosclerotic lesion development.

In conclusion, this study identified MRC1, CLEC4N, SORT1, and SCARF2 as novel candidate genes modulating macrophage foam cell formation and the initiation of atherosclerotic lesion development. Studies using knockout mice or siRNA for these specific genes is necessary to clarify the exact role of these genes in atherosclerosis.

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