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

Meurs, I.

Chapter 5

Macrophage ABCB1 deficiency increases serum cholesterol levels but does not promote atherosclerosis in LDL receptor knockout mice on Western-type diet

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Submitted for publication

ABSTRACT

Objective- Many different ABC transporters are associated with the transport of sterols and other lipids both *in vitro* and *in vivo*. Also class 1 P-glycoprotein [Pgp; MDR1 (ABCB1) in humans, mdr1a and mdr1b in mice] has recently been implicated in cholesterol homeostasis. However, its exact role in lipid metabolism and the molecular mechanisms involved are largely unclear. The aim of this study was to assess the effects of macrophage ABCB1 deficiency on lipid metabolism and atherosclerosis in LDL receptor deficient ($LDLr^{-/-}$) mice.

Methods and Results- Chimeras with dysfunctional macrophage ABCB1 ($ABCB1^{-/-}\rightarrow LDLr^{-/-}$) were generated by transplantation of bone marrow from $ABCB1^{-/-}$ mice into irradiated $LDLr^{-/-}$ mice. The transplanted $LDLr^{-/-}$ mice were fed a Western-type diet (WTD), containing 0.25% cholesterol and 15% fat for 9 weeks to induce atherosclerosis. On WTD, disruption of macrophage ABCB1 resulted in a significant increase in serum total cholesterol levels (1.3-fold compared to $ABCB1^{+/+}\rightarrow LDLr^{-/-}$ mice, $p<0.05$), which was due to an increase in both VLDL and LDL cholesterol levels (2.0-fold, $p<0.001$ and 1.3-fold, $p<0.05$ compared to control mice, respectively). Nevertheless, macrophage ABCB1 deficiency did not significantly affect atherosclerotic lesion development ($260\pm34\times10^3$ μm^2 for $ABCB1^{-/-}\rightarrow LDLr^{-/-}$ mice compared to $190\pm24\times10^3$ μm^2 for $ABCB1^{+/+}\rightarrow LDLr^{-/-}$ mice; $p=0.11$). In addition, an *in vitro* study using bone marrow-derived macrophages from $ABCB1^{-/-}\rightarrow LDLr^{-/-}$ mice showed that ABCB1 deficiency does not affect cholesterol efflux to HDL, while a tendency to increased apoA-I efflux was observed.

Conclusion- Macrophage ABCB1 deficiency does not affect the development of atherosclerotic lesions in $LDLr^{-/-}$ mice fed a Western-type diet, despite a significant increase in the pro-atherogenic lipoproteins VLDL and LDL.

INTRODUCTION

Several members of the ATP-binding cassette (ABC) superfamily are involved in the transport of xenobiotic compounds between major organs in the body. [1] An example is class I P-glycoprotein [Pgp; MDR1 (ABCB1) in humans, mdr1a and mdr1b in mice], which is a ~170-kDa integral membrane protein that originally was identified in multidrug-resistant (MDR) tumor cells, where it reduces intracellular concentrations of structurally diverse toxic xenobiotics. [2] In addition to tumors, ABCB1 is also expressed in a number of different tissues, including liver, adrenal, placenta, intestine, kidney, and endothelial cells at the blood-brain barrier. [3,4] The physiologic function of ABCB1 in these tissues, however, remains poorly characterized and is still the subject of discussion.

Several lines of evidence indicate a role for ABCB1 in cholesterol-related processes in the cell. First, ABCB1 expression is regulated in response to the cellular content of cholesterol. Human monocyte-derived macrophages incubated with HDL for cholesterol depletion or with acetylated LDL for cholesterol loading displayed upregulation and downregulation of ABCB1 expression levels, respectively. [5] Furthermore, Langmann *et al.* [6] showed that ABCB1 mRNA expression was induced in monocyte-derived macrophages upon stimulation with the nuclear receptors liver X receptor (LXR) and retinoid X receptor (RXR) agonists T0901317 and 9-cis retinoic acid. Thus, although regulation of ABCB1 expression is opposite to the regulation of ABCA1, an ABC transporter essentially involved in the efflux of cholesterol from cells, upon cellular cholesterol loading and deloading, ABCB1 is regulated similarly to ABCA1, upon stimulation with LXR/RXR agonists.

Second, cholesterol is recognized and transported as an endogenous substrate of ABCB1 [7] and stimulates basal (i.e. without any drugs) ABCB1 ATPase activity. [8-10] It is involved in the transport of free cholesterol from the plasma membrane to the endoplasmic reticulum (ER), the site of cholesterol esterification by acyl-CoA:cholesterol acyltransferase (ACAT). [11-13] In agreement, the presence of ABCB1 enhances cellular cholesterol esterification, while the addition of ABCB1 inhibitors blocks the process of cholesterol esterification. ABCB1 is thus required for cholesterol transport and esterification. [11-13]

Third, relative increases in ABCB1 expression within a given cell type *in vitro* are associated with increased accumulation of cholesterol [14], suggesting a role for ABCB1 in the uptake of cholesterol. However, in mice, independent studies have agreed [15] or disagreed [16] with the hypothesis that ABCB1a/1b is involved in cholesterol uptake. Plosch *et al.* [15] reported that ABCB1a/1b-deficient mice displayed a significant decrease in total plasma cholesterol and liver cholesteryl ester concentrations compared to wild-type mice following 15 days standard mouse diet feeding. Based on these observations a role for ABCB1 in the intestinal absorption of cholesterol was proposed. On the contrary, Luker *et al.* [16] demonstrated that absorption of orally administered cholesterol was not affected in ABCB1a/1b-deficient mice compared to control wild-type mice, indicating that ABCB1 is not essential for overall absorption of cholesterol through the intestine. However, Luker *et al.* [16] did find a decrease in hepatic cholesterol, which was suggested to be independent of effects on the intestinal absorption of cholesterol, but rather due to reduced transport/trafficking of orally administered cholesterol to the liver. Interestingly, in Brazilian hypercholesterolemic

patients from European descent, high baseline serum total and LDL cholesterol levels were associated with a linked C3435T and G2677T ABCB1 polymorphism [17], suggesting that this specific ABCB1 haplotype may contribute to increased plasma cholesterol levels.
Importantly, ABCB1 polymorphisms were associated with an increased risk for coronary artery disease. [18] Additionally, mRNA levels of ABCB1 were higher in atherosclerotic lesions from human arteries when compared to control arteries. [13] In these atherosclerotic lesions, mRNA levels of ABCB1 and ACAT were positively correlated with the cholesteryl ester content, implying that the ABCB1 gene is involved in the process of cholesterol esterification in atherosclerotic lesions. [13, 19] It is currently however unknown if ABCB1 expression by macrophages indeed influences the pathogenesis of atherosclerosis.
The aim of the present study was to gain insight into the role of macrophage ABCB1 in atherosclerotic lesion development. Mice with a genetic disruption of ABCB1a and ABCB1b ($ABCB1a^{-/-}/ABCB1b^{-/-}$) appear phenotypically normal and were fertile under laboratory conditions. [20] To study the role of macrophage ABCB1 in atherosclerotic lesion development $LDLr^{-/-}$ mice were transplanted with bone marrow from these $ABCB1a^{-/-}/ABCB1b^{-/-}$ mice.

MATERIALS AND METHODS

Animals

$ABCB1a/1b^{+/-}$ FVB mice, kindly provided by Dr. A.H. Schinkel (The Netherlands Cancer Institute, The Netherlands) and back-crossed on a C57Bl/6 background for 3 generations, were cross-bred to generate $ABCB1a^{+/+}/1b^{+/+}$ ($ABCB1^{+/+}$) and $ABCB1a^{-/-}/1b^{-/-}$ ($ABCB1^{-/-}$) littermates. Homozygous LDL receptor knockout ($LDLr^{-/-}$) mice were obtained from The Jackson Laboratory (Bar Harbor, Me) as mating pairs and bred at the Gorlaeus Laboratory (Leiden, The Netherlands). Mice were housed in sterilized filter-top cages in a temperature-controlled room with a 12-h light/dark cycle and food and water were provided *ad libitum*. Mice were maintained on sterilized regular chow containing 4.3% (w/w) fat and no cholesterol (RM3, Special Diet Services, Witham, UK) or fed Western-type diet (WTD) containing 15% (w/w) cacaobutter and 0.25% (w/w) cholesterol (Diet W, Abdiets, Woerden, The Netherlands). Drinking water was supplied with antibiotics (83 mg/L ciprofloxacin and 67 mg/L polymyxin B sulfate) and 6.5 g/L sucrose. 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 and carried out in compliance with the Dutch government guidelines.

Bone marrow transplantation

To induce bone marrow aplasia, male $LDLr^{-/-}$ recipient mice of 13-15 weeks old were exposed to a single dose of 9 Gy (0.19 Gy/min, 200 kV, 4 mA) total body irradiation using an Andrex Smart 225 Röntgen source (YXLON International, Hamburg, Germany) with a 6-mm aluminum filter 1 day before the transplantation (n=14-15 per group). Bone marrow

was isolated by flushing the femurs and tibias from the donor ABCB1$^{+/+}$ and ABCB1$^{-/-}$ mice with phosphate-buffered saline (PBS). Single-cell suspensions were obtained by passing the cells through a 70 µm cell strainer (Falcon, The Netherlands). Irradiated recipients received 5x10^{6} bone marrow cells by intravenous injection into the tail vein. After a bone marrow transplantation (BMT) recovery period of 8 weeks mice were fed WTD for 9 weeks, after which the animals were sacrificed. Body mass was recorded weekly throughout the study.

Assessment of successful bone marrow reconstitution

The hematologic chimerism of the LDLr$^{-/-}$ mice was determined using genomic DNA from bone marrow by polymerase chain reaction (PCR) at 17 weeks after transplant. The forward and reverse primers 5'-GTGCATAGACCACCCTCAAGG-3' and 5'-GTCATGCACATCAAACCAGCC-3' for mouse endogenous ABCB1 gene; 5'-GTGCATAGACCACCCTCAAGG-3' and 5'-GGAGCA AAGCTGCTATTGGC-3' for mouse targeted ABCB1 gene were used.

Serum lipid and lipoprotein analyses

After an overnight fast, ≈100 µL of blood was drawn from each mouse by tail bleeding. Serum concentrations of free cholesterol were determined by enzymatic colorimetric assays with 0.048 U/mL cholesterol oxidase (Sigma) 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). For the determination of total cholesterol, 0.03 U/mL cholesteryl esterase (Seikagaku, Tokyo, Japan) was added to the reaction solution. The concentrations of phospholipids and triglycerides in serum were determined using enzymatic colorimetric assays (Spinreact S.A., Girona, Spain and Roche Diagnostics, Mannheim, Germany, respectively). Precipath I (Roche Diagnostics, Mannheim, Germany) was used as an internal standard. Absorbance was read at 490 nm. The distribution of lipids over the different lipoproteins in serum was determined by fractionation of 30 µl serum of each mouse using a Superose 6 column (3.2x300mm, Smart-system, Pharmacia, Uppsala, Sweden). Cholesterol contents in the effluent were determined as above.

Macrophage cellular cholesterol efflux

Bone marrow cells, isolated from ABCB1$^{+/+}$→LDLr$^{-/-}$ and ABCB1$^{-/-}$→LDLr$^{-/-}$ mice, were cultured for 7 days in complete RPMI medium supplemented with 20% fetal calf serum (FCS) and 30% L929 cell-conditioned medium, as the source of macrophage colony-stimulating factor (M-CSF), to generate bone marrow-derived macrophages. Bone marrow-derived macrophages were subsequently incubated with 0.5 µCi/mL ^{3}H-cholesterol in DMEM/0.2% bovine serum albumin (BSA) for 24 hours at 37°C. To determine cholesterol loading, cells were washed 3 times with washing buffer (50mmol/L $CaCl_2$, pH 7.4), lysed in 0.1 mol/L NaOH, and the radioactivity was determined by liquid scintillation counting. Cholesterol efflux was studied by incubation of the cells with DMEM/0.2% BSA alone or supplemented with either 10 µg/mL apolipoprotein (apo) A-I (Calbiochem) or 50 µg/mL human HDL (density 1.063 to 1.21 g/mL), isolated according to Redgrave *et al.*.[21] Radioactivity in the medium and the cells was determined by scintillation counting after 24 hours of incubation. The cholesterol

efflux percentages are calculated as the amount of radioactivity in the medium compared to the total amount of radioactivity measured in the medium plus the cells.

Histological analysis of the aortic root and other organs

To analyze lipid accumulation in the different tissues, mice were sacrificed after 9 weeks (17 weeks after BMT) on WTD. After *in situ* perfusion, organs were excised and stored in 3.7% neutral-buffered formalin (Formal-fixx; Shandon Scientific Ltd, UK). Atherosclerotic lesion development was quantified in oil red O-stained cryosections of the aortic root of $LDLr^{-/-}$ mice transplanted with $ABCB1^{+/+}$ or $ABCB1^{-/-}$ bone marrow. Mean lesion area (in μm^2) was calculated from 10 sections at 20 μm intervals, starting at the appearance of the tricuspid valves. Sections were stained immunohistochemically for the presence of macrophages using a rat MOMA-2 antibody, dilution 1:50 (Serotec Ltd., Oxford, UK). The percentage of macrophages in the lesion was calculated as a ratio of the macrophage-stained area and the lesion area. Masson Trichrome-staining (Sigma Diagnostics, USA) was used to determine the collagen content of the plaque. In addition, 7 μm cryosections of formalin-fixed lung, liver, and spleen of $ABCB1^{+/+}\rightarrow LDLr^{-/-}$ and $ABCB1^{-/-}\rightarrow LDLr^{-/-}$ mice were prepared and stained for lipid accumulation using oil red O staining. Hematoxylin (Sigma Diagnostics, St. Louis, MO, USA) was used to stain the nuclei in the different organs. All quantifications were performed blinded by using the Leica image analysis system, consisting of a Leica DMRE microscope coupled to a video camera and Leica Qwin Imaging software (Leica Ltd., Cambridge, UK).

Hepatic lipid analyses

Extraction of hepatic lipids was performed according to the Bligh and Dyer protocol [22]. Concentrations of free cholesterol, cholesteryl ester, total cholesterol, phospholipids and triglycerides were determined as described above. A BCA protein assay was performed to determine the amount of protein present (Pierce).

Gene mRNA expression analysis

Guanidium thiocyanate-phenol was used to extract total RNA from livers. cDNA was generated using RevertAid M-MuLV reverse transcriptase (Fermentas, Burlington, Canada) according to manufacturer's protocol. Quantitative gene expression analysis was performed using the SYBR-Green method on a 7500 fast Real-time PCR machine (Applied Biosystems, Foster City, CA). PCR primers were designed using Primer Express Software according to the manufacturer's default settings. Primer sequences are available upon request. Hypoxanthine Guanine Phosphoribosyl Transferase (HPRT), β-actin, and acidic ribosomal phosphoprotein PO (36B4) were used as the standard housekeeping genes. Relative gene expression was calculated by subtracting the threshold cycle number (Ct) of the target gene from the average Ct of housekeeping genes and raising two to the power of this difference.

Peritoneal leukocyte analysis

On sacrifice the peritoneal cavity of the mice was lavaged with 10 mL cold PBS to collect peritoneal leukocytes for quantification of macrophage foam cells using an automated Sysmex XT-2000iV analyzer (Goffin Meyvis, Etten-Leur, The Netherlands).

Data analyses

Statistical analyses were performed using GraphPad Instat software. Data are expressed as mean±SEM P values <0.05 were considered significant.

RESULTS

Generation of LDLr$^{-/-}$ mice with a deletion of ABCB1 in bone marrow-derived cells

To investigate the role of macrophage ABCB1 in lipoprotein metabolism and atherogenesis, ABCB1 was selectively disrupted in hematopoietic cells by transplantation of bone marrow from ABCB1$^{+/+}$ or ABCB1$^{-/-}$ mice into LDLr$^{-/-}$ mice. Succesful reconstitution of the recipients with donor bone marrow was confirmed by analysis of ABCB1 transcripts in genomic DNA from bone marrow of the transplanted mice (Fig. 1). Bone marrow from LDLr$^{-/-}$ mice transplanted with ABCB1$^{+/+}$ bone marrow showed only the 610kb ABCB1 endogenous-specific band, while bone marrow of ABCB1$^{-/-}$→LDLr$^{-/-}$ mice displayed the 423kb band indicative for the disrupted ABCB1 gene. After a recovery period of 8 weeks the diet was switched from a regular chow diet to Western-type diet (WTD), containing 15% (w/w) cacaobutter and 0.25% (w/w) cholesterol. During the course of the experiment, the weight gain curve did not show significant differences between ABCB1$^{+/+}$→LDLr$^{-/-}$ and ABCB1$^{-/-}$ →LDLr$^{-/-}$ mice (data not shown).

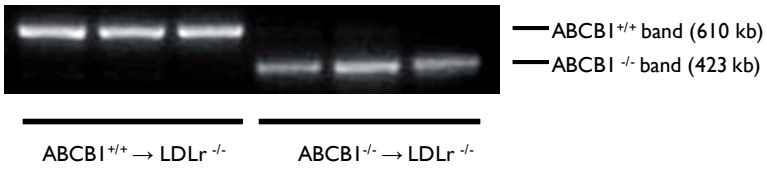

Fig. 1. Verification of success of bone marrow transplantation.
Verification of successful reconstitution with donor bone marrow cells by polymerase chain reaction amplification of the endogenous (610 kb) and targeted (423 kb) ABCB1 gene at 17 weeks after bone marrow transplantation.

The effect of macrophage ABCB1 deletion on plasma lipid levels in LDLr$^{-/-}$ mice

After a recovery period of 8 weeks on a regular chow diet, no differences in lipid concentrations between LDLr$^{-/-}$ mice transplanted with bone marrow of ABCB1$^{+/+}$ and ABCB1$^{-/-}$ mice were observed (Fig. 2A). Furthermore, the distribution of lipids over the different lipoprotein fractions of ABCB1$^{+/+}$→LDLr$^{-/-}$ and ABCB1$^{-/-}$→LDLr$^{-/-}$ mice showed no significant differences (Fig. 2B). Upon challenging the mice with a high cholesterol, high fat WTD, serum total cholesterol levels were increased by approximately 3-fold at 17 weeks after BMT (i.e., 9 weeks WTD feeding). The absence of macrophage ABCB1 in LDLr$^{-/-}$ mice resulted in 30% higher serum total cholesterol (TC) levels (1656±104 mg/dL compared to 1262±123 mg/dL for controls, $p<0.05$), which was due to a 30% increase in serum free cholesterol (FC) levels

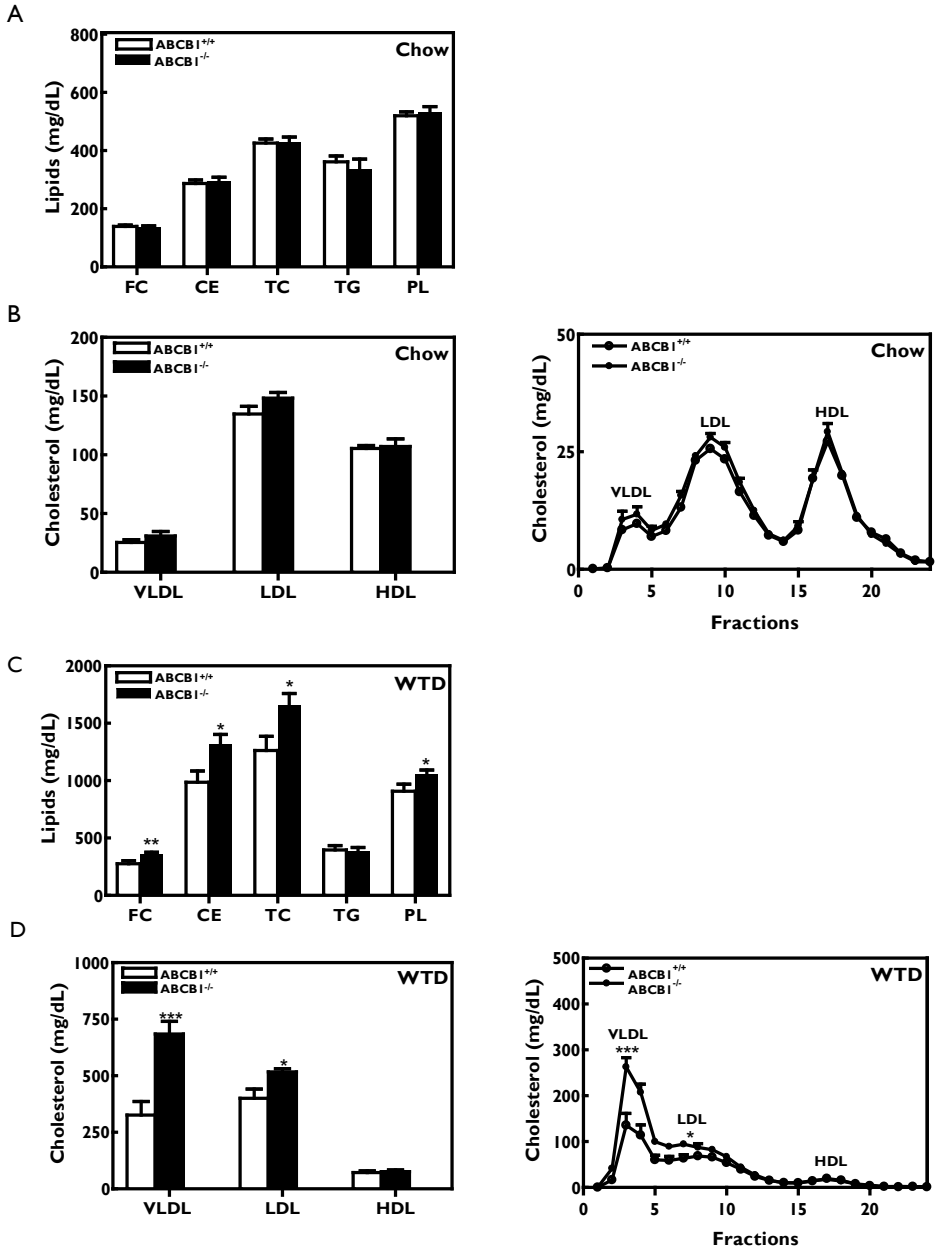

Fig. 2. Serum lipid levels and cholesterol lipoprotein distribution profile on chow diet and after 9 weeks of WTD feeding in LDLr$^{-/-}$ mice reconstituted with ABCB1$^{+/+}$ or ABCB1$^{-/-}$ bone marrow.
Blood samples were drawn after an overnight fast. The concentrations of free cholesterol (FC), cholesterol esters (CE), total cholesterol (TC), triglycerides (TG), and phospholipids (PL) in serum of transplanted mice on chow (A) and after WTD feeding (C) were determined using enzymatic colorimetric assays. The distribution of cholesterol over the different lipoproteins was determined by fractionation of serum from individual transplanted mice on chow (B) and after WTD feeding (D) using a Superose 6 column. Fractions 2 to 5 represent VLDL; fraction 6 to 14, LDL; and fractions 15 to 20, HDL, respectively. Values represent the mean±SEM of 12-15 mice per group. Statistically significant difference *p<0.05, **p<0.01, and *** p<0.001

(362±15 mg/dL compared to 276±26 mg/dL for controls, p<0.01) and a 34% increase in serum cholesteryl ester (CE) levels (1318±84 mg/dL compared to 986±98 mg/dL for control animals, p<0.05) (Fig. 2C). In addition, serum phospholipid (PL) levels in ABCB1$^{-/-}$→LDLr$^{-/-}$ mice were 16% higher (p<0.05, compared to ABCB1$^{+/+}$→LDLr$^{-/-}$ mice), whereas triglyceride (TG) levels were not affected. Fractionation of serum lipoproteins showed that the increase in serum cholesterol levels induced by WTD feeding of ABCB1$^{-/-}$→LDLr$^{-/-}$ mice was caused by an increase in VLDL and LDL cholesterol (VLDL: 684±56 mg/dL compared to 326±60 mg/dL for ABCB1$^{+/+}$→LDLr$^{-/-}$ mice, p<0.001; and LDL: 517±15 compared to 400±41 mg/dL for ABCB1$^{+/+}$→LDLr$^{-/-}$ mice, p<0.05) (Fig. 2D).

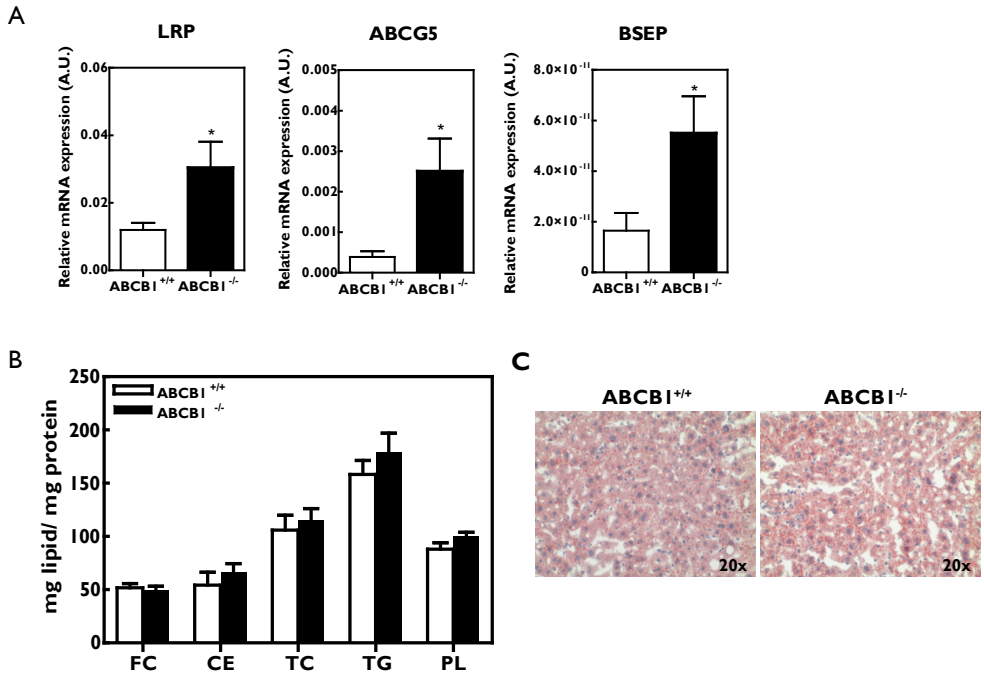

Fig. 3. Effect of macrophage ABCB1 disruption on hepatic gene expression and lipid homeostasis. A) mRNA expression levels of genes involved in cholesterol homeostasis in livers of LDLr$^{-/-}$ mice reconstituted with ABCB1$^{+/+}$ or ABCB1$^{-/-}$ bone marrow. Values represent the mean ± SEM of 6 mice per group. Statistically significant difference *p<0.05. B) Extraction of hepatic lipids was performed according to the Bligh and Dyer protocol. [22] Subsequently, the concentrations of hepatic free cholesterol (FC), cholesterol esters (CE), total cholesterol (TC), triglycerides (TG), and phospholipids were determined using enzymatic colorimetric assays. Values represent the mean±SEM of 12-14 mice per group. C)Representative photomicrographs of oil red O stained liver sections of ABCB1$^{+/+}$→LDLr$^{-/-}$ and ABCB1$^{-/-}$→LDLr$^{-/-}$ mice after 9 weeks of WTD feeding.

The effect of macrophage ABCB1 disruption on hepatic gene expression and lipid homeostasis

Hepatic mRNA expression levels of genes involved in cholesterol homeostasis were determined to investigate the underlying cause of the observed increase in serum cholesterol levels upon WTD feeding. mRNA expression levels of ABCA1, ABCB4, ABCG8, Acetyl-

Coenzyme A acetyltransferase (ACAT), ApoA-I, ApoE, adipose triglyceride lipase (ATGL), cholesteryl ester hydrolase (CEH), CYP7A1, CYP27A1, farnesoid X receptor (FXR), HMG-CoA reductase (HMGCR), hormone-sensitive lipase (HSL), LXR, microsomal triglyceride transfer protein (MTP), PDZK, stearoyl-CoA desaturase-1 (SCD1), and scavenger receptor class B type I (SR-BI) were not affected in ABCB1$^{-/-}$→LDLr$^{-/-}$ compared to ABCB1$^{-/-}$→LDLr$^{-/-}$ livers (data not shown). Deletion of macrophage ABCB1, however, induced mRNA expression levels of LDL receptor-related protein 1 (LRP1), ABCG5, and bile salt export pump (BSEP) (2.5-fold increase, $p<0.05$; 6-fold increase, $p<0.05$; 3.3-fold increase, $p<0.05$, respectively) (Fig. 3A), suggesting an increase in VLDL/LDL cholesterol uptake by LRP1 and induced excretion of cholesterol via ABCG5 or bile acids via BSEP into the bile.

As ABCB1 is proposed to be involved in cholesterol esterification, a hepatic lipid extraction was performed to determine the lipid composition of the liver of ABCB1$^{+/+}$→LDLr$^{-/-}$ and ABCB1$^{-/-}$→LDLr$^{-/-}$ mice. However, no differences in FC, CE, TC, TG, and PL contents were observed between livers of ABCB1$^{+/+}$→LDLr$^{-/-}$ and ABCB1$^{-/-}$→LDLr$^{-/-}$ mice (Fig. 3B). In line, staining of neutral lipids in liver by oil red O revealed no differences in lipid accumulation (Fig. 3C).

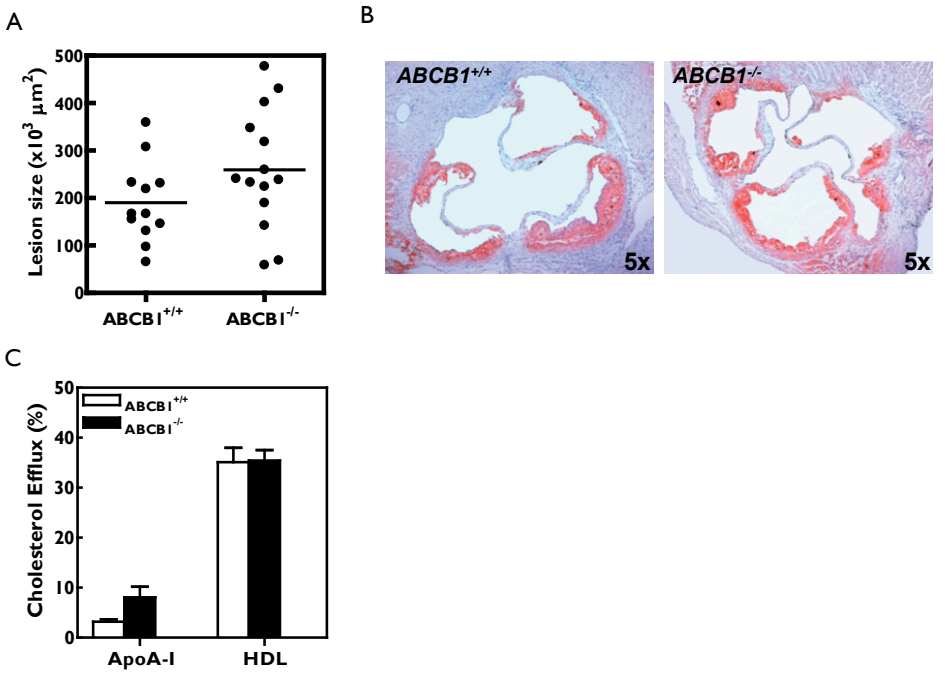

Fig. 4. Effect of macrophage ABCB1 deficiency on atherosclerotic lesion development and cholesterol efflux.

A) Atherosclerotic lesion formation was determined in the aortic root at the level of the tricuspid valves of ABCB1$^{+/+}$→LDLr$^{-/-}$ and ABCB1$^{-/-}$→LDLr$^{-/-}$ mice fed a WTD for 9 weeks. Mean lesion area of each individual mouse is shown. Horizontal lines represent the mean of 12-14 animals B) Representative photomicrographs of oil red O-stained lesions are shown. C)ApoA-I (10 µg/mL) and HDL (50 µg/mL) induced cellular cholesterol efflux from ^{3}H-cholesterol-labeled bone marrow-derived macrophages of ABCB1$^{+/+}$→LDLr$^{-/-}$ and ABCB1$^{-/-}$→LDLr$^{-/-}$ mice. Basal efflux to BSA (in the absence of added acceptors) has been subtracted from the data shown. Values represent the mean±SEM of 6 animals.

Effect of macrophage ABCB1 disruption on atherosclerotic lesion formation

To define the role of macrophage ABCB1 in atherogenesis, atherosclerotic lesion development was analyzed in the aortic root of ABCB1$^{+/+}$→LDLr$^{-/-}$ and ABCB1$^{-/-}$→ LDLr$^{-/-}$ mice after 9 weeks of WTD feeding. Higher serum cholesterol levels are often associated with a more rapid development of atherosclerotic lesions. Interestingly, although ABCB1$^{-/-}$→LDLr$^{-/-}$ mice exhibited a signicifant increase in serum cholesterol levels, no significant difference in atherosclerotic lesion development was observed (260±34x10^3 μm^2 [n=14] for ABCB1$^{-/-}$→LDLr$^{-/-}$ mice compared to 190±24x10^3 μm^2 [n=12] for ABCB1$^{+/+}$→LDLr$^{-/-}$ mice; p=0.11)(Fig. 4A). Representative photomicrographs of the aortic root of control mice and mice deficient for macrophage ABCB1 are shown in Fig. 4B. Furthermore, lesion composition was determined by staining (immuno)histochemically for the presence of macrophages and collagen content. Deletion of macrophage ABCB1 did not result in differences in macrophage and collagen content compared to ABCB1$^{+/+}$→LDLr$^{-/-}$ mice (data not shown).

In vitro studies using bone marrow-derived macrophages showed that the percentage of cholesterol efflux to HDL did not differ significantly between ABCB1$^{+/+}$→LDLr$^{-/-}$ and ABCB1$^{-/-}$→LDLr$^{-/-}$ mice (p=0.9), while a tendency to increased apoA-I induced efflux was observed (2.5-fold; p=0.07)(Fig. 4C).

DISCUSSION

Besides the important role of the integral membrane ABC transporter ABCB1 in chemoresistance in cancer cells, ABCB1 has also been implicated in cholesterol homeostasis via several mechanisms, including cholesterol esterification [11, 12, 23], transport [7-10], absorption [15, 16], and efflux [5]. Therefore, it is assumed that ABCB1 could also play a role in the development of atherosclerosis. In the current study using the bone marrow transplantation technique, we show that the effect of macrophage specific ABCB1 deficiency in LDLr$^{-/-}$ mice augments the WTD-induced increase in serum total cholesterol levels, which was caused by a significant increase in the amount of cholesterol transported by apoB-containing lipoproteins. Interestingly, studies in humans showed that increased ABCB1 mRNA and protein expression found in ABCB1 haplotype carriers was associated with high levels of serum total and LDL cholesterol, suggesting that this specific ABCB1 haplotype may contribute to increased plasma cholesterol levels [17].

The liver plays a pivotal role in systemic lipid homeostasis. Therefore, hepatic mRNA expression levels of genes involved in cholesterol homeostasis were determined in ABCB1$^{+/+}$→LDLr$^{-/-}$ and ABCB1$^{-/-}$→LDLr$^{-/-}$ mice. However, no significant differences were found which may be explain the observed increase in serum total cholesterol. Interestingly, ABCB1$^{-/-}$→LDLr$^{-/-}$ mice did exhibit significantly higher mRNA expression levels of LRP1, ABCG5, and BSEP, suggesting an increase in LDL cholesterol uptake by the liver via LRP1 and induced excretion of cholesterol via ABCG5 or bile acids via BSEP into the bile. In agreement, Thornton *et al.* [24] recently reported that total body ABCB1-deficient mice show an increase in fecal total cholesterol content, which may be due to increased cholesterol efflux from the liver via the bile (mediated by the heterodimeric pair of proteins ABCG5 and

8). They demonstrated that mice lacking ABCB1 do not exhibit alterations in hepatic total cholesterol storage and circulating plasma total cholesterol levels either on a normal fat diet or a high fat diet [24]. Therefore, the increase in fecal cholesterol content in ABCB1-deficient mice may be due to increased cholesterol elimination via the liver into the bile or due to the decreased uptake of dietary and biliary cholesterol.

Different studies using ABCB1-deficient mice or patients with a ABCB1 haplotype, however, show differential effects of ABCB1 on serum cholesterol levels varying from an increase [17] to no effect [24] and even a decrease [15]. Clearly, further research is necessary to unravel the mechanisms underlying the effects of ABCB1 in the different organs on serum cholesterol levels.

During atherogenesis, the primary cell that is overloaded with cholesterol is the macrophage. Maintenance of normal cholesterol homeostasis in macrophages is therefore essential to prevent foam cell formation and atherosclerotic lesion development. Klucken *et al.* [5] have reported that ABCB1 mRNA can be downregulated *in vitro* by incubation of monocyte-derived macrophages with acetylated LDL and upregulated by induction of cholesterol efflux to HDL. ABCA1 is up-regulated in response to cholesterol loading [25, 26], consistent with its function as a cholesterol efflux transporter. ABCB1 expression, however, was regulated in the opposite direction to that of ABCA1, supporting a role for ABCB1 in maintaining cellular cholesterol levels. On the contrary, Langmann *et al.*[6] showed that ABCB1, like ABCA1, mRNA expression was induced in macrophages upon stimulation with LXR/RXR agonists. In addition, also a role was suggested for ABCB1 in the transport of cholesterol from the plasma membrane to the ER for esterification of plasma membrane-derived cholesterol by ACAT [11, 12]. Batteta *et al.* [13] demonstrated that high expression mRNA expression levels of ABCB1 were positively correlated with high levels of ACAT mRNA expression and cholesteryl ester content in atherosclerotic lesions of human arteries. These findings suggest a role for ABCB1 in macrophage cholesterol homeostasis and thus in atherosclerotic lesion development. However, atherosclerotic lesion development did not differ significantly between ABCB1$^{-/-}$→LDLr$^{-/-}$ and ABCB1$^{+/+}$→LDLr$^{-/-}$ mice, despite the observed increase in serum lipid levels. Interestingly, we observed a tendency to increased cholesterol efflux from macrophages of ABCB1$^{-/-}$→LDLr$^{-/-}$ mice to apoA-I. ABCB1 was proposed to redistribute cholesterol in the plasma membrane, suggesting that ABCB1 could modulate cholesterol efflux to cholesterol acceptors [27]. In line, Le Goff *et al.* [28] showed that cholesterol efflux to methyl-b-cyclodextrin (CD) was 4-fold higher in ABCB1 expressing cells compared with control cells lacking ABCB1, indicating that the accessible pool of plasma membrane cholesterol was indeed increased by ABCB1 expression [28]. ApoA-I-induced cholesterol efflux via ABCA1 plays an important protective role against atherosclerosis [29]. It is interesting to speculate that the increased cholesterol efflux from macrophages to apoA-I might thus have prevented an anticipated increased susceptibility to athersclerosis as a result of the increase in pro-atherogenic apoB-lipoproteins upon disruption of ABCB1 in bone marrow-derived cells.

In conclusion, macrophage ABCB1 does not affect atherosclerotic lesion development, despite its modulatory effects on serum lipid levels.

ACKNOWLEDGEMENTS

This research was supported by The Netherlands Organization for Scientific Research (Grant 917.66.301 (I.M. and M.V.E.)). M.V.E. is an Established Investigator of the Netherlands Heart Foundation (Grant 2007T056).

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