ABC transporters and scavenger receptor BI : important mediators of lipid metabolism and atherosclerosis
Meurs, I.

Chapter 3

Macrophage ABCA5 deficiency influences cellular cholesterol efflux and increases susceptibility to atherosclerosis in female LDLr knockout mice

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ABSTRACT

Objectives- To determine the role of macrophage ATP-binding cassette transporter A5 (ABCA5) in cellular cholesterol homeostasis and atherosclerotic lesion development.
Methods and results- Chimeras with dysfunctional macrophage ABCA5 (ABCA5$^{-M/-M}$) were generated by transplantation of bone marrow from ABCA5 knockout (ABCA5$^{-/-}$) mice into irradiated LDLr$^{-/-}$ mice. In vitro, bone marrow-derived macrophages from ABCA5$^{-M/-M}$ chimeras exhibited a 29% (P<0.001) decrease in cholesterol efflux to HDL, whereas a 21% (P=0.07) increase in cholesterol efflux to apoA-1 was observed. Interestingly, expression of ABCA1, but not ABCG1, was up-regulated in absence of functional ABCA5 in macrophages. To induce atherosclerosis, the transplanted LDLr$^{-/-}$ mice were fed a high-cholesterol Western-type diet (WTD) for 6, 10, or 18 weeks, allowing analysis of effects on initial as well as advanced lesion development. Atherosclerosis development was not affected in male ABCA5$^{-M/-M}$ chimeras after 6, 10, and 18 weeks WTD feeding. However, female ABCA5$^{-M/-M}$ chimeras did develop significantly (P<0.05) larger aortic root lesions as compared with female controls after 6 and 10 weeks WTD feeding.
Conclusions- ABCA5 influences macrophage cholesterol efflux, and selective disruption of ABCA5 in macrophages leads to increased atherosclerotic lesion development in female LDLr$^{-/-}$ mice.

INTRODUCTION

Reverse cholesterol transport (RCT) is an important mechanism by which high-density lipoprotein (HDL) and its major protein apolipoprotein A-I (apoA-I) protect against atherosclerosis.[1] Several genes have been implicated in RCT, including ATP-binding cassette (ABC) transporters ABCA1 and ABCG1, as well as scavenger receptor class B type I (SR-BI). ABCA1 mediates cholesterol efflux from macrophages to lipid-free apoA-I[2,3],while ABCG1 and SR-BI facilitate the efflux of cholesterol from macrophages to mature HDL.[4-6]

In addition to ABCA1, several other members of the ABC sub-family A have been implicated in lipid translocation or homeostasis, including ABCA2[7] and ABCA7.[8] Recent evidence suggests a physiological role for ABCA5. The cDNA cloning of human and rat ABCA5 was firstly described by Petry et al.[9] ABCA5 knockout (ABCA5$^{-/-}$) mice have been generated, and these mice exhibited symptoms of lysosomal disease in the heart, developed dilated cardiomyopathy, and died at approximately 10 weeks of age, due to depression of the cardiovascular system.[10] Additionally, ABCA5$^{-/-}$ mice displayed exophthalmos and collapse of the thyroid gland. Currently, neither the exact functions nor the substrate spectrum of ABCA5 is known. Immunohistochemical studies revealed that ABCA5 is highly expressed in cardiomyocytes of the heart, oligodendrocytes and astrocytes of the brain, alveolar type II cells of the lung, and Leydig cells of the testis.[11] The pronounced expression of ABCA5 in Leydig cells, which are known to process cholesterol and synthesize essential steroid hormones such as testosterone, indicates that ABCA5 may play a role in intracellular sterol/steroid trafficking. Furthermore, ABCA5 mRNA is expressed in human monocytes and macrophages.[12] Klucken et al. have reported that ABCA5 mRNA can be upregulated in vitro by incubation of monocyte-derived macrophages with acetylated LDL and downregulated by induction of cholesterol efflux to HDL$_3$.[13] Recently, we identified ABCA5 as a new transporter which is highly expressed by Kupffer cells, resident macrophages of the liver. Of note, in response to Western-type diet, ABCA5 expression was induced especially in Kupffer cells.[14] It is thus conceivable that ABCA5 may play a potential role in macrophage cholesterol homeostasis. However, the evidence for a role of macrophage ABCA5 in atherogenesis is presently lacking.

In this study, we investigated the effects of selective disruption of ABCA5 in bone marrow-derived cells and thus macrophages on cellular cholesterol homeostasis, lipoprotein metabolism, and atherosclerotic lesion development by using bone marrow transplantation (BMT). We show that ABCA5 potentially influences macrophage cholesterol efflux. Disruption of ABCA5 in macrophages did not affect atherosclerotic lesion development in male LDL receptor knockout (LDLr$^{-/-}$) mice. However, atherosclerosis in female LDLr$^{-/-}$ mice, which are more susceptible to atherosclerosis, was significantly increased in absence of functional macrophage ABCA5.

MATERIALS AND METHODS

For detailed methodology, please refer to the data supplement.

Chimeric mice with a selective deficiency of ABCA5 in hematopoietic cells, including macrophages, were generated by using the BMT technique. Briefly, LDLr$^{-/-}$ mice (C57Bl/6J N5) were exposed to a single dose of 9 grays (Gy) (0.19 Gy/min, 200 kV, 4 mA) X-ray total body irradiation, using an Andrex Smart 225 Röntgen source (YXLON International, Copenhagen, Denmark). Irradiated male and female recipients were transplanted by intravenous injection of 0.5x10^7 bone marrow cells, isolated from male and female ABCA5$^{-/-}$ mice[10], respectively, or from wild-type littermates. After that, transplanted animals (from now indicated as ABCA5$^{-M/-M}$ and ABCA5$^{+M/+M}$ mice, respectively) were maintained on sterilized regular chow diet (RM3, Special Diet Services, Witham, UK) for 8 weeks to allow the mice recover from the BMT. To induce atherosclerosis development, the mice were fed Western-type diet (WTD), containing 15% (w/w) fat and 0.25% (w/w) cholesterol (Diet W, Ab diets, Woerden, The Netherlands) for 6, 10, or 18 (only in the male group) after which the mice were euthanized and atherosclerotic lesion development was quantified.

At 14 weeks posttransplant (i.e., after 6 weeks WTD feeding), bone marrow cells were isolated from male ABCA5$^{-M/-M}$ and ABCA5$^{+M/+M}$ mice, bone marrow-derived macrophages were generated, and macrophage cholesterol efflux was evaluated. In addition, these macrophages were incubated with βVLDL (50 µg/ml) or ox-LDL (20 µg/ml) for 24 hours. Lipid accumulation was visualized with oil red O staining, and gene mRNA and protein expression was determined. Furthermore, in vivo foam cell formation was determined in the peritoneal cavity of ABCA5$^{-M/-M}$ mice and ABCA5$^{+M/+M}$ controls at 14 weeks posttransplant, measured on a Sysmex XT-2000i analyzer (Goffin Meyvis, the Netherlands).

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.

RESULTS

Effect of ABCA5 deficiency on macrophage cholesterol metabolism

Cholesterol efflux from ^{3}H-cholesterol-loaded ABCA5$^{+/+}$ and ABCA5$^{-/-}$ bone marrow-derived macrophages was compared (Fig. 1A). Disruption of ABCA5 resulted in a 29% (n=5; P<0.001) decrease in macrophage cholesterol efflux to HDL, whereas a 21% (n=5; P=0.07) increase in cellular cholesterol efflux to apoA-I was observed.

Next, the impact of ABCA5 expression on macrophage foam cell formation was investigated upon loading with βVLDL or ox-LDL for 24 hours. Interestingly, the use of different cholesterol sources led to different patterns of lipid accumulation in macrophages. As shown, lipid droplets unite and form large intracellular lipid deposits in macrophages after VLDL loading, while loading with ox-LDL resulted in a more diffuse lipid distribution (Fig. 1B). No apparent difference in cytoplasmic lipid accumulation was found between ABCA5$^{+/+}$ and ABCA5$^{-/-}$ macrophages upon loading with βVLDL. However, loading with ox-LDL did lead to clearly less extensive lipid staining in ABCA5$^{-/-}$ macrophages as compared to ABCA5$^{+/+}$ macrophages.

Furthermore, quantitative RT-PCR analysis revealed that ABCA5 mRNA expression was

not significantly regulated upon incubation with βVLDL, but it was 4.3-fold (P<0.05) up-regulated in response to ox-LDL (Fig. 1C). ABCA1 mediates macrophage cholesterol efflux to apoA-I. Interestingly, ABCA1 mRNA expression was significantly (P<0.01) higher in ABCA5$^{-/-}$ macrophages as compared to ABCA5$^{+/+}$ macrophages upon either βVLDL or ox-LDL loading. Western-blot analysis showed a 1.7-fold increase in ABCA1 protein expression in ABCA5$^{-/-}$ macrophages as compared to ABCA5$^{+/+}$ macrophages upon ox-LDL loading (Fig. 1D). However, no difference in ABCA1 protein was observed between ABCA5$^{-/-}$ and ABCA5$^{+/+}$ macrophages upon βVLDL loading (Fig. 1D), which is most likely caused by the fact that ABCA1 has already been maximally upregulated in both types of macrophages in response to the extensive lipid loading with βVLDL. ABCG1 and SR-BI facilitate cholesterol efflux to HDL. ABCG1 mRNA expression did not differ between ABCA5$^{+/+}$ and ABCA5$^{-/-}$ macrophages in response to either βVLDL or ox-LDL (Fig. 1C). No mRNA expression of SR-BI could be detected in bone marrow-derived macrophages after culture (data not shown). In addition, mRNA expression of genes which are involved in cholesterol biosynthesis [i.e., Hydroxymethylglutaryl-CoA (HMG-CoA) and HMG-CoA reductase (HMGR)] and lipoprotein uptake (i.e., SR-A, CD36, VLDL receptor, and LDL receptor-related protein), did not significantly differ between ABCA5$^{+/+}$ and ABCA5$^{-/-}$ macrophages in response to either βVLDL or ox-LDL loading (data not shown). These observations thus suggest that disruption of ABCA5 in macrophages may directly or indirectly (via compensatory upregulation of ABCA1) affect cellular cholesterol efflux.

Effect of macrophage ABCA5 deficiency on serum lipid levels in LDLr$^{-/-}$ mice

To evaluate the effects of macrophage ABCA5 expression in vivo, ABCA5 was selectively disrupted in hematopoietic cells, thus including macrophages, by transplantation of bone marrow from ABCA5$^{-/-}$ mice into LDLr$^{-/-}$ mice. After a recovery period of 8 weeks on regular chow diet, the transplanted mice were challenged with a high cholesterol, high fat Western-type diet (WTD) for 6, 10, and 18 weeks (only in male recipients). During the course of the experiment, the weight gain curve did not show significant changes between ABCA5$^{-M/-M}$ mice and ABCA5$^{+M/+M}$ controls, either in the male or the female transplanted groups (Fig. S1).

On regular chow diet, serum total cholesterol, phospholipids, and triglyceride levels were similar between ABCA5$^{+M/+M}$ and ABCA5$^{-M/-M}$ mice, both in the male and the female transplanted groups (Table 1). The distribution of lipids over the different lipoprotein fractions of the control and experimental groups was essentially identical (data not shown).

On challenging the mice with WTD, serum total cholesterol levels were increased by ≈3-fold at 14 weeks after BMT (i.e., 6 weeks WTD feeding) (Table 1). However, no significant effect of macrophage ABCA5 deficiency was observed on serum total cholesterol levels (male ABCA5$^{-M/-M}$: 1252±54 mg/dL, n=11 vs. male ABCA5$^{+M/+M}$: 1089±75 mg/dL, n=11, P=0.40; and female ABCA5$^{-M/-M}$: 769±44 mg/dL, n=9 vs. female ABCA5$^{+M/+M}$: 878±47 mg/dL, n=7, P=0.12). Fractionation of serum lipoproteins showed that VLDL and/or LDL cholesterol levels were increased in ABCA5$^{-M/-M}$ mice as compared to ABCA5$^{+M/+M}$ controls, in both the male and the female recipients. HDL cholesterol levels did not significantly change in the male
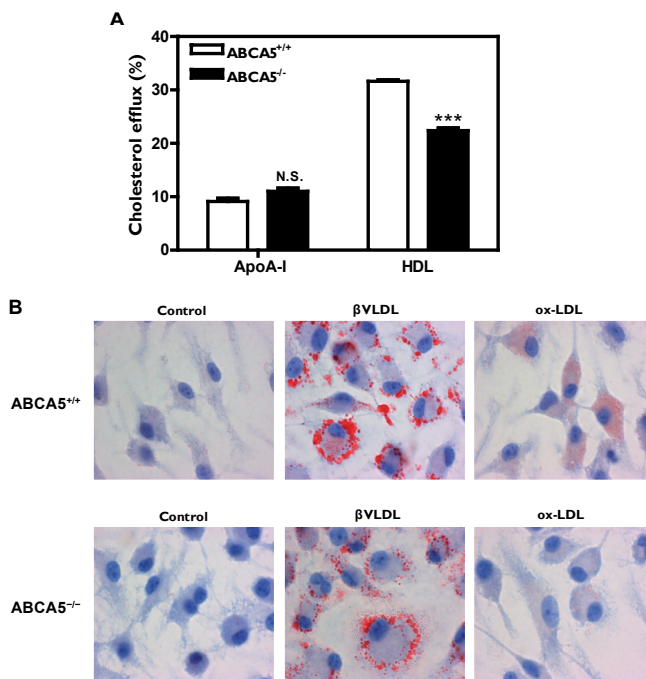
Fig. 1. Effect of macrophage ABCA5 deficiency on cholesterol efflux

Bone marrow cells were isolated from male recipients at 14 weeks posttransplant. A) ApoA-I (10 µg/mL) and HDL (50 µg/mL) induced cellular cholesterol efflux from ^{3}H-cholesterol-labeled ABCA5$^{+/+}$ (open bars) and ABCA5$^{-/-}$ (closed bars) bone marrow-derived macrophages. Results are the mean±SEM of n=5 per group. Statistically significant difference ***p<0.001 vs. ABCA5$^{+/+}$ macrophages. N.S. = non-significant. B) ABCA5$^{+/+}$ and ABCA5$^{-/-}$ bone marrow-derived macrophages were incubated with βVLDL (50 µg/ml) or oxidized-LDL (ox-LDL; 20 µg/ml) for 24 hours. Lipid accumulation was visualized with oil red O staining. Original magnification x400. C) mRNA expression of ABCA5, ABCA1, and ABCG1 in ABCA5$^{+/+}$ and ABCA5$^{-/-}$ bone marrow-derived macrophages upon incubation with either βVLDL or ox-LDL, determined by quantitative RT-PCR. **p<0.01 vs. ABCA5$^{+/+}$ macrophages upon cholesterol loading. $^{#}$p<0.05 vs. ABCA5$^{+/+}$ macrophages upon control loading. N.S. = non-significant. D)Western blots showing ABCA1 protein in cell lysates of ABCA5$^{+/+}$ and ABCA5$^{-/-}$ bone marrow-derived macrophages. Equal amounts of cellular proteins (15 µg) were loaded on a 7.5% SDS-PAGE gel. A representative immunoblot of ABCA5$^{+/+}$ and ABCA5$^{-/-}$ macrophages under various loading conditions (i.e., control, βVLDL, and ox-LDL) is shown. A single band of ABCA1 protein (~230kDa) was detected with different abundance in all tested samples. α-tubulin (~50kDa) was used to control equal loading. Numbers below the blot indicate changes of ABCA1 protein expression between ABCA5$^{-/-}$ and ABCA5$^{+/+}$ macrophages after adjustment for the internal control.

ABCA5$^{-M/-M}$ mice (82±11 mg/dL, n=6 vs. 78±10 mg/dL, n=6 for the male controls), whereas markedly (P<0.01) lower HDL cholesterol levels were observed in the female ABCA5$^{-M/-M}$ mice (36±2 mg/dL, n=6 vs. 63±3 mg/dL, n=6 for the female controls) (Fig. 2). In addition, macrophage ABCA5 deficiency resulted in 1.2-fold (P<0.05) higher serum phospholipid levels (male ABCA5$^{-M/-M}$: 725±23 mg/dL, n=11 vs. male ABCA5$^{+M/+M}$: 589±56 mg/dL, n=11; and female ABCA5$^{-M/-M}$: 823±48 mg/dL, n=9 vs. female ABCA5$^{+M/+M}$: 676±40 mg/dL, n=7) (Table I). The increased phospholipid levels were mainly caused by an increase in the VLDL fraction (Fig. 2). Moreover, serum triglyceride levels did not differ between the male ABCA5$^{-M/-M}$ mice and the male ABCA5$^{+M/+M}$ controls, whereas 2.5-fold (P<0.05) higher triglyceride levels were observed in the female ABCA5$^{-M/-M}$ mice (171±38 mg/dL, n=9 vs. 68±9 mg/dL, n=7 for the

female controls) (Table I). The increased triglyceride levels in the female ABCA5$^{-M/-M}$ mice were mainly due to increases in the VLDL/LDL fractions (data not shown). Thus, macrophage ABCA5 deficiency resulted in increased serum lipid levels associated with VLDL and/or LDL particles in LDLr$^{-/-}$ recipients, while HDL cholesterol was specifically reduced in the female transplanted LDLr$^{-/-}$ mice on WTD.
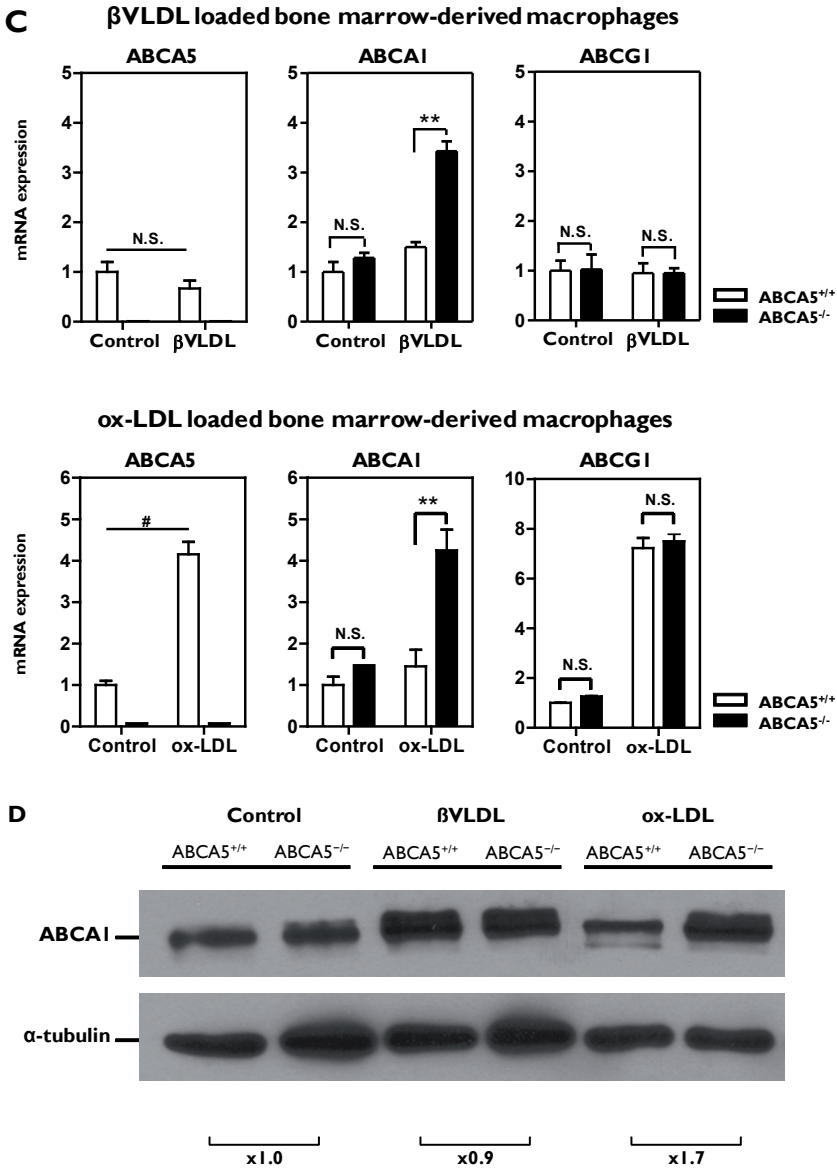

C βVLDL loaded bone marrow-derived macrophages

ox-LDL loaded bone marrow-derived macrophages

D

Fig. 1. Continued

Table 1. The effect of macrophage ABCA5 deficiency on serum lipid levels in transplanted LDLr$^{-/-}$ mice

Male	Time (weeks)	Diet	Total Cholesterol (mg/dL)	HDL Cholesterol (mg/dL)	Phospholipids (mg/dL)	Triglycerides (mg/dL)
ABCA5$^{+M/+M}$	Baseline	Chow	416±12	ND	524±20	217±19
	8	Chow	317±20	104±11	644±26	158±24
	14	WTD	1089±75	78±10	589±56	444±88
ABCA5$^{-M/-M}$	Baseline	Chow	419±10	ND	547±14	242±11
	8	Chow	303±15	122±5	718±25	193±16
	14	WTD	1252±54	82±11	725±23*	477±62

Female	Time (weeks)	Diet	Total Cholesterol (mg/dL)	HDL Cholesterol (mg/dL)	Phospholipids (mg/dL)	Triglycerides (mg/dL)
ABCA5$^{+M/+M}$	Baseline	Chow	330±11	ND	519±16	143±13
	8	Chow	244±8	88±4	508±20	90±8
	14	WTD	769±44	63±3	676±40	68±9
ABCA5$^{-M/-M}$	Baseline	Chow	329±14	ND	497±18	143±14
	8	Chow	251±12	87±3	474±17	118±10
	14	WTD	878±47	36±2**	823±48*	171±38*

Blood samples were drawn after an overnight fast. Serum lipid concentrations, including total cholesterol, phospholipids, and triglycerides, were measured in ABCA5$^{+M/+M}$ and ABCA5$^{-M/-M}$ mice before transplantation (baseline), at 8 weeks post transplant while feeding regular chow (Chow), and at 14 weeks after transplantation after 6 weeks of feeding a high-cholesterol Western-type diet (WTD). Data represent mean±SEM of ≥8 mice per group, either in the male or the female transplanted groups. *p<0.05, **p<0.01 vs. ABCA5$^{+M/+M}$ controls. ND indicates not determined.

Effect of macrophage ABCA5 deficiency on in vivo foam cell formation and atherosclerotic lesion development in LDLr$^{-/-}$ mice

To investigate whether disruption of ABCA5 in macrophages would affect in vivo foam cell formation, peritoneal leukocytes were isolated from ABCA5$^{-M/-M}$ and ABCA5$^{+M/+M}$ mice at 14 weeks after BMT. Foam cell counts did not significantly differ between the male ABCA5$^{-M/-M}$ mice (3.8±0.7×10^4, n=5) and the male ABCA5$^{+M/+M}$ controls (4.3±0.9×10^4, n=5). However, a 2.7-fold (P<0.05) increase in foam cell formation was detected in the female ABCA5$^{-M/-M}$ mice (7.1±1.1×10^4, n=6) as compared with the female ABCA5$^{+M/+M}$ controls (2.7±1.2×10^4, n=6) (Fig. 3). In agreement, microscopic visualization using oil red O-stained cytospins also showed more peritoneal foam cells in the female ABCA5$^{-M/-M}$ chimeras, but not in the male ABCA5$^{-M/-M}$ mice.

Macrophage ABCA5 deficiency led to a 1.5-fold (P<0.05) increase in the mean aortic lesion area in the female recipients after 6 weeks WTD feeding (female ABCA5$^{-M/-M}$: 382±29x10^3 μm^2, n=9 vs. female ABCA5$^{+M/+M}$: 260±32x10^3 μm^2, n=7), and a moderate 1.3-fold (P=0.18) increase in lesion size after 10 weeks WTD feeding (female ABCA5$^{-M/-M}$: 659±73x10^3 μm^2, n=7 vs. female ABCA5$^{+M/+M}$: 516±77x10^3 μm^2, n=6) (Fig. 4A). Two-way ANOVA analysis
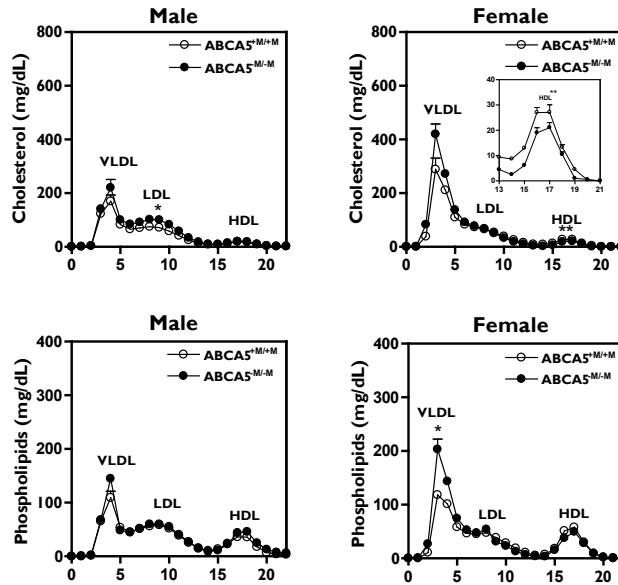
Fig. 2. Effect of macrophage ABCA5 deficiency on serum cholesterol and phospholipid distribution in transplanted LDLr$^{-/-}$ mice

Blood samples were drawn after an overnight fast in ABCA5$^{+M/+M}$ and ABCA5$^{-M/-M}$ mice (male: left; female: right) at 14 weeks posttransplant [i.e., after 6 weeks of feeding a high cholesterol Western-type diet (WTD)]. Sera from individual mice were loaded onto a Superose 6 column, and fractions were collected. Fractions 2 to 5 represent VLDL; fraction 6 to 14, LDL; and fractions 15 to 20, HDL, respectively. The distribution of cholesterol and phospholipid over the different lipoproteins in ABCA5$^{+M/+M}$ (○) and ABCA5$^{-M/-M}$ (●) chimeras is shown. Values represent the mean±SEM of ≥6 mice per group. Statistically significant difference *p<0.05, and **p<0.01 vs. ABCA5$^{+M/+M}$ controls.

showed a significant effect of ABCA5 deficiency on atherosclerosis development in the female recipients ($F_{[1,29]}$ = 7.13, P=0.0123). Lesion composition did not significantly differ between the female ABCA5$^{-M/-M}$ mice and the ABCA5$^{+M/+M}$ controls after 6 weeks (the macrophage content: 90±2.4% and 92±1.8%, respectively; the collagen content: 1.0±0.3% and 1.1±0.4%, respectively) and 10 weeks WTD feeding (the macrophage content: 50±2.8% and 53±3.2%, respectively; the collagen content: 9.9±2.0% and 8.9±3.0%, respectively). Furthermore, macrophage ABCA5 deficiency did not significantly affect atherosclerosis development in the male recipients after 6 weeks (male ABCA5$^{-M/-M}$: 118±10x10^3 μm^2, n=10; male ABCA5$^{+M/+M}$: 104±16x10^3 μm^2, n=11) and 10 weeks WTD feeding (male ABCA5$^{-M/-M}$: 254±45x10^3 μm^2, n=11; male ABCA5$^{+M/+M}$: 219±31x10^3 μm^2, n=11) (Fig. 4B). Of note, even after 10 weeks on WTD, the male transplanted animals developed initial lesions, primarily composed of macrophage-derived foam cells. Therefore, we prolonged the WTD feeding period to 18 weeks. Again, no change was found between the experimental and control groups (male ABCA5$^{-M/-M}$: 543±28x10^3 μm^2, n=4 vs. ABCA5$^{+M/+M}$: 563±103x10^3 μm^2, n=7). Two-way ANOVA analysis showed no significant effect of ABCA5 deficiency on atherosclerosis development in the male recipients ($F_{[1,48]}$ = 0.07, P=0.7996). Lesion composition did not significantly differ between the male ABCA5$^{-M/-M}$ mice and the

ABCA5$^{+M/+M}$ controls after 6 weeks (the macrophage content: 94±2.4% and 93±1.8%, respectively; the collagen content: 0.9±0.2% and 1.0±0.4%, respectively), 10 weeks (the macrophage content: 84±3.4% and 87±2.8%, respectively; the collagen content: 2.8±0.3% and 3.3±1.0%, respectively), and 18 weeks WTD feeding (the macrophage content: 36±3.0% and 35±2.9%, respectively; the collagen content: 7.6±0.8% and 6.1±1.4%, respectively). Thus, macrophage ABCA5 deficiency significantly increased atherosclerosis in female LDLr$^{-/-}$ mice, whilst atherosclerotic lesion development was largely unaffected in male LDLr$^{-/-}$ mice on WTD.
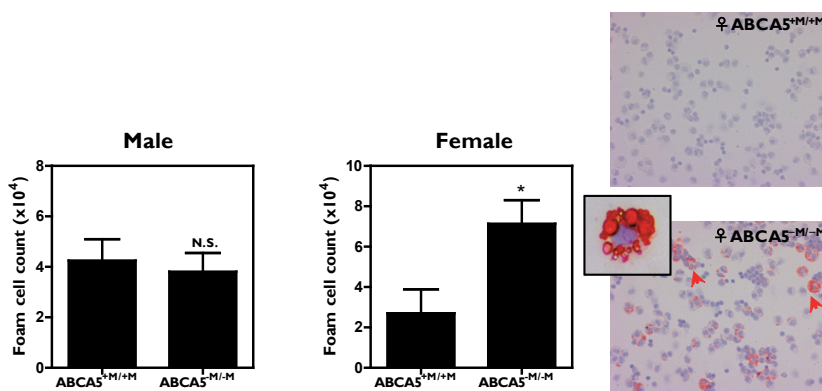

Fig. 3. *In vivo* foam cell formation
Peritoneal leukocytes were isolated from both male (left) and female (right) recipients at 14 weeks posttransplant. Peritoneal foam cells were counted. Values represent the mean±SEM of ≥5 mice per group. *p<0.05 vs. ABCA5$^{+M/+M}$ controls. N.S. = non-significant. Lipid accumulation in peritoneal leukocytes from female ABCA5$^{+M/+M}$ mice and female ABCA5$^{-M/-M}$ mice was visualized with oil red O staining in order to detect foam cells (red arrows). Original magnification x400.

DISCUSSION

Macrophages cannot limit their uptake of cholesterol via scavenger receptors.[15] Therefore, cholesterol efflux is a vital mechanism to maintain cholesterol homeostasis in macrophages. The central importance of ABCA1, ABCG1, and SR-BI in facilitating cholesterol efflux from macrophages has been clearly established.[5,16] In this study, we demonstrate for the first time that another novel mediator, ABCA5, may also affect macrophage cholesterol efflux, as ABCA5 deficiency significantly suppressed macrophage cholesterol efflux to HDL (by 29%). RT-PCR analysis showed that ABCG1 mRNA expression did not differ between macrophages with and without functional ABCA5 upon either βVLDL or ox-LDL loading, while no SR-BI mRNA expression could be detected in macrophages after culture. Thus, the effect of ABCA5 deficiency to reduce macrophage cholesterol efflux to HDL appears to be independent of ABCG1 and SR-BI. Contrary to reduced cholesterol efflux to HDL, a tendency to increased cholesterol efflux to apoA-I (by 21%) was observed in ABCA5-deficient macrophages. Gene expression analysis indicated a compensatory up-regulation of ABCA1 in absence of ABCA5 in macrophages, indicative for a functional relation between these two ABCA transporters.

Fig. 4. Macrophage ABCA5 deficiency increases susceptibility to atherosclerosis in female transplanted LDLr^{–/–} mice, but not in male transplanted LDLr^{–/–} mice

Formation of atherosclerotic lesions in female (**A**) and male (**B**) transplanted mice was determined at 14, 18, or 26 weeks posttransplant [i.e., after 6, 10, or 18 weeks of feeding a high-cholesterol Western-type diet (WTD)]. The mean lesion area (μm^2) was calculated from oil red O-stained cross-sections of the aortic root at the level of the tricuspid valves. Representative pictures are shown. Original magnification x50. Values represent the mean±SEM of 4-11 mice per group. Statistically significant difference *p<0.05 vs. ABCA5[+M/+M] controls. N.S. = non-significant.

Interestingly, ABCA1 and ABCA5 show similar subcellular localization. ABCA1 is sited in lysosomes and late endosomes, and mediates vesicular trafficking from these intracellular compartments to the plasma membrane.[17-18] Tangier disease fibroblasts lacking functional ABCA1 display defective endocytic trafficking, leading to the accumulation of cholesterol in late endosomes.[19-21] ABCA5 is also located in lysosomes and late endosomes, and ABCA5-deficient mice exhibited symptoms similar to those of several lysosomal diseases in the heart.[10] Previous studies have suggested that effects of single transporter deficiency can be compensated by upregulation of the other transporter(s). For instance, ABCA1 and ABCG1 function cooperatively, and ABCG1 deletion led to compensatory upregulation of ABCA1 and vice versa.[22,23] It is thus likely that ABCA5 utilizes the same intracellular route as ABCA1, and ABCA1 may compensate for the lack of functional ABCA5, thereby directly or indirectly impacting macrophages cholesterol homeostasis.

To study the role of macrophage ABCA5 expression in atherogenesis, ABCA5 was selectively disrupted in hematopoietic cells, thus including macrophages, by transplantation of bone marrow from ABCA5$^{-/-}$ mice into LDLr$^{-/-}$ mice. In contrast to ABCA5$^{-/-}$ mice, which have been reported to develop dilated cardiomyopathy and die at approximately 10 weeks of age[10], ABCA5$^{-M/-M}$ chimeras did survive even at 26 weeks after transplantation, showing normal weight gain and absence of cardiomyopathy. Disruption of ABCA5 in macrophages increased atherosclerotic lesion development in female transplanted LDLr$^{-/-}$ mice, but not in male transplanted animals. In agreement, in vivo foam cell formation was only increased in females and not in males. In vitro studies showed impaired cholesterol efflux to HDL from ABCA5-deficient macrophages. Enhanced foam cell formation locally in the vessel wall as a result of impaired cholesterol efflux might thus have contributed to the observed increase in atherosclerosis development in female ABCA5$^{-M/-M}$ chimeras. However, it does not explain why no increase in atherosclerosis susceptibility was found in the male recipients. Furthermore, foam cell formation in vitro was either not affected (in response to b-VLDL) or even reduced (in response to ox-LDL). It is thus more likely that systemic differences between males and females are responsible for the differential effects on atherogenesis. As described in this study, both male and female ABCA5$^{-M/-M}$ chimeras displayed higher levels of pro-atherogenic VLDL lipids, but the effects were more pronounced in the females. Furthermore, significantly lower levels of anti-atherogenic HDL cholesterol were specifically observed in the female ABCA5$^{-M/-M}$ mice. The liver plays a pivotal role in systemic lipid homeostasis, and Kupffer cells (resident macrophages in the liver) that are predominantly of donor bone marrow origin are rapidly replaced after bone marrow transplantation.[24] Since ABCA5 is highly expressed by Kupffer cells, it is likely that ABCA5 expression could be significantly decreased in the livers of ABCA5$^{-M/-M}$ mice, thereby directly or indirectly influencing hepatic lipoprotein biosynthesis or secretion. The direct cause for the differential findings between male and female recipients is currently unknown. Recently, Yang et al have reported that in mice many hepatic genes show sexual dimorphism (~70%). Furthermore, the highest changes (>3-fold) in gene expression between females and males were observed in genes involved in steroid and lipid metabolism.[25] Thus, gender differences may have contributed to the differential effects of macrophage ABCA5 deficiency on serum lipid levels and atherogenesis in male and female transplanted LDLr⁻In summary, we demonstrate here that ABCA5 influences macrophage cholesterol efflux. However, the compensatory

up-regulation of ABCA1 in macrophages lacking functional ABCA5 might have limited the effects of macrophage ABCA5 deficiency on atherosclerosis. Therefore, the generation of ABCA1 and ABCA5 double knockout mouse is worth exploiting to clarify the exact role of macrophage ABCA5 in atherosclerosis.

ACKNOWLEDGEMENTS

Chapter 3

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SUPPLEMENTARY APPENDIX

MATERIALS AND METHODS

Lipoprotein isolation

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.[1] 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.[2] Furthermore, low-density lipoprotein (LDL) (density 1.063 to 1.019 g/mL) and HDL (density 1.063 to 1.21 g/mL) were isolated from plasma of healthy subjects by ultracentrifugation in a KBr discontinuous gradient and dialysed against PBS/1mM EDTA according to Redgrave et al[1]. For generation of oxidized-LDL (ox-LDL), LDL was oxidatively modified by incubation of 200 μg/ml of LDL with 10 μM $CuSO_4$ at 37°C for 20 h.

Generation of bone marrow-derived macrophages in vitro

Bone marrow cells, isolated from male ABCA5$^{-/M/-M}$ and ABCA5$^{+M/+M}$ mice at 14 weeks posttransplant, 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.

Macrophage cholesterol efflux studies

Bone marrow-derived macrophages were incubated with 0.5 μCi/mL ^{3}H-cholesterol in DMEM/0.2% bovine serum albumin (BSA; fatty acid free) for 24 hours at 37°C. To determine cholesterol loading, cells were washed 3 times with washing buffer (50 mmol/L Tris containing 0.9% NaCl, 1 mmol/L EDTA, and 5 mmol/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 apoA-I (Calbiochem) or 50 μg/mL human HDL. Radioactivity in the medium was determined by scintillation counting after 24 hours of incubation.

Gene mRNA and protein expression analysis

Guanidium thiocyanate-phenol was used to extract total RNA from macrophages. 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 (Table S1) were designed using Primer Express Software according to the manufacturer's default settings. Hypoxanthine Guanine Phosphoribosyl Transferase (HPRT), b-actin, and acidic ribosomal phosphoprotein PO (36B4) were used as the standard housekeeping genes. Relative gene expression was calculated by subtracting

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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, in order to exclude the possibility that changes in the relative expression were caused by variations in the separate housekeeping gene expression.

Immunoblotting on protein from bone marrow-derived macrophages was performed as described previously.[3] In short, after running equal amounts of total cell protein (15 µg) on a 7.5% SDS-PAGE gel, ABCA1 was detected using murine monoclonal ABCA1 primary antibody (AC-10; a kind gift from Dr. M. Hayden, Vancouver, Canada) and a peroxidase-conjugated goat-anti-mouse IgG secondary antibody (Jackson ImmunoResearch). Monoclonal anti-α-tubulin antibody (Sigma) was used as a loading control. Finally, immunolabeling was detected by enhanced chemiluminescence (Thermo).

Serum lipid and lipoprotein analyses

At 8 weeks posttransplant when the mice were on regular chow diet and at 14, 18, or 26 weeks posttransplant when the animals were fed WTD, blood was drawn after an overnight fasting-period for determination of serum lipid levels and their distribution over different lipoproteins. In detail, after an overnight fast, ≈100 µL of blood was drawn from each mouse by tail bleeding. Total cholesterol in serum was determined using enzymatic colorimetric assays (Roche Diagnostics, Mannheim, Germany), with 0.03 U/ml cholesterol oxidase (Sigma), 0.065 U/ml peroxidase, and 15 µg/ml cholesteryl esterase (Seikagaku, Tokyo, Japan) 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). The concentrations of phospholipids and triglycerides in serum were determined using enzymatic colorimetric assays (Spinreact S.A. and Roche Diagnostics, respectively). Precipath I (Roche Diagnostics) was used as an internal standard. Absorbance was read at 490 nm. The distribution of lipids over the different lipoproteins was determined by fractionation of 30 ml serum of each mouse using a Superose 6 column (3.2x300mm, Smart-system, Pharmacia, Uppsala, Sweden). Total cholesterol, phospholipids and triglyceride contents in the effluent were determined as above.

Statistical analyses

Statistical analyses were performed using one- and two-way ANOVA using Graphpad Prism Software (Graphpad Software, Inc.; http://www.graphpad.com). The level of statistical significance was set at $P < 0.05$.

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Table S1: Primers for quantitative real-time PCR analysis

| Gene	GenBank Accession	Forward primer	Reverse Primer
β-actin	X03672	AACCGTGAAAAGATGACCCAGAT	CACAGCCTGGATGGCTACGTA
36B4	X15267	GGACCCGAGAAGACCTCCTT	GCACATCACTCAGAATTTCAATGG
HPRT	J00423	TTGCTCGAGATGTCATGAAGGA	AGCAGGTCAGCAAAGAACTTATAG
ABCA5	NM_147219	TCATGGGAGAGGCCGCT	GCGAGAAGGCTATGACGAGGT
ABCA1	NM_013454	GGTTTGGAGATGGTTATACAATAGTTGT	TTCCCGGAAACGCAAGTC
ABCG1	NM_009593	AGGTCTCAGCCTTCTAAAGTTCCTC	TCTCTCGAAGTGAATGAAATTTATCG
SR-BI	NM_016741	GGCTGCTGTTTGCTGCG	GCTGCTTGATGAGGGAGGG
SR-A	L04274	GGTGGTAGTGGAGCCCATGA	CCCGTATATCCCAGCGATCA
CD36	NM_007643	GTTCTTCCAGCCAATGCCTTT	ATGTCTAGCACACCATAAGATGTACAGTT
LRP	NM_008512	TGGGTCTCCCGAAATCTGTT	ACCACCGCATTCTTGAAGGA
VLDLr	NM_013703	TGGAGATGAAGACTGTGCGG	CGAAGTCAGACTCAGCACACG
HMG-CoA	NM_008256	TTTCATTCCGAGTGTCCAAGG	CTGACACACTAGACACCAGTTTCTCC
HMGR	M62766	TCTGGCAGTCAGTGGGAACTATT	CCTCGTCCTTCGATCCAATTT
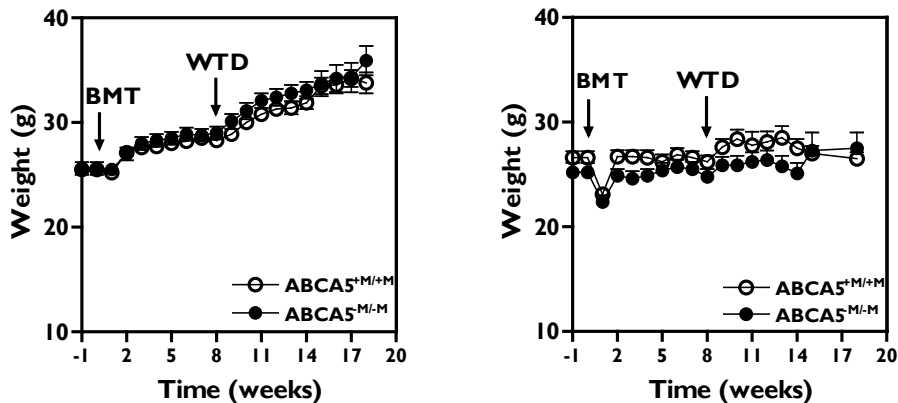
Fig. 1S. Effect of macrophage ABCA5 deficiency on weight gain in transplanted LDLr$^{-/-}$ mice
The body weight was measured in male (left) and female (right) LDLr$^{-/-}$ mice one week before bone marrow transplantation (BMT), and in ABCA5$^{+M/+M}$ (○) and ABCA5$^{-M/-M}$ (●) chimeras up to 18 weeks after BMT. Data represent mean±SEM of ≥20 mice per group.