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Macrophage ABCA2 deletion modulates intracellular cholesterol deposition, affects macrophage apoptosis, and decreases early atherosclerosis in LDL receptor knockout mice

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ABSTRACT

Objective: The ABCA2 transporter shares high structural homology to ABCA1, which is crucial for the removal of excess cholesterol from macrophages and, by extension, in atherosclerosis. It has been suggested that ABCA2 sequesters cholesterol inside the lysosomes, however, little is known of the macrophage-specific role of ABCA2 in regulating lipid homeostasis *in vivo* and in modulating susceptibility to atherosclerosis.
Methods: Chimeras with dysfunctional macrophage ABCA2 were generated by transplantation of bone marrow from ABCA2 knockout (KO) mice into irradiated LDL receptor (LDLr) KO mice.
Results: Interestingly, lack of ABCA2 in macrophages resulted in a diminished lesion size in the aortic root (−24.5%) and descending thoracic aorta (−36.6%) associated with a 3-fold increase in apoptotic cells, as measured by both caspase 3 and TUNEL. Upon oxidized LDL exposure, macrophages from wildtype (WT) transplanted animals developed filipin-positive droplets in lysosomal-like compartments, corresponding to free cholesterol (FC) accumulation. In contrast, ABCA2-deficient macrophages displayed an abnormal diffuse distribution of FC over peripheral regions. The accumulation of neutral sterols in lipid droplets was increased in ABCA2-deficient macrophages, but primarily in cytoplasmic clusters and not in lysosomes. Importantly, apoptosis of oxLDL loaded macrophages lacking ABCA2 was increased 2.7-fold, probably as a consequence of the broad cellular distribution of FC.
Conclusions: Lack of functional ABCA2 generates abnormalities in intracellular lipid distribution/trafficking in macrophages consistent with its lysosomal sequestering role, leading to an increased susceptibility to apoptosis in response to oxidized lipids and reduced atherosclerotic lesion development.

1. Introduction

The excessive accumulation of cholesterol in macrophages can lead to their transformation into foam cells contributing to the initiation and progression of atherosclerotic lesions. ATP-Binding Cassette (ABC) transporters constitute a large highly conserved superfamily of membrane molecules that use the energy of ATP hydrolysis to transport a wide variety of substrates across the cell membrane [1]. To date, there are 52 characterized human ABC transporter genes, which can be divided into seven classes (A–G), and mutations in these genes cause several human genetic disorders [1]. Several ABC transporters exert major functions in macrophage lipid and lipoprotein trafficking being intimately involved in the pathogenesis of atherosclerosis [2,3]. In this context, ABCA1 has a particularly important role in mediating cholesterol and phospholipid efflux from macrophages in the presence of apolipoprotein (apo) acceptors like apoA–I [4]. Apart from ABCA1, other members of the ABC transporter superfamily play a significant role in protection against atherosclerosis by

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facilitating the removal of excess cholesterol from macrophages, such as ABCG1 and ABCB4 [5,6].

ABCA2 is a full-size ABC transporter of 270 kDa which is located on chromosome 9q close to ABCA1 [7,8]. Both members share a high degree of structural homology, strongly suggesting a duplication event during evolution [9]. Analysis of the ABCA2 promoter region exposed multiple potential binding sites for transcription factors with potential roles in the differentiation and activation of macrophages, myeloid and neural cells [7,10,11]. Moreover, ABCA2 contains promoter target sites for SP1, a transactivator which can modulate the promoter activity of the cholesterol-responsive transporter ABCA1 and half-size transporter ABCG1 [7], as well as the presence of a lipocalin signature motif coding a conserved group of proteins which bind and transport sterols [12,13]. It is thus conceivable that ABCA2, like ABCA1, can function as a facilitator of cholesterol lateral translocation. In fact, it was reported that ABCA2 is involved in the intracellular metabolism of sphingolipids in the brain and may serve as a regulator of cholesterol and phospholipid flux to the myelin membrane in oligodendrocytes [14,15]. ABCA2 is detected in endosomes, lysosomes and trans-Golgi network organelles. It is found predominantly in the brain and to a lesser extent in kidney, uterus, liver, heart, thymus, and myeloid cells, including monocytes [7,9,12,16]. Interestingly, ABCA2 expression is upregulated during monocyte differentiation into macrophages [19].

ABCA2 is a sterol-sensitive gene which is overexpressed by sustained cholesterol influx in the presence of enzymatically modified LDL in macrophages [7], showing a regulatory response similar to transporters ABCA1 and ABCG1 [17,18]. A functional role for the ABCA2 transporter in the intracellular cholesterol trafficking, delivering low-density lipoprotein (LDL)-derived free cholesterol (FC) to the endoplasmic reticulum for esterification, has been described in Chinese hamster ovary (CHO) cells and neuroblastoma cells [19–21]. Stable overexpression of ABCA2 in CHO cells showed cholesterol sequestration in lysosomes and positively regulated other genes that increase cellular cholesterol levels (LDL receptor (LDLr) and 3-hydroxy-3-methylglutaryl CoA synthase (HMGCoA S)) whereas it reduced the esterification of LDL-derived FC [19]. Recently, ABCA2 was shown to regulate cholesterol homeostasis and LDLr metabolism in neuronal-type-like cells [21].

Given the importance of cholesterol homeostasis in macrophages for prevention of foam cell formation and the anti-atherogenic effects of leukocyte ABCA1 in hyperlipidemic mice [22,23], ABCA2 represents a potentially attractive target for atherosclerosis intervention. The data presented in the current study provide, to our knowledge, the first assessment of the effect of hematopoietic ABCA2 deficiency on atherosclerosis and lipid metabolism in mice using the bone marrow (BM) transplantation strategy.

2. Methods

An expanded Methods section is available in the data supplement.

2.1. Animals

ABCA2 knockout (KO) and ABCA2 wildtype (WT) mice were bred and maintained as previously described [15]. Homozygous LDLr KO mice were obtained from The Jackson Laboratory (Bar Harbor, Me) as mating pairs and bred at the Gorlaeus Laboratory (Leiden, The Netherlands).

2.2. Bone marrow transplantation

To induce bone marrow aplasia, LDLr KO recipient mice of 10–12 weeks old were exposed to a single dose of 9 Gy 1 day before the transplantation ($n = 13–14$ per group). Bone marrow was isolated from the donor ABCA2 KO and WT mice with phosphate-buffered saline (PBS). Irradiated recipients received 5×10^6 bone marrow cells by intravenous injection into the tail vein.

2.3. Serum lipid and lipoprotein analyses

The concentrations of total cholesterol (TC), phospholipids (PL) and triglycerides (TG) in serum were determined using enzymatic colorimetric assays 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. After 10 weeks WTD feeding, total blood cells were quantified using an automated Sysmex XT-2000iV analyzer (Goffin Meyvis, Etten-Leur, The Netherlands).

2.4. Flow cytometry analysis

After sacrificing the mice, spleen, liver, and peritoneal macrophages were isolated and single cell suspensions were obtained. Subsequently, 300,000 cells were stained with the appropriate antibodies.

2.5. Lipoprotein isolation and generation of bone marrow-derived macrophages in vitro

Please see Supplementary methods.

2.6. Gene mRNA and protein expression analysis

Quantitative gene expression analysis was performed using the SYBR-Green method on an Applied Biosystems Prism 7500 sequence detection system (Applied Biosystems, Foster City, CA). PCR primers (Supplemental Table 1) were designed using Primer Express Software according to the manufacturer's default settings. Immunoblotting on protein from peritoneal macrophages was performed as described previously [24].

2.7. Foam cell formation studies

BM-derived macrophages were incubated with oxLDL (20 μg/mL) for 24 h. Lipid accumulation was visualized with Oil-red O staining. Cellular lipids were extracted with isopropyl alcohol-hexane (2:3 v/v), dried with nitrogen and reconstituted with isopropyl alcohol–0.5% sodium cholate [25], as previously described. Shingomyelin species were analyzed using liquid chromatography/mass spectrometry methodology.

For analysis of *in vivo* foam cell formation, 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. Samples were cytospun and stained with Oil-red O for detection of lipid accumulation.

2.8. Histological analysis of the aortic root and tissues

Cryosections of formalin-fixed lung, liver, brain and spleen from ABCA2 KO and WT transplanted animals 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. Atherosclerotic mean lesion area (in μm^2) was quantified in the aortic root from ten Oil-red O-stained sections for each animal, starting at the appearance of the tricuspid valves, as well as in the thoracic descending aorta and the aortic arches. The macrophage infiltration, the collagen content and the apoptotic cells in the atherosclerotic lesions were determined.

2.9. Design and cloning of short hairpin RNA (shRNA) directed against murine ABCA2, and silencing effect of pSUPER-H1.shABCA2

A pair of 64-nucleotide shRNA oligonucleotides which contained extra BglII and HindIII restriction sites to facilitate cloning, was designed targeting murine ABCA2. The 64-nucleotide oligonucleotides were annealed and cloned into de BglII and HindIII sites of the pSUPER vector. The lentiviral expression vectors pRR1-cPPt-H1.PreSIN (H1.Empty) and pRR1-cPPt-H1.shABCA2-PreSIN (H1.shABCA2) were constructed by removing the cytomegalovirus (CMV) promoter from the expression vector pRR1-cPPt-CMV.PreSIN using ClaI and PstI digestion, followed by insertion of the H1 promoter or the complete H1.shABCA2 construct. Target cells (thioglycollate-elicited peritoneal macrophages) were transiently cotransfected with either H1.Empty or H1.shABCA2 lentivirus vectors (MOI 15) using Lipofectamine 2000 (Invitrogen). After 24 h, quantitative analysis of ABCA2 expression was determined.

2.10. Macrophage cholesterol efflux studies

Wild-type (C57BL/6) and ABCA1 KO (with a C57BL/6 background) thioglycollate-elicited peritoneal macrophages were transiently cotransfected with either H1.Empty or H1.shABCA2 lentivirus vectors (MOI 15) for 24 h. Subsequently, cells were incubated with oxLDL/0.5 μCi/mL ^{3}H-cholesterol in DMEM/0.2% BSA (fatty acid free) for 24 h at 37 °C. Cholesterol efflux was studied by incubation of the cells with DMEM/0.2% BSA alone or supplemented with either 10 μg/mL apo-AI (Calbiochem) or 50 μg/mL human HDL.

2.11. Fluorescence microscopy

WT and ABCA2 KO BM-derived macrophages were seeded in control medium or supplemented with oxLDL (20 μg/mL) for 24 h. After treatment FC, neutral sterols and PL distribution were detected with lipidTOX according to the manufacturers protocol (Invitrogen, Carlsbad, CA, USA). Cleaved caspase 3 antibody detecting endogenous levels of the large fragment of activated caspase 3 resulting from cleavage adjacent to Asp175 was used (Cell Signalling Technology, The Netherlands). Image acquisition was performed using a Nikon TE2000 combined with a Prior stage. All images were processed and quantitative image analysis was performed using Image-Pro® Plus (Version 5.1; Media Cybernetics).

2.12. Transmission electron microscopy

For electron microscopy, WT and ABCA2 KO BM-derived macrophages were plated in control medium or supplemented with oxLDL (20 μg/mL) for 24 h. Subsequently, cells were fixed and embedded in an epoxy resin. Ultrathin sections were post stained with uranyl acetate and lead citrate and viewed and imaged with an FEI Tecnai 12 transmission electron microscope, operated at 120 kV and equipped with an Eagle 4kx4k camera (FEI, Eindhoven, The Netherlands).

2.13. Data analyses

Data are expressed as mean ± S.E.M. Comparison of data for two groups was performed by Student's *t*-test or Mann–Whitney *U* test, depending on whether the distribution of data was Gaussian or not. To compare differences among groups One-way ANOVA with Newman–Keuls post-test was performed using GraphPad Prism version 4.00 for Windows (GraphPad Software, San Diego, CA, USA, www.graphpad.com). A P value <0.05 was considered statistically significant.

3. Results

3.1. Effect of macrophage ABCA2 deficiency on serum lipid levels and circulating cells in LDLr KO mice

ABCA2 was selectively disrupted in hematopoietic cells, thus including macrophages, by transplantation of BM from ABCA2 KO mice into LDLr KO mice, which represent a recognized model for the development of atherosclerosis. After a recovery period of 8 weeks on regular murine chow diet, the transplanted mice were challenged with a high cholesterol, high fat WTD for 10 weeks. During the course of the experiment, the weight gain curve was unaffected between ABCA2 KO and WT mice (data not shown).

On regular chow diet, serum TC, TG, and PL levels were similar between WT and ABCA2 KO transplanted mice (Supplemental Fig. IA). Furthermore, the distribution of lipids over the different lipoprotein fractions of both experimental groups was essentially the same. Switching from a regular chow diet to a WTD resulted in an approximately 2.3-fold increase in serum TC levels in both types of chimeric mice (Supplemental Fig. IB). After 10 weeks WTD feeding, no significant effect of macrophage ABCA2 deficiency was observed on serum TC levels (828 ± 89 mg/dL vs. 1008 ± 88 mg/dL in WT transplanted mice; $P = 0.16$), TG (142 ± 10 mg/dL vs. 150 ± 13 mg/dL in transplanted WT mice; $P = 0.61$) or PL (793 ± 79 mg/dL vs. 727 ± 34 mg/dL in transplanted WT mice; $P = 0.47$). Fractionation of serum lipoproteins for TC (Supplemental Fig. I), TG and PL (data not shown) did not show significant changes in lipoprotein distribution patterns between groups.

Bone marrow ABCA2-deficiency did not result in changes in the number of circulating blood cells (Supplemental Fig. II). Thus, ABCA2 KO chimera mice showed similar counts than WT transplanted mice for total leukocytes ($1.8 \pm 0.2 \times 10^9$ cells/L vs. $1.4 \pm 0.2 \times 10^9$ cells/L), neutrophils ($0.6 \pm 0.1 \times 10^9$ cells/L vs. $0.4 \pm 0.1 \times 10^9$ cells/L), lymphocytes ($1.1 \pm 0.1 \times 10^9$ cells/L vs. $0.8 \pm 0.1 \times 10^9$ cells/L) and monocytes ($0.1 \pm 0.04 \times 10^9$ cells/L vs. $0.1 \pm 0.02 \times 10^9$ cells/L).

3.2. Effect of macrophage ABCA2 deficiency on immune cells and lipid homeostasis in tissues

No morphological changes in tissues rich in macrophages, such as liver, spleen, lung and brain, were observed in ABCA2 KO transplanted animals compared to their control littermates. Accordingly, no differences in tissue weight were found as a consequence of leukocyte ABCA2 deficiency (data not shown). This fact was consistent with the similar extent of neutral lipid accumulation between ABCA2 KO and WT transplanted mice visualized, as indicated by Oil-red O staining (Supplemental Fig. IIIA–D) and confirmed by extracting hepatic lipids with organic solvents (Supplemental Fig. IIIE). No inflammation was observed in the liver. In line, the expression of CD68, TNF-a, and IL-6 was not changed (Supplementary Fig. IIIF). In light of the predominant expression of ABCA2 in myeloid cell types, it is conceivable that this transporter is involved in processes associated with immune functions. The effect of macrophage ABCA2 deficiency on cellular composition in liver, spleen and peritoneal cells was measured by flow cytometry (Supplemental Fig. IV). Overall, the loss of function of macrophage ABCA2 did not modify the percentage of the cell subsets studied. However, there was a slight increase in the percentage values of $CD4^+$ and $CD8^+$ lymphocytes in spleens of animals which were transplanted with BM deficient for the ABCA2 transporter (30.8 ± 1.2% vs. 25.9 ± 1.6% in WT transplanted mice; $P = 0.04$ and 15.0 ± 0.8% vs. 12.7 ± 0.6% in transplanted WT mice; $P = 0.04$, respectively), although no difference

was observed for the total $CD3^+$ lymphocyte population count (Supplemental Fig. IVB).

3.3. Effect of macrophage ABCA2 deficiency on in vivo foam cell formation and atherosclerosis

The transformation of macrophages into foam cells upon lipid loading is considered a crucial process in the formation of atherosclerotic lesions. Therefore, to investigate whether disruption of ABCA2 in macrophages would affect foam cell formation, resident peritoneal leukocytes were isolated from mice transplanted with WT and ABCA2 KO bone marrow after 10 weeks WTD feeding. Foam cell counts did not significantly differ between both experimental groups (0.3 ± 0.04% vs. 0.3 ± 0.05% in WT transplanted mice; $P = 0.43$) (Fig. 1A). Microscopic visualization using Oil-red O-stained cytospins, as well as the intracellular lipid content did not show significant changes (data not shown). Interestingly, the mRNA expression of ABCA1 was 1.3-fold ($P = 0.036$) upregulated in the ABCA2 KO group (Fig. 1B). In line, Western-blot analysis showed a 1.9-fold increase ($P = 0.04$) in ABCA1 protein expression in the ABCA2 KO macrophages as compared to WT cells (Fig. 1C).

Next, the susceptibility to atherosclerosis in the aortic root and thoracic aorta of LDLr KO mice reconstituted with WT and ABCA2 KO bone marrow after 10 weeks WTD feeding was analyzed (Fig. 2). Dissection of the descending thoracic aorta and the aortic arch and *en face* examination showed less Oil-red O staining in the ABCA2 KO transplanted group (−36.6%) as compared to the WT transplanted controls (3.8 ± 0.4% and 6.0 ± 1.1% respectively, $P < 0.05$) (Fig. 2D and E). In line with the findings in the descending aorta, lack of ABCA2 in macrophages resulted in a diminished (−24.5%) mean atherosclerotic lesion size in the aortic root (163140 ± 19786 μm^2 vs. 216,143 ± 20482 μm^2 in WT transplanted mice; $P < 0.05$)(Fig. 2A). Because the composition of atherosclerotic lesions, along with lesion size, is critically important in atherogenesis, cell and matrix components of the lesions were also characterized by analyzing macrophage content and collagen deposition (Fig. 2B and C). Macrophage ABCA2 deficiency did not affect atherosclerotic lesion composition (the macrophage content: 39.4 ± 2.4% vs. 39.3 ± 2.2% in WT transplanted mice; the collagen content: 8.0 ± 0.7% vs. 7.9 ± 0.8% in WT transplanted mice). To determine apoptosis, atherosclerotic lesions from both groups of animals were stained for caspase 3 activity. The data in Fig. 3A demonstrate unequivocally that lesions in the aortic roots of ABCA2 KO transplanted mice displayed a 3-fold increase in cleaved caspase 3-positive apoptotic cells as compared with mice receiving WT bone marrow ($P = 0.007$), while also the percentage of TUNEL positive cells in lesions was also significantly increased (Supplementary data V).

3.4. Effect of ABCA2 deficiency on macrophage apoptosis susceptibility

To investigate whether the increase in apoptotic cells observed in lesions of LDLr KO mice transplanted with ABCA2 KO bone marrow was a macrophage-intrinsic response, BMDM from WT and ABCA2 KO mice were loaded in the presence of oxLDL, the prominent lipoprotein present in atheromas, for 24 h (Fig. 3B). Interestingly, there was a 2.7-fold increase in the number of ABCA2 KO cells undergoing apoptosis as compared with WT macrophages (36.3 ± 1.9 vs. 13.35 ± 3.5; $P < 0.001$).

3.5. Effect of macrophage ABCA2 deficiency on efflux of free cholesterol

In the absence of macrophage ABCA2, the expression of the crucial lipid efflux transporter ABCA1 was upregulated in peritoneal macrophages, possibly to compensate for the loss of function of ABCA2. In order to reveal the intrinsic role of ABCA2 on cellular efflux of free cholesterol an approach using lentiviral vectors to reduce ABCA2 expression in ABCA1-deficient macrophages was carried out to minimize the ABCA1-dependent effect.

Knockdown of ABCA2 was achieved by means of lentiviral vectors encoding shRNA against murine ABCA2 (H1.shABCA2) in

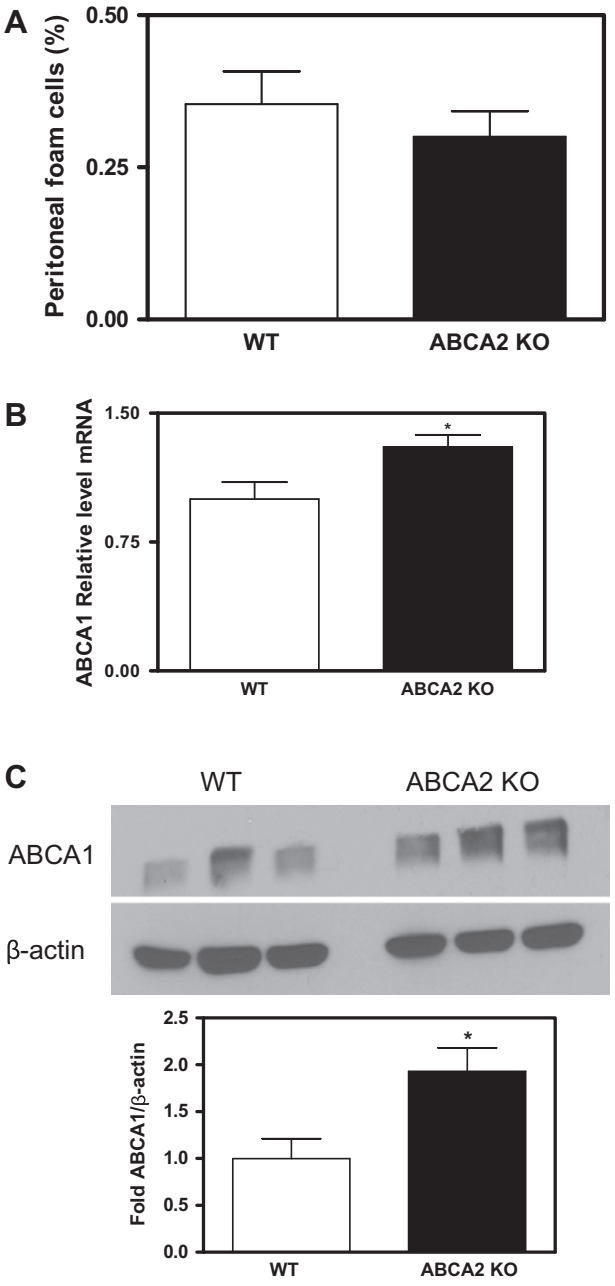

Fig. 1. Effect of macrophage ABCA2 deficiency on *in vivo* foam cell formation of LDLr KO mice reconstituted with WT and ABCA2 KO bone marrow. (A) Peritoneal leukocytes from transplanted mice were analyzed using a hematology analyzer and the number of macrophage foam cells was quantified as percentage of the total amount of isolated cells. Values represent the mean ± SEM of 13 mice per group. (B) Total RNA from cells and ABCA1 mRNA expression was determined by real-time RT-PCR. HPRT, β-actin and GAPDH were used as the standard housekeeping genes. Values represent the mean ± SEM of 4–6 mice per group. (C) Western blots showing ABCA1 protein in cell lysates of WT and ABCA2 KO peritoneal macrophages. Equal amounts of cellular protein extracts were loaded on a 7.5% SDS-PAGE gel. A single band of ABCA1 protein (~230 kDa) was detected with different abundance in all tested samples. β-actin (~40 kDa) was used to control equal loading. Statistically significant difference $^*P < 0.05$.

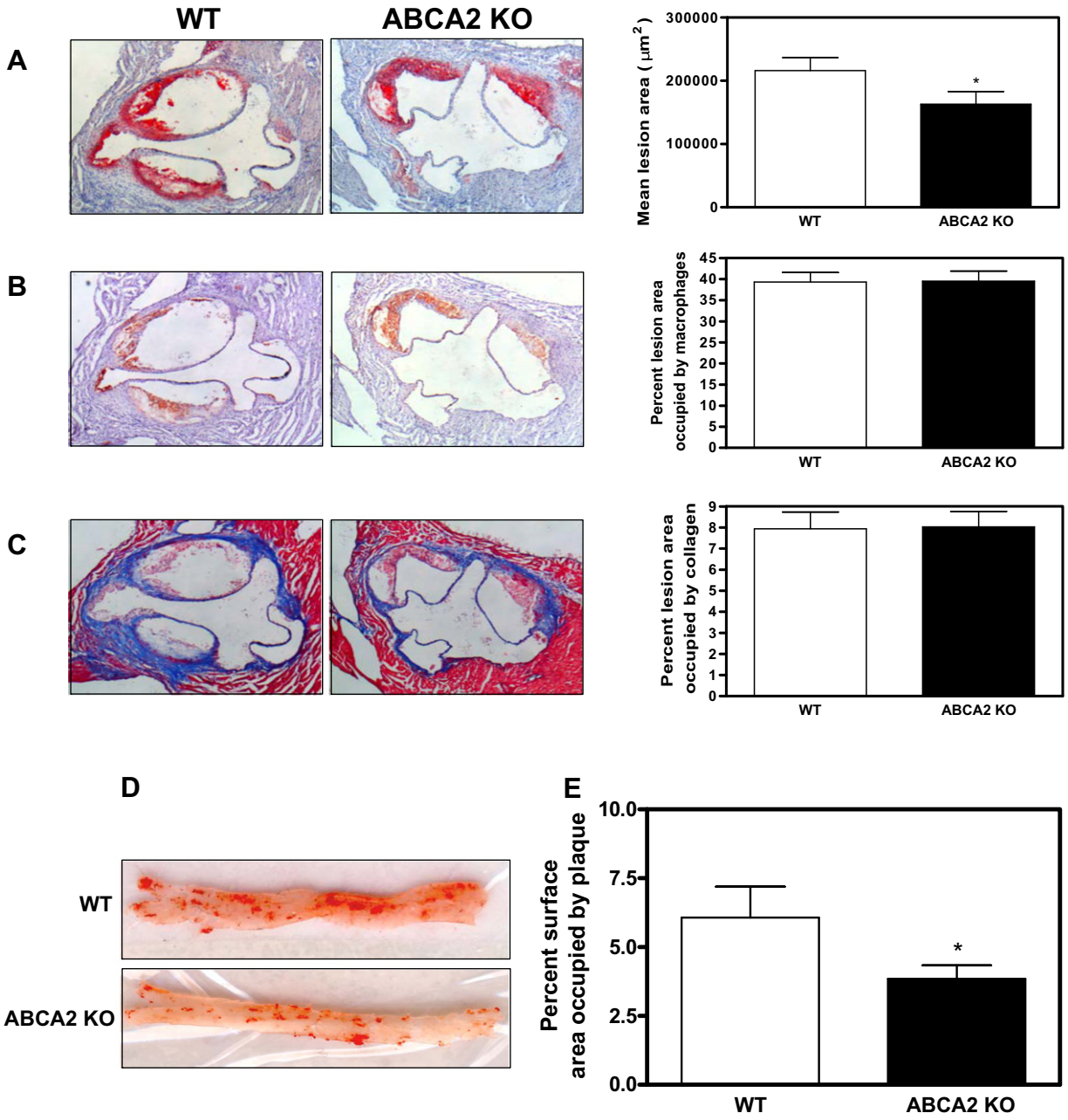

Fig. 2. Effect of macrophage ABCA2 deficiency on atherosclerotic lesion development and composition in the aortic root and the descending thoracic aorta of LDLr KO mice reconstituted with WT and ABCA2 KO bone marrow after 10 weeks on WTD. (A) The mean lesion area was calculated from Oil-red O-stained cross-sections of the aortic root at the level of the tricuspid valves (original magnification ×50). (B) Macrophage content measured by a MOMA2 staining (original magnification ×50), (C) collagen content measured by a Masson's Trichrome staining (original magnification ×50). Representative photomicrographs are shown in all cases. Values represent the mean ± SEM of 12–13 mice per group. (D) Representative Oil-red O-stained thoracic aortas and the aortic arch from LDLr KO mice reconstituted with WT (top) and ABCA2 KO (bottom) BM after 10 weeks on WTD. (E) Percent thoracic aorta and the aortic arch surface area occupied by plaque. Values represent the mean ± SEM of 7–8 mice per group. Statistically significant difference $^{*}P < 0.05$.

peritoneal macrophages. A 79% decrease in ABCA2 expression was found in WT H1.shABCA2-transfected cells (referred as ABCA2 knockdown (KD) macrophages)($P = 0.0015$), whereas an 80% decline was observed in ABCA1 KO H1.shABCA2-transfected cells (referred as the ABCA1 KO/A2 KD group)($P = 0.0016$), both compared to WT macrophages (WT H1.Empty-transfected cells). ABCA1 mRNA expression was ~1.7-fold increased upon knockdown of ABCA2 in WT macrophages (ABCA2 KD vs. WT macrophages; $P = 0.0014$). No effects on ABCG1 expression were found (data not shown).

Secondly, the percentage of ^{3}H-cholesterol efflux from oxLDL-laden WT, ABCA1 KO, ABCA2 KD and ABCA1 KO/A2 KD peritoneal macrophages to human HDL did not differ significantly between the experimental groups (Supplemental Fig. VI). However, a marked reduction in cholesterol efflux was observed from ABCA1 KO and ABCA1 KO/A2 KD macrophages when using apo-AI as exogenous lipid acceptor (1.2 ± 0.1% and 1.1 ± 0.2% vs. 4.5 ± 1.1% in WT macrophages, respectively; $P < 0.05$). Efflux to apo-AI from ABCA2 KD macrophages was not modified when compared to WT cells (3.4 ± 0.6% and 4.5 ± 1.1%, respectively; $P = 0.4$). Importantly, the increased apoptosis susceptibility of macrophages lacking ABCA2 is thus probably not the consequence of increased FC accumulation in the cell as a result of impaired cholesterol efflux. In agreement, cellular FC concentrations were unaffected by macrophage ABCA2

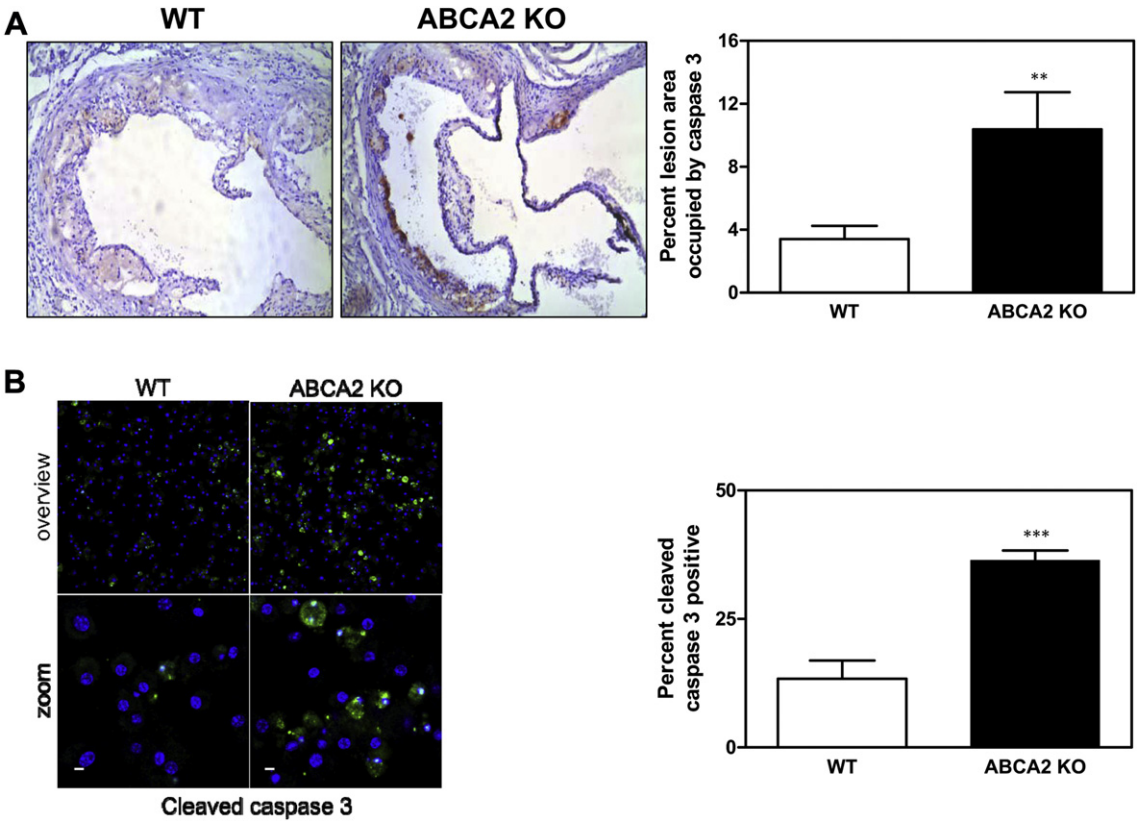

Fig. 3. Effect of macrophage ABCA2 deficiency on apoptosis *in vivo* and *in vitro*. (A) Sections of aortic roots of WT and ABCA2 KO transplanted animals ($n = 8$/genotype) were immunostained for the presence of apoptotic cells with cleaved caspase 3 (original magnification ×200). (B) Fluorescence photomicrographs of cleaved caspase 3-stained WT and ABCA2 KO BMDM cultured in the presence of oxLDL (20 μg/mL) for 24 h, and graphs show the quantitation of the image analysis of random fields (percent of apoptotic-positive cells). Values represent the mean ± SEM. Statistically significant difference $^{**}P < 0.01$, $^{***}P < 0.001$. Bar, 10 μm.

deficiency upon oxLDL loading (FC: 255.2 ± 85.95 μg/ng vs. 296.4 ± 48.91 μg/ng in WT transplanted mice) (Supplemental Fig. VIIA). In addition, no differences were found in the content of neutral sterols (CE: 226.8 ± 54.96 μg/ng vs. 199.4 ± 31.24 μg/ng and TG: 24.5 ± 11.08 μg/ng vs. 26.28 ± 18.92 μg/ng in WT transplanted mice, respectively) and phospholipids (PL: 225.2 ± 81.97 μg/ng vs. 274.0 ± 33.41 μg/ng in WT transplanted mice).

3.6. *Effect of macrophage ABCA2 deficiency on intracellular macrophage cholesterol metabolism*

To gain further insight into the impact of ABCA2 expression on macrophage cholesterol homeostasis, intracellular cholesterol metabolism was studied in more detail. Hereto, the genes expression of key genes in cholesterol metabolism was assessed in BM-derived macrophages upon oxLDL exposure. As expected, deletion of macrophage ABCA2 in oxLDL-laden macrophages resulted in a complete absence of ABCA2 mRNA (Supplemental Fig. VIIB). Potential compensatory expression of other ABCA transporters in KO cells was examined by quantitative real-time RT-PCR analysis. In basal media conditions, in line with previous data, a ∼1.5-fold upregulation of ABCA1 mRNA expression was found in ABCA2 KO macrophages (data not shown), similarly as in peritoneal macrophages (Fig. 1B), which was even more accentuated upon oxLDL exposure (∼3-fold; $P = 0.046$) (Supplemental Fig. VIIB). In addition, oxLDL-stimulated ABCA2 KO macrophages showed a down-regulation of HMG-CoA reductase (∼1.7-fold; $P = 0.03$) and Sp1 transcription factor (2-fold; $P = 0.02$), and an upregulation of CD36 scavenger receptor (∼1.7-fold; $P < 0.001$).

Next, fluorescent microscopy was employed to examine the distribution of lipids within the cells. Interestingly, noticeable differences were apparent between WT and ABCA2 KO macrophages in lipid distribution pattern, both in control media and after incubation with oxLDL. WT cells showed bright FC clusters, whereas ABCA2-deficient foam cells mainly displayed a diffuse distribution of FC with significantly reduced number of vesicles per nuclei in both control (11.8-fold less) and oxLDL loading (2-fold less) conditions (Fig. 4A). Furthermore, under the same experimental settings, ABCA2 KO cells exhibited more numerous neutral sterol lipid droplets as compared to WT macrophages (Fig. 4B)(3-fold for control and 2.3-fold for oxLDL exposure). No difference in the PL pattern between lipid-laden WT and ABCA2 KO was observed, although, as previously described [26,27], oxLDL induced phospholipidiosis in macrophages (Fig. 4B insets). Exactly the same observations for FC, neutral sterols and PL distribution were found when methyl-beta-cyclodextrin, which depletes cholesterol from the cell membrane, was added (data not shown).

Next, transmission electron microscopy offered an additional perspective of the effects caused by macrophage ABCA2 deficiency, particularly on the intracellular storage/trafficking of oxLDL-derived lipids (Fig. 5). Lipid-laden WT macrophages showed the occurrence of endosomal/lysosomal-like vesicles, whereas its presence was low in ABCA2-deficient cells. Instead, ABCA2-deficient macrophages exhibited cytoplasms filled with lipid droplets (Fig. 5). The foam cells in the aortic arch of ABCA2-transplanted animals showed more widespread lipid accumulation (Fig. 6). Consequently, the size of the macrophages in the atherosclerotic lesions was increased (Supplementary Fig. VIII).

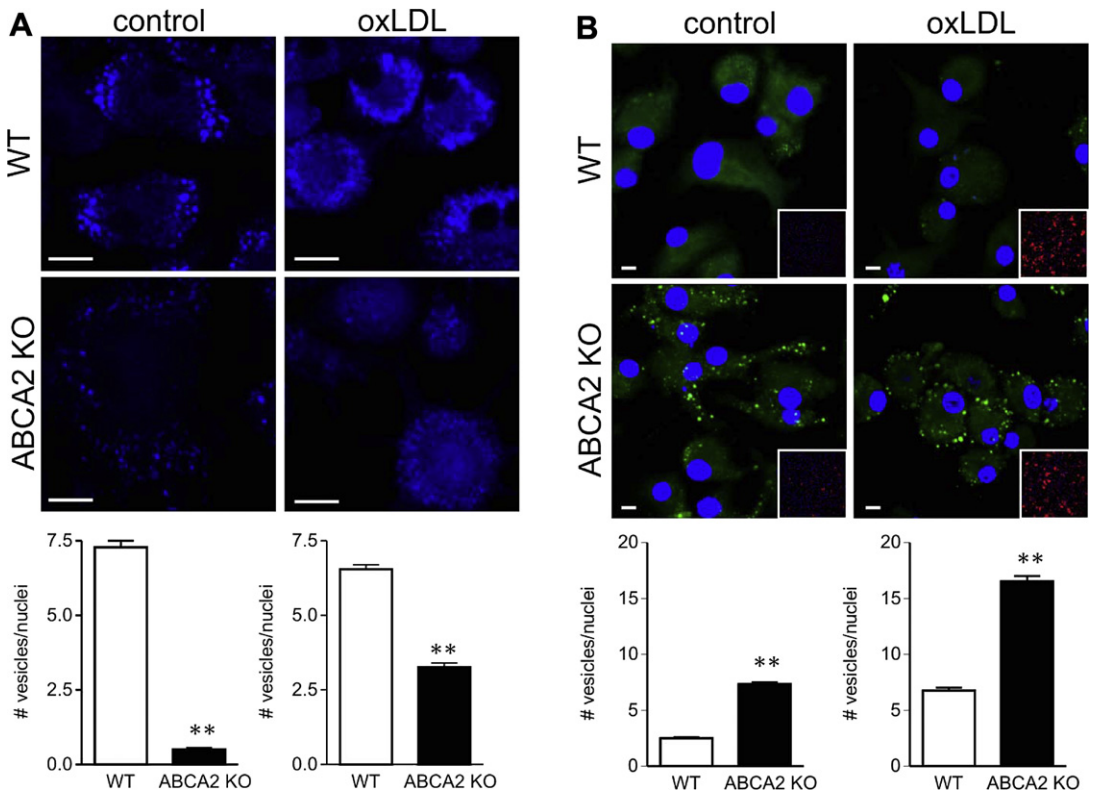

Fig. 4. Effect of macrophage ABCA2 deficiency on lipid deposits in BM-derived macrophages upon oxLDL exposure. WT and ABCA2 KO BM-derived macrophages were incubated in serum free control medium or supplemented with oxLDL (20 μg/mL) for 24 h. Fluorescence photomicrographs of filipin-stained FC (A) and LipidTOX staining for neutral sterols (B), and graphs show the quantitation of the image analysis (#vesicles/nuclei). Inset, fluorescent phospholipid staining for all conditions. Values represent the mean ± SEM. Statistically significant difference $^{**}P < 0.01$. Bar, 10 μm.

4. Discussion

In the present study, we have shown that the absence of leukocyte ABCA2 results in smaller atherosclerotic lesions in LDLr deficient mice accompanied with and/or caused by increased apoptosis. Furthermore, our results support a role for ABCA2 in intracellular lipid trafficking/distribution in macrophages consistent with its lysosomal sequestering role, rather than having a direct impact on cholesterol efflux.

Atherosclerosis is characterized by inflammation and cholesterol deposition in the arterial wall. Oxidation of LDL accompanies the disease process and oxidized lipoproteins are indeed present *in vivo*, particularly in arterial lesions [28]. Excess cholesterol ester accumulation in macrophages of the vessel occurs as a result of an imbalance between delivery and removal and leads to formation of lipid-laden foam cells. Macrophages are incapable of limiting the uptake of lipids via scavenger receptors and, therefore, largely depend on cholesterol efflux pathways to maintain cellular lipid homeostasis, mainly through ATP-binding cassette transporters. Among them, ABCA1 appears to be crucial for cellular cholesterol and phospholipids efflux to lipid-poor apoA-I [22,23] and, interestingly, ABCA2 is a close relative [9]. However, there is only limited information available to date on the function of ABCA2 in macrophages, where this transporter is abundantly expressed [7]. The goal of the present study was to test whether ABCA2 contributes to the regulation of macrophage sterol homeostasis in the vessel wall and modulates the susceptibility to atherosclerosis. We examined the effect of ABCA2 deficiency on lipid metabolism in BM-derived macrophages and on atherogenesis in LDLr-deficient mice, an established model for atherosclerosis studies.

We found that loss of ABCA2 function in macrophages disturbed lipid homeostasis by affecting the intracellular lipid distribution. Control and oxLDL-laden ABCA2 KO cells mostly exhibited a diffuse FC distribution over peripheral regions, probably in internal membranes and/or coupled to cytosolic carriers [29]. However, discrete fluorescent filipin-positive droplets were clearly observed in WT cells. It has been shown that in macrophages the FC generated from the hydrolysis of oxLDL cholesteryl esters is trapped in the lysosomes [30]. The lysosomal cholesterol enrichment coincides with a reduction in acid sphingomyelinase activity, which is inhibited by 7-ketocholesterol present in oxLDL, leading to lysosomal accumulation of sphingomyelin (SM) which traps the FC [31]. Accordingly, abundant lysosome-like compartments were observed in oxLDL-laden WT macrophages by electron microscopy. In this regard, reduced SM content has been described in ABCA2-null brain [14], though the concentration of SM species determined by liquid chromatography/mass spectrometry analysis did not differ significantly between WT and ABCA2 KO cells in our study ($P > 0.3$ for all species; data not shown) or in CNS tissue of WT and ABCA2 KO littermates [15].

Interestingly, a higher occurrence of discrete cytoplasmic neutral sterol droplets was quantified in ABCA2 KO macrophages as compared to WT cells, what was confirmed by ultrastructural microscopy. ABCA2 localizes to a variety of cellular compartments fundamental for intracellular cholesterol dynamics, primarily the endolysosomal and trans-Golgi membranes network [32]. Presumably, ABCA2 may facilitate FC uptake/storage into perinuclear vesicles, which transport a variety of substrates across intracellular membranes for vectorial transfer or ultimate fusion with a membrane destination, similarly to other ATP-dependent

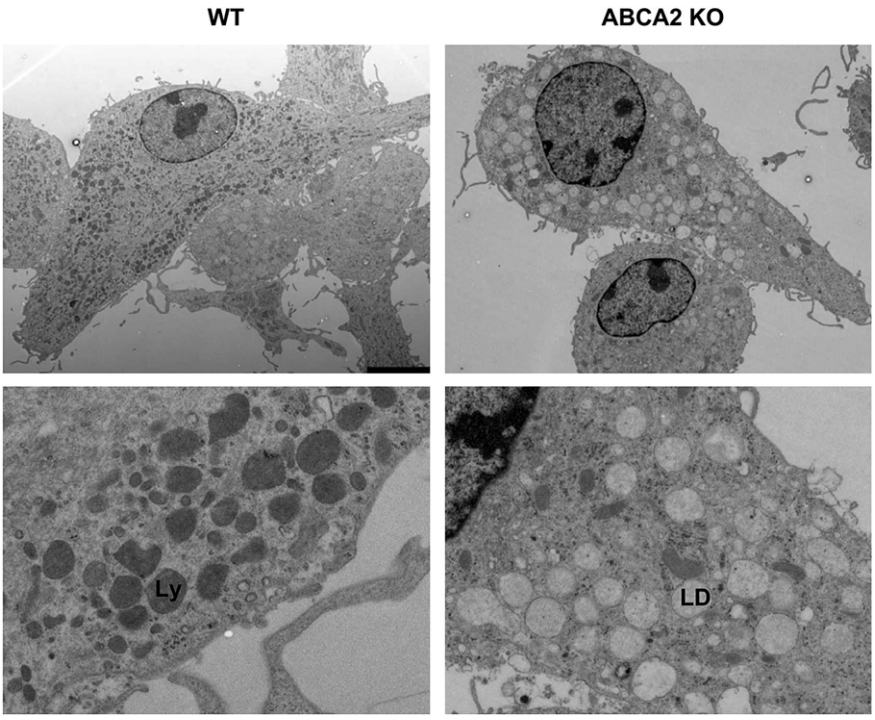

Fig. 5. Effect of macrophage ABCA2 deficiency on conventional transmission electron microscopy of macrophages lipid laden by treatment with oxLDL for 24 h. WT macrophages showed abundant cytoplasmic vesicular endosomal/lysosomal-like compartments (Ly), whereas ABCA2 KO cells contained rich accumulations of lipid droplets (LD). Original magnification ×6000.

transporters, such as ABCA3 or ABCA12 [33,34]. Accordingly, constitutive overexpression of ABCA2 protein in CHO and neuroblastoma cells impaired the delivery of LDL-derived FC to the endoplasmic reticulum for esterification and resulted in cholesterol sequestration in lysosomes [19,21]. Our data are in agreement with this lysosomal oriented sequestration role of ABCA2.

The comparable relative cholesterol distribution of oxLDL-loaded cells and control cells (no added lipoproteins) might indicate that under these latter conditions a similar intracellular route for cholesterol transport is utilized as with oxLDL loaded cells. Interestingly, the widespread fluorescent filipin–sterol complexes and the higher occurrence of neutral sterol lipid droplets in ABCA2-deficient macrophages did not affect quantitatively the total cholesterol content (sum of FC and cholesteryl esters (CE)) detected by the cholesterol oxidase method in oxLDL-laden cells. In the same line, leukocyte ABCA2 deficiency did not modify the lipid staining in organs with high prevalence of macrophages, which correlates with unaffected central nervous system tissue lipid composition in ABCA2 total deficient mice [15]. Therefore, ABCA2 loss seems to dysregulate cholesterol distribution/localization, whereas overall lipid concentrations are unchanged. Previous data showed that ABCA2 expression is coordinately regulated with genes whose expression responds to changes in cellular cholesterol availability [19]. Thus, overexpression of ABCA2 positively regulated sterol-responsive genes which are elevated under sterol-deprived conditions in CHO and neuroblastoma cells [19,21]. Accordingly, in the current study, ABCA2 deficiency in lipid-laden macrophages showed downregulation of the rate limiting enzyme for cholesterogenesis,

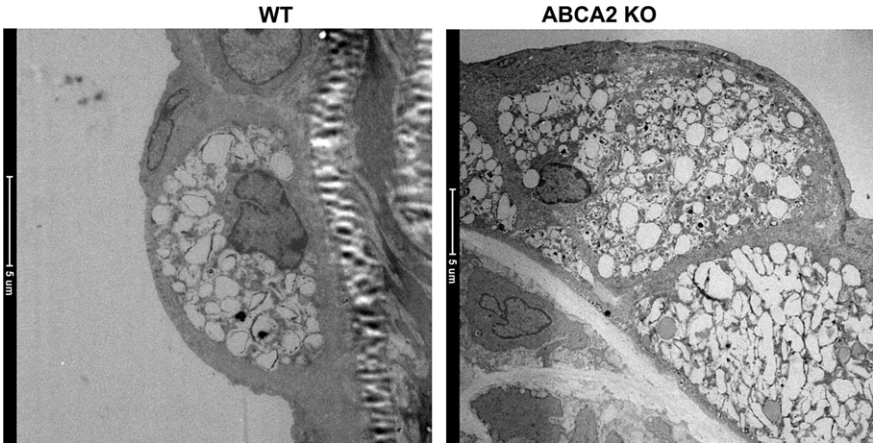

Fig. 6. Effect of macrophage ABCA2 deficiency on foam cell formation in the atherosclerotic lesions. Foam cell formation in the aorta arch from LDLr KO mice transplanted with bone marrow from WT and ABCA2 KO mice was visualized by conventional transmission electron microscopy. 5 μm-long ruler was inserted in the picture.

HMG-CoA reductase. The trend to reduced SREBP2 gene expression in these cells might have contributed to the downregulation of HMG-CoA reductase [35]. Of note, the proximal promoter of the ABCA2 gene contains binding sites for the Sp1 transcription factor [12]. In this regard, a 2-fold reduction in the Sp1 gene expression, which also controls SREBP2 transcription, was detected in ABCA2 KO macrophages as compared to WT controls.

The elucidation of the role of macrophage ABCA2 in atherogenesis is important, not only for its relatedness to ABCA1 but also for its impact on intracellular cholesterol homeostasis and trafficking. Actually, recent data demonstrate that abnormalities in cellular cholesterol trafficking, for instance loss of Niemann-Pick C1 (NPC1) function in BM-derived cells, can directly modulate atherosclerosis susceptibility [36]. Absence of ABCA2 in leukocytes reduced atherosclerosis development in LDLr KO mice in the aortic root (−24.5%) and the thoracic aorta (−36.6%). It has previously been suggested that the ABCA2 transporter may have a role in cholesterol efflux, as it is a cholesterol-responsive gene with highly conserved identity in a C-terminal region associated with the ABCA1-apoA-I interaction and lipid efflux [7,37]. However, analogous efflux rates to apoA-I and HDL were observed between single ABCA1 and combined ABCA1/ABCA2 deficiency indicating that defective ABCA2 did not have measurable effects on this parameter in macrophages, at least not in the currently employed assays. In line, a lack of impact of transient ABCA2 expression on either cholesterol efflux and Aβ peptide secretion in CHO cells, or cholesterol efflux to apoE discs in neuronal cell lines has been previously described [38]. In the same line, it is unlikely that ABCA1 upregulation is the primary cause for the decreased atherosclerosis susceptibility of ABCA2 KO transplanted animals as no effects on cellular cholesterol efflux were observed. However, ABCA2 may interact with intracellular ABCA1 in cholesterol transport as suggested by Ouimet and Marcel [39]. In paticular, the shuttling of ABCA1 for the efflux of cholesterol out of endosomal compartments maybe functionally important. Furthermore, macrophage ABCA1 overexpression does not affect on the development of initial lesions and only inhibits lesion progression in later stages in LDLr KO mice (≥9 weeks on high fat diet) [40].

Apoptosis plays an important role in the development of atherosclerotic lesions [41,42]. Interestingly, an increase in macrophage apoptosis in early lesions has been associated with decreased lesion progression [41], while in advanced lesions apoptosis does lead to larger lesions [43]. Caspase-dependent apoptosis was significantly increased in lesions of recipient mice that had undergone transplantation with bone marrow from ABCA2 KO mice. The observed increase in apoptosis might account for relatively a fewer number of larger macrophages in atherosclerotic lesions of ABCA2 KO transplanted animals. Furthermore, based on our *in vitro* studies, we conclude that ABCA2 deficient macrophages are more prone than WT cells to undergo apoptosis in response to oxidative lipids. Oxidative lipids exert a clear stimulation of autophagy and apoptosis [39]. Thus, inactive ABCA2 might increase cell death due to abnormalities in lipid distribution and trafficking through the lysosomal pathway, presumably, as a result of the broad distribution of unesterified FC, which is known to stimulate proapoptotic processes [44,45]. A different way of cholesterol accumulation inside the cell may help uncover the specific role of ABCA2 in the prevention of apoptosis more clearly. Direct transport studies with fluorescent lipids in ABCA2 KO macrophages will be needed to investigate the relation between intracellular cholesteorl deposition and apoptosis in the future.

In conclusion, the current study suggests that macrophage ABCA2 deletion modulates intracellular cholesterol distribution/trafficking, leading to increased susceptibility to apoptosis and reduced development of early atherosclerotic lesions.

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Disclosures

Nothing to disclose.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.atherosclerosis.2012.05.039.

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