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Impaired Development of Atherosclerosis in *Abcg1*$^{-/-}$*Apoe*$^{-/-}$ Mice; Identification of Specific Oxysterols that both Accumulate in *Abcg1*$^{-/-}$*Apoe*$^{-/-}$ Tissues and Induce Apoptosis

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Abstract

Objective—ABCG1 is highly expressed in macrophages and endothelial cells, two cell types that play important roles in the development of atherosclerosis. We generated *Abcg1*$^{-/-}$*Apoe*$^{-/-}$ mice in order to understand the mechanism and cell types involved in changes in atherosclerosis following loss of ABCG1.

Methods and Results—*Abcg1*$^{-/-}$*Apoe*$^{-/-}$ and *Apoe*$^{-/-}$ mice, and recipient *Apoe*$^{-/-}$ mice that had undergone transplantation with bone marrow from *Apoe*$^{-/-}$ or *Abcg1*$^{-/-}$*Apoe*$^{-/-}$ mice were fed a western diet for 12–16 weeks prior to quantification of atherosclerotic lesions. These studies demonstrated that loss of ABCG1 from all tissues, or from only hematopoietic cells, was associated with significantly smaller lesions that contained increased numbers of TUNEL- and cleaved caspase-3-positive apoptotic *Abcg1*$^{-/-}$ macrophages. We also identified specific oxysterols that accumulate in the brain and macrophages of the *Abcg1*$^{-/-}$*Apoe*$^{-/-}$ mice. These oxysterols promoted apoptosis and altered the expression of pro-apoptotic genes when added to macrophages *in vitro*.

Conclusion—Loss of ABCG1 from all tissues or from only hematopoietic cells, results in smaller atherosclerotic lesions populated with increased numbers of apoptotic macrophages, by processes independent of ApoE. Specific oxysterols identified in tissues of *Abcg1*$^{-/-}$*Apoe*$^{-/-}$ mice may be critical as they induce macrophage apoptosis and the expression of pro-apoptotic genes.

Keywords

ABCG1; apolipoprotein E; atherosclerosis; apoptosis; Bid; Bok; oxysterols

Introduction

The ATP binding cassette transporter, subfamily G, member 1 (ABCG1) is one member of a large superfamily of membrane proteins that function to transport substrates across specific

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Disclosures

None

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membranes.[1, 2] Studies with *Abcg1*$^{-/-}$*LacZ* knock-in mice have demonstrated that ABCG1 is expressed in numerous organs and cell types with particularly high expression in macrophages, endothelial and epithelial cells and neurons.[3–7]

Numerous studies have shown that ABCG1 can function to efflux cholesterol and/or other sterols from cells to various exogenous acceptors, including HDL.[8] Studies with *Abcg1*$^{-/-}$ mice demonstrated that pulmonary macrophages accumulate massive levels of cholesterol and sterol esters, consistent with these cells being particularly sensitive to loss of function of this transporter.[3, 6, 9, 10] These lipid-loaded *Abcg1*$^{-/-}$ pulmonary macrophages also undergo increased apoptosis.[11] These data are consistent with the normal role of pulmonary macrophages in clearing cholesterol-containing surfactant from the extracellular space[12] with ABCG1 then functioning to eliminate the sterols from the cells to maintain cellular sterol homeostasis.

Loss of ABCG1 from macrophages results in increased expression of multiple inflammatory genes, consistent with a stimulatory effect of the accumulating cellular sterols on inflammation.[9, 11, 13] Earlier studies demonstrated that endothelial cells, like macrophages, express particularly high levels of ABCG1.[14] Interestingly, administration of a western diet to *Abcg1*$^{-/-}$ mice was recently shown to increase the inflammatory status and sterol levels of endothelial cells.[13] Taken together, these data suggest that loss of ABCG1 results in subtle or gross changes in cellular sterols that may result in induction of inflammatory genes and/or increased apoptosis in two cell types (endothelial cells and macrophages) that are known to play critical roles in the development of atherosclerosis.

Atherosclerosis is a complex disease that is characterized in the early stages by the accumulation of lipid-loaded macrophages (foam cells) in the intima.[15] The initial findings that loss of ABCG1 led to the accumulation of sterol-loaded macrophages in the lungs of *Abcg1*$^{-/-}$ mice[3, 6, 10, 11] suggested that hyperlipidemic mice lacking functional ABCG1 would exhibit accelerated atherosclerosis. However, *Abcg1*$^{-/-}$ mice have a normal plasma lipoprotein profile and thus do not develop significant atherosclerotic lesions even when fed a western diet.[16] To assess the role of ABCG1 in the development of atherosclerosis, three groups independently performed bone-marrow transplant studies using donor cells from wild-type or *Abcg1*$^{-/-}$ C57BL/6 mice and recipient hyperlipidemic *Ldlr*$^{-/-}$ mice. Although the protocols used in these studies were similar, the conclusions were not; one group reported that transplantation of *Abcg1*$^{-/-}$ bone marrow led to either a modest but significant increase in atherosclerotic lesions in the *Ldlr*$^{-/-}$ recipient mice[6, 17] or to no change in lesion size.[17, 18] In contrast, Baldan et al.[16] and Ranalleta et al.[19] observed a significant decrease in lesion size in *Ldlr*$^{-/-}$ mice receiving *Abcg1*$^{-/-}$ cells. It remains to be determined whether these inconsistencies result from differences in the genetic backgrounds of the mice, from different concentrations of cholesterol in the diet or different treatment times that affect lesion progression.

Alternative mechanisms, that are not necessarily exclusive, were invoked to explain the unexpected decrease in lesion size noted in two of the transplantation studies.[16, 19] Ranalletta et al.[19] proposed that the *Abcg1*$^{-/-}$ macrophages secreted increased amounts of ApoE protein, a known anti-atherogenic protein. It was also suggested that increased expression of a second sterol transporter, ABCA1, in the *Abcg1*$^{-/-}$ macrophages might reduce sterol accumulation in the foam cells and thus impair lesion development.[19] In contrast, Baldan et al.[16] proposed that the smaller lesions were a result of increased apoptosis of the *Abcg1*$^{-/-}$ macrophages that populated the atherosclerotic lesions of *Ldlr*$^{-/-}$ mice. A role for ABCG1 in protection against apoptosis is consistent with studies showing that the lungs of *Abcg1*$^{-/-}$ mice contain increased TUNEL-positive apoptotic cells[11] and overexpression of ABCG1 in cultured cells attenuates

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oxysterol-induced cell death possibly by stimulating the efflux of either 7β-hydroxycholesterol[20] or 7-ketocholesterol[21] to exogenous HDL.

Apoptosis plays an important role in the development of atherosclerotic lesions.[22–24] An increase in macrophage apoptosis in early lesions has been associated with decreased lesion progression.[22] In contrast, an increase in macrophage apoptosis in advanced lesions is thought to promote the development of the necrotic core, a key factor in vulnerable plaque formation and acute thrombosis.[24] The increase in apoptotic cells in lesions may result from the accumulation of unesterified cholesterol and/or oxysterols as these lipids are known to stimulate pro-apoptotic processes.[25, 26] Support for a role for ABCG1 in preventing apoptosis[16] came from studies showing that *Abcg1*$^{-/-}$ or *Abcg1*$^{-/-}$ *Abca1*$^{-/-}$ macrophages exhibit increased apoptosis *in vitro* compared to wild type cells after a challenge with oxLDL.[27, 28]

We now report that hyperlipidemic *Apoe*$^{-/-}$ mice lacking ABCG1 in all tissues or in hematopoietic cells only, exhibit decreased lesions, decreased aortic lesion calcification, and increased macrophage apoptosis as a result of the accumulation of specific pro-apoptotic oxysterols.

Materials and Methods

We fed a western diet for 12–16 weeks to *Abcg1*$^{-/-}$*Apoe*$^{-/-}$ and *Apoe*$^{-/-}$ mice, and to recipient *Apoe*$^{-/-}$ mice that had undergone transplantation with bone marrow from *Apoe*$^{-/-}$ or *Abcg1*$^{-/-}$*Apoe*$^{-/-}$ mice. Atherosclerotic lesion size, apoptosis and oxidized sterol concentrations in macrophages in these mice were determined. For details and further methods please see the online supplemental material (http://atvb.ahajournals.org).

Results

Characterization of *Abcg1*$^{-/-}$*Apoe*$^{-/-}$ Mice

Endothelial cells of *Abcg1*$^{-/-}$ mice fed a western diet accumulate 7-ketocholesterol, a non-enzymatic product of cholesterol autoxidation.[13] *Abcg1*$^{-/-}$ mice also exhibit decreased endothelium-dependent vasodilatation and decreased eNOS activity.[13] Macrophages from *Abcg1*$^{-/-}$ mice also exhibit increased expression of inflammatory genes and accumulate intracellular 7-ketocholesterol.[9, 11] In addition, loss of ABCG1 from macrophages has been reported to result in increased secretion of ApoE protein[19], an anti-atherosclerotic protein.[29]

Consequently, to better understand the effect of loss of function of ABCG1 from all cell types, including macrophages and endothelial cells, and to remove any confounding effects that could arise from altered secretion of ApoE from macrophages, we generated *Abcg1*$^{-/-}$*Apoe*$^{-/-}$ double knockout (DKO) mice. Analysis of the plasma showed that, compared to *Apoe*$^{-/-}$ mice, DKO mice had increased hemoglobin (14.0±0.67 vs. 12.68±0.96; $p<0.04$; n=10) and hematocrit values (40.42±1.94 vs. 36.8±2.14; $p<0.03$), but that lipid and lipoprotein levels and a broad array of hematology values did not significantly differ between the two genotypes (data not shown).

To accelerate the development of atherosclerosis, DKO and *Apoe*$^{-/-}$ mice were challenged with a western diet (21% fat and 0.2% cholesterol). After 16 weeks, analysis of plasma from DKO and *Apoe*$^{-/-}$ mice indicated that there was no significant difference in lipid levels (Supplemental Table I) or lipoprotein profile (Supplemental Fig. IA). Lungs of the DKO, but not the *Apoe*$^{-/-}$ mice appeared white and stained positive with Oil Red O, especially in areas enriched in Lac Z-expressing macrophages (Supplemental Fig. II, data not shown). In addition, red and white pulp of spleens of the DKO, but not *Apoe*$^{-/-}$ mice, contained cells that were

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positive for both Lac Z and Oil Red O (Supplemental Fig. II). Thus, multiple tissues of the DKO mice exhibited evidence of neutral lipid accumulation.

Abcg1$^{-/-}$Apoe$^{-/-}$ Mice have Decreased Atherosclerotic Lesions Containing Increased Numbers of Apoptotic Macrophages

After 16 weeks on the western diet atherosclerotic lesions were determined both by *en face* analysis of the Sudan IV-stained descending aorta or following analysis of stained frozen sections (25–30 sections/mouse) of the aortic root.

The data show that *Abcg1$^{-/-}$Apoe$^{-/-}$* mice had significantly smaller lesions than *Apoe$^{-/-}$* mice in both the aortic root (Fig. 1A; Fig. 1B, panel b vs. a) and in the descending aorta (Fig. 1D, E). The lesions of the DKO mice contained numerous LacZ-positive macrophages (Supplemental Fig. III), thus ruling out the possibility that DKO macrophages do not enter the subendothelial space. Calcified deposits in the lesions of the aortic root, identified following staining of sections with either von Kossa or Oil Red O, hematoxylin and fast green, were significantly reduced in the *Abcg1$^{-/-}$Apoe$^{-/-}$* mice (Fig. 1C; Fig. 1B, panel d vs. c and b vs. a), consistent with the smaller lesions in the DKO mice. Photomicrographs taken at higher magnification illustrate that the calcium deposition occurs within the aortic lesions, adjacent to the medial layer (Supplemental Fig. IV).

Loss of ABCG1 from Hematopoietic Cells Delays the Development of Atherosclerosis and Increases Apoptotic Macrophages in the Lesions, Independent of ApoE

To determine the relative importance of hematopoietic and non-hematopoietic *Abcg1$^{-/-}$* cells on the observed changes in atherosclerosis noted in the *Abcg1$^{-/-}$Apoe$^{-/-}$* mice (Fig. 1), we performed bone marrow transplant studies wherein bone marrow from either *Apoe$^{-/-}$* or DKO mice was transplanted into recipient *Apoe$^{-/-}$* animals. After a 4 week recovery period, the mice were fed a western diet for 12 weeks. Analysis of the lungs of the *Apoe$^{-/-}$* recipients indicated that mice that had been transplanted with DKO donor cells, but not those receiving *Apoe$^{-/-}$* cells, contained Lac Z-positive cells and white patches consistent with lipid deposition in macrophages (data not shown).

Neither plasma lipid levels (Supplemental Table II) nor plasma lipoprotein profiles (Supplemental Fig. IB), were significantly different between the two groups of recipient *Apoe$^{-/-}$* mice. Compared to wild type mice, all transplanted mice contained elevated levels of VLDL and LDL and low levels of HDL independent of the genotype of the donor cells (Supplemental Fig. IB; data not shown).

Quantification of the atherosclerotic lesions showed that they were significantly smaller in mice transplanted with DKO as compared to *Apoe$^{-/-}$* donor bone marrow (Fig. 2A; Fig. 2B, panel b vs. a). Interestingly, and in agreement with the studies utilizing whole body DKO mice, calcification in the lesions of the aortic root was also significantly decreased in mice transplanted with *Abcg1$^{-/-}$Apoe$^{-/-}$* donor bone marrow (Fig. 2C; Fig. 2B, panel d vs. c and b vs. a), consistent with smaller lesions in the latter mice.

En face analysis of the descending aorta indicated a trend towards lower lesions in those mice receiving bone marrow from *Abcg1$^{-/-}$Apoe$^{-/-}$* mice, although the difference just failed to reach statistical significance (Fig. 2D, E). However, lesion coverage in the thoracic and abdominal sections, but not the proximal sections, were significantly smaller in mice transplanted with DKO cells (Supplemental Fig. V) consistent with slower lesion progression in mice receiving DKO donor cells.

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Increased Macrophage Apoptosis in Lesions of DKO Mice

After 16 weeks on the western diet the aortic root lesions of DKO mice contained significantly greater numbers of TUNEL-positive cells, often present as multi-cell aggregates (Fig. 3A). A similar difference was seen in the bone marrow transplant studies wherein we observed a 22-fold increase in TUNEL-positive cells in lesions of *Apoe*$^{-/-}$ mice transplanted with DKO as compared to *Apoe*$^{-/-}$ donor cells (Fig. 3C).

One of the late events in apoptosis involves cleavage of the precursor form of caspase-3 to form an active protease.[30] To identify cells undergoing apoptosis within the lesions of the aortic root, frozen sections from the aortic roots of mice were immunostained with antibodies to macrophages and to the cleaved form of caspase-3. Analysis of multiple stained sections indicated that lesions of *Apoe*$^{-/-}$ mice had few active caspase-3-positive cells, whereas numerous active caspase-3-positive cells, often present as aggregates, were present in the lesions of DKO mice (Fig. 3B) and in *Apoe*$^{-/-}$ mice that were the recipients of the DKO bone marrow (Fig. 3D). These tissue sections also stained positive for macrophages when co-stained with anti-mac3 (Fig. 3B–D). Analysis of the merged figures showed that cleaved caspase-3-positive and anti-mac3-positive cells co-localize in the lesions of mice lacking ABCG1 (Figs. 3B, D), thus identifying the apoptotic cells as macrophages. Interestingly, cleaved caspase-3- or TUNEL-positive endothelial cells were never observed in any section suggesting that loss of ABCG1 from endothelial cells did not result in accelerated apoptosis *in vivo* (data not shown).

Taken together, the data from studies with whole body DKO mice and following bone marrow transplantation demonstrate that loss of ABCG1 from hematopoietic cells alone is sufficient to slow the progression of atherosclerotic lesions. This is associated with an increase in the number of apoptotic cells in the lesions and decreased calcification within the lesion. All these changes occur by mechanisms that are independent of ApoE.

Identification of Specific Oxysterols Accumulating in *Abcg1*$^{-/-}$*Apoe*$^{-/-}$ Macrophages

Identification of specific sterols that accumulate within macrophages in atherosclerotic lesions is complicated by the inability to obtain sufficient numbers of cells. Consequently, we performed bronchoalveolar lavage on *Apoe*$^{-/-}$ and *Abcg1*$^{-/-}$*Apoe*$^{-/-}$ mice and recovered alveolar macrophages. Analyses of these samples using isotope dilution mass spectrometry identified a number of oxysterols, including 24-, 25-, and 27-hydroxycholesterols that accumulate in the *Abcg1*$^{-/-}$*Apoe*$^{-/-}$ and *Abcg1*$^{-/-}$ cells compared to wild type or *Apoe*$^{-/-}$ cells (Table 1). We also show that, compared to *Apoe*$^{-/-}$ mice, 25- and 27-hydroxycholesterol levels are significantly increased in the brains of the DKO mice (Table 1). Hence, the increase in the levels of these enzymatically synthesized oxysterols is not limited to macrophages.

Abcg1$^{-/-}$ Bone Marrow-derived Macrophages Display a Pro-apoptotic Phenotype and Altered Sensitivity to Oxysterols

The data of Fig. 4A show that after 7 days in culture, *Abcg1*$^{-/-}$ and *Abcg1*$^{-/-}$*Apoe*$^{-/-}$ bone marrow derived macrophages (BMDM) exhibited a 3- to 6-fold increase in TUNEL-positive cells, as compared to wild type or *Apoe*$^{-/-}$ cells. Although exposure of all these cells to oxidized LDL (oxLDL) for 8 h increased TUNEL staining, the highest levels of apoptosis/TUNEL staining were seen when cells lacked ABCG1 (Fig. 4A), consistent with the proposal that ABCG1 is critical for limiting apoptosis in response to lipid-loading. As expected, oxLDL treatment of wild type, *Apoe*$^{-/-}$, *Abcg1*$^{-/-}$ or *Abcg1*$^{-/-}$*Apoe*$^{-/-}$ BMDMs increased the expression of the LXR target genes *Abca1* and *Srebp1c* and the anti-apoptotic gene *Aim* (Supplemental Fig. VI A–C).

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Based on the finding that specific oxysterols accumulate in alveolar macrophages and the brains of *Abcg1*$^{-/-}$*Apoe*$^{-/-}$ mice (Table 1), we next investigated whether cells lacking ABCG1 and/or ApoE are particularly sensitive to these same oxysterols. In the absence of added oxysterols, the number of BMDMs undergoing apoptosis was 4-fold greater in *Abcg1*$^{-/-}$*Apoe*$^{-/-}$ as compared to *Apoe*$^{-/-}$ cells (Fig. 4B, DMSO). Addition of 10 μM 7-ketocholesterol, 25-hydroxycholesterol or 27-hydroxycholesterol increased the number of TUNEL-positive cells (Fig. 4B). Importantly, the percent of apoptotic cells was greatest following addition of oxysterols to the DKO BMDMs (Fig. 4B).

The increased sensitivity of cells lacking ABCG1 to oxysterol-induced apoptosis suggested that these cells might also exhibit altered expression of genes involved in apoptosis. Consequently, we performed a PCR-based screen to identify apoptotic genes that were altered after exposure of cells to 50 μg/ml ox-LDL (data not shown). Confirmation of altered gene expression came from subsequent RT-qPCR analysis that showed that incubation of cells with specific oxysterols increased the expression of *Bid* and *Bok*, two members of the Bcl-2 pro-apoptotic gene family (Fig. 4C, D). Importantly, expression of *Bid* and *Bok* was higher in DKO or *Abcg1*$^{-/-}$ BMDMs as compared to wild type or *Apoe*$^{-/-}$ cells (Fig. 4C, D).

Discussion

We report here on the generation and initial characterization of *Abcg1*$^{-/-}$*Apoe*$^{-/-}$ mice. Studies with both whole body *Abcg1*$^{-/-}$*Apoe*$^{-/-}$ mice and following bone marrow transplantation into *Apoe*$^{-/-}$ mice demonstrate that atherosclerotic lesion progression is reduced when mice lack ABCG1 either in all tissues or in macrophages and other hematopoietic cells (Figs. 1, 2). In preliminary studies we also noted that neutrophils accumulated in the adventitia, adjacent to lesions of *Apoe*$^{-/-}$ and *Abcg1*$^{-/-}$*Apoe*$^{-/-}$ transplanted mice (data not shown) consistent with increased inflammation. Despite this latter finding, and the observation that endothelial cells lacking ABCG1 exhibit increased inflammatory properties[13], the current data suggest that the decrease in lesion size is dependent upon loss of ABCG1 from hematopoietic cells and occurs by processes independent of ApoE. Whether the profound decrease in calcium deposition in the atherosclerotic lesions (Fig. 1, 2 and Supplemental Fig. IV) is simply a consequence of the smaller lesions or is a consequence of the increase in apoptotic macrophages in the lesions will require additional studies.

The importance of macrophage apoptosis in affecting early lesion development was initially reported by Arai et al.[22] as a result of studies with mice lacking the anti-apoptotic gene *Aim*. Importantly, the expression of *Aim* is largely restricted to macrophages.[22] Arai et al. demonstrated a remarkable (>90%) attenuation of atherosclerotic lesions in hyperlipidemic *Aim*$^{-/-}$*Ldlr*$^{-/-}$ mice, as compared to *Aim*$^{+/+}$*Ldlr*$^{-/-}$ mice.[22] These data suggested that increased apoptosis of macrophages limited early development and progression of atherosclerotic lesions.[22] Strikingly, in the current study we show that cells lacking ABCG1 are more susceptible to oxysterols/induced apoptosis despite increased expression of *Aim* mRNA.

The current data extend a previous report where it was shown that atherosclerotic lesions were decreased in *Apoe*$^{-/-}$ recipient mice following repopulation with *Abcg1*$^{-/-}$*Apoe*$^{+/+}$ cells, as compared to *Abcg1*$^{+/+}$*Apoe*$^{+/+}$ (wild type) bone marrow.[16] However, the interpretation of the latter result was complicated by the fact that expression of ApoE in the donor marrow cells is sufficient to attenuate atherosclerosis in recipient *Apoe*$^{-/-}$ mice.[31] It was further complicated by the report that loss of ABCG1 from macrophages resulted in increased secretion of ApoE.[18] Although we have not been able to confirm the latter finding, the current data demonstrate that the decrease in lesion progression following deletion of ABCG1 can occur independent of ApoE.

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Lammers et al.[17] recently reported that atherosclerotic lesions of *Ldlr*$^{-/-}$*Apoe*$^{+/+}$ mice were increased following transplantation with *Abcg1*$^{-/-}$*Apoe*$^{-/-}$ bone marrow.[17] However the finding that the transplanted mice expressed ApoE in many non-hematopoietic cells makes comparison with the current studies difficult. This same group has previously reported either no change or an increase[6, 18] in lesion size following transplantation of *Abcg1*$^{-/-}$ bone marrow into hyperlipidemic *Ldlr*$^{-/-}$ mice. Whether these differences in lesion progression relate to differences in serum cholesterol levels[32] or to differences in genetic background of the mice, length of time on different diets, and the extent of the disease remains unclear at the current time.

The result of the present, as well as previous work are consistent with a more important role of the ABCG1 transporter for efflux of a number of oxysterols than for efflux of cholesterol.[5, 20, 21, 33, 34] Loss of this transporter thus leads to a higher accumulation of 7-β-hydroxycholesterol, 7-ketocholesterol, 24-, 25- and 27-hydroxycholesterol than of cholesterol. The accumulation of the side-chain oxidized oxysterols is surprising in view of their physiochemical properties which allow them to pass biomembranes at a much higher rate than cholesterol.[35] Not only oxysterols, but also desmosterol, an intermediate in the cholesterol biosynthetic pathway, has been shown to accumulate in ABCG1 deficient cells.[34] Cytotoxic and apoptotic properties of the side-chain oxidized oxysterols are well documented[35, 36] and it is shown here that exposure of macrophages lacking ABCG1 to 7-ketocholesterol, 25-hydroxycholesterol and 27-hydroxycholesterol leads to increased apoptosis.

To summarize, our results are consistent with the possibility that at least part of the apoptotic effects of a loss of the ABCG1 transporter is due to the accumulation of oxysterols. We also demonstrate that loss of ABCG1 from macrophages results in increased expression of the two pro-apoptotic genes *Bid* and *Bok*. Whether such changes in pro-apoptotic genes is sufficient to contribute to the increased apoptosis, despite an increase in the expression of the anti-apoptotic gene *Aim* is unknown.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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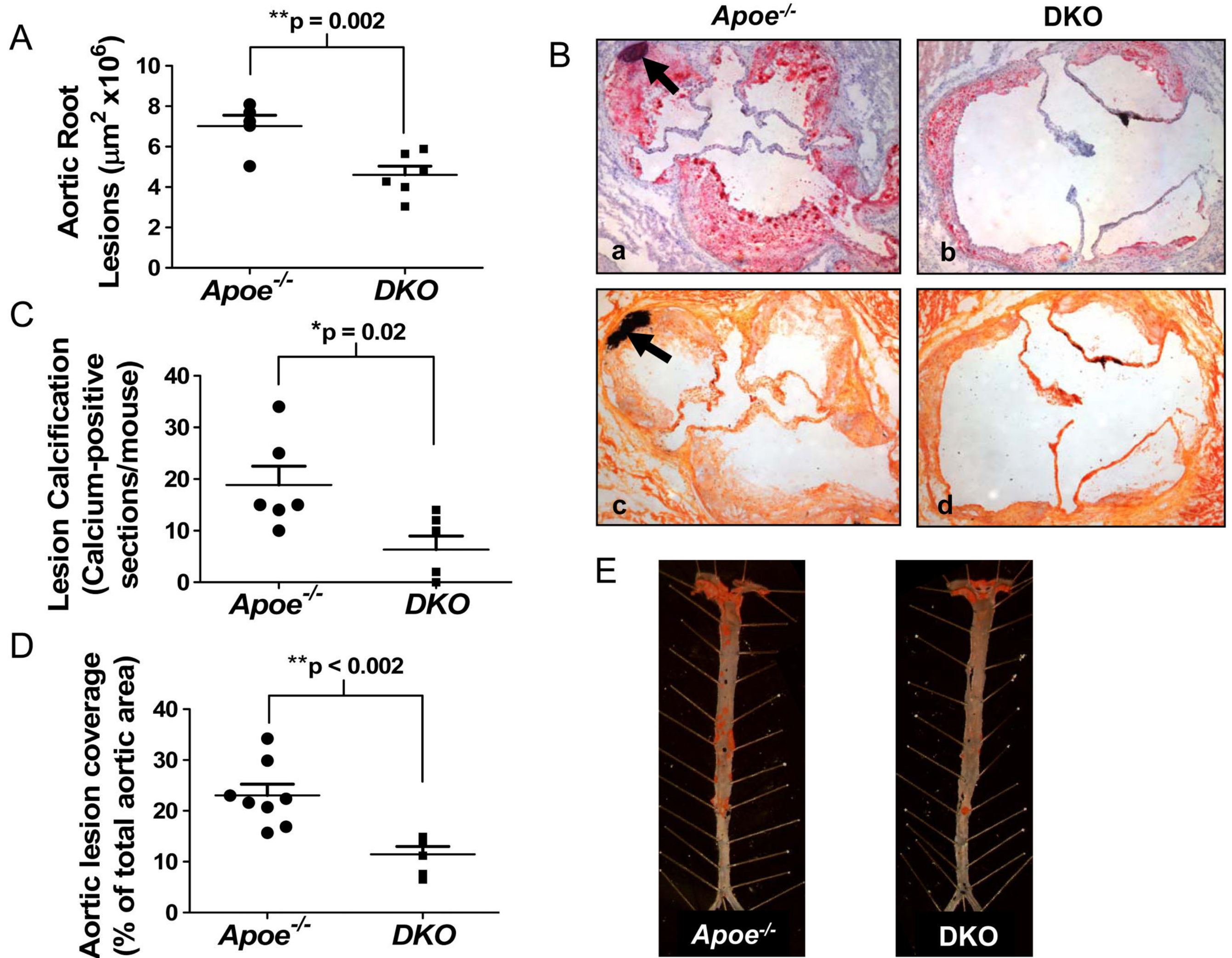

Figure 1.

Atherosclerosis and lesion calcification are reduced in *Abcg1*$^{-/-}$*Apoe*$^{-/-}$ as compared to *Apoe*$^{-/-}$ mice. DKO and *Apoe*$^{-/-}$ mice (6–8 mice per group) were fed a western diet for 16 weeks. (A) Frozen sections (25–30 sections/mouse; n=6 mice/group) from the aortic root were stained with Oil Red O and counter stained with hematoxylin and fast green before lesion areas were determined as described in the Methods. Each point represents an individual mouse. **p<0.01 (B) Shows representative Oil Red O-stained sections counter stained with hematoxylin and fast green (panels a and b) and adjacent sections stained with von Kossa stain (panels c and d). Calcification/calcium phosphate deposits, are indicated by arrows in panels a and c. (C) Quantification of lesion calcification. *p<0.05 (D) Lesions in the descending aorta were identified by *en face* analysis and quantified as described in the Methods (n=8 mice/group). **p<0.01 (E) Representative Sudan IV-stained aortas are shown from the two genotypes.

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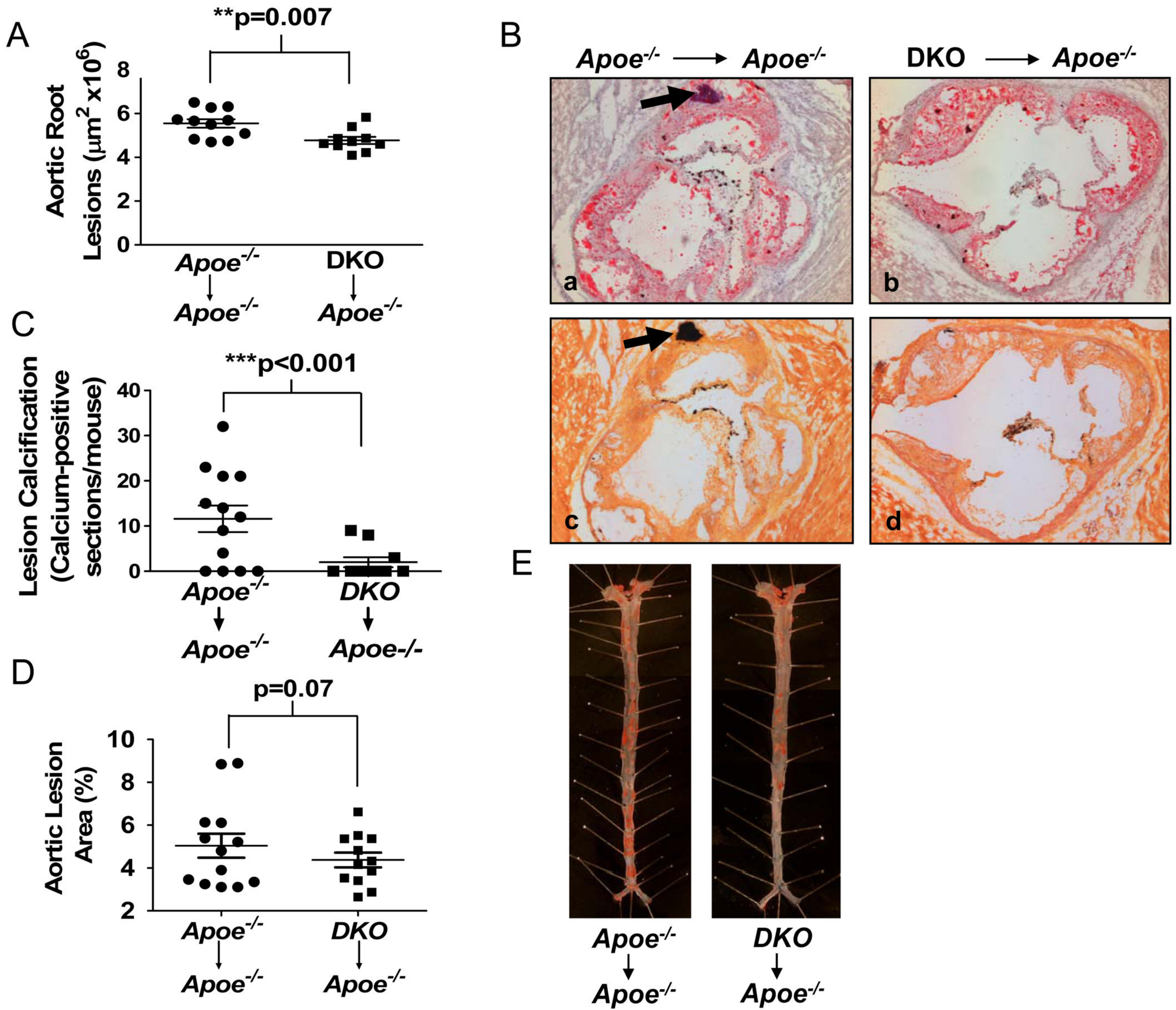

Figure 2.

Apoe$^{-/-}$ mice lacking ABCG1 in hematopoietic cells have reduced atherosclerotic lesions. *Apoe*$^{-/-}$ mice were transplanted with bone marrow from *Apoe*$^{-/-}$ or DKO mice before being fed a western diet for 12 weeks. All analyses were performed as described in Fig. 1. Lesion size (A) and calcification (C) in the aortic root sections (10–13 mice per group), and lesion size in the descending aorta (D) (13–16 mice per group) are shown, with each point representing one mouse. (B) Representative sections from the aortic root after staining with Oil Red O and counterstaining with hematoxylin and fast green (panels a and b) or adjacent sections stained with von Kossa stain (panels c, d). Arrows identify calcium deposits. (E) Representative Sudan IV-stained sections of the descending aorta. **p<0.01, ***p<0.001.

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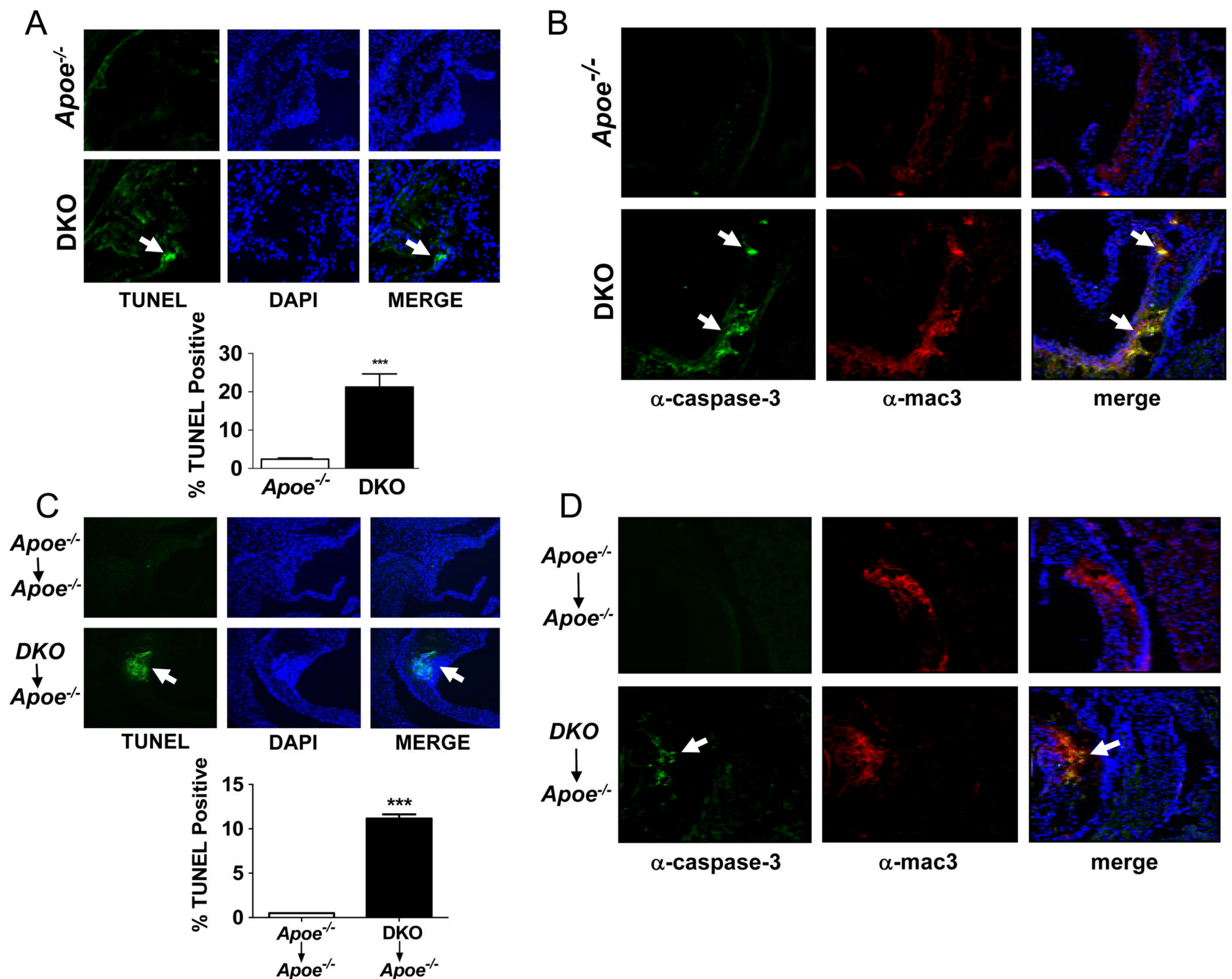

Figure 3.

ABCG1 deficiency results in increased numbers of apoptotic macrophages in atherosclerotic lesions. The indicated whole body knockout mice (A, B) or bone marrow transplanted mice (C, D) were fed a western diet as described in Figs. 1–2. TUNEL- and DAPI-positive cells (green and blue, respectively) were determined in adjacent sections of the aortic roots (A, C) of the indicated mice. Aggregated TUNEL-positive cells are indicated by arrows. Graphs show the percentage of TUNEL-positive cells in the lesions. (B, D) Adjacent frozen sections were also stained with either antibody to cleaved caspase-3 (green foci marked by arrows) or macrophages (red). The merged images are also shown (B and D). The data are representative of multiple stained sections (n=15/sections/mouse; 3 mice/genotype). Data are expressed as mean ±SEM. *** $p<0.001$.

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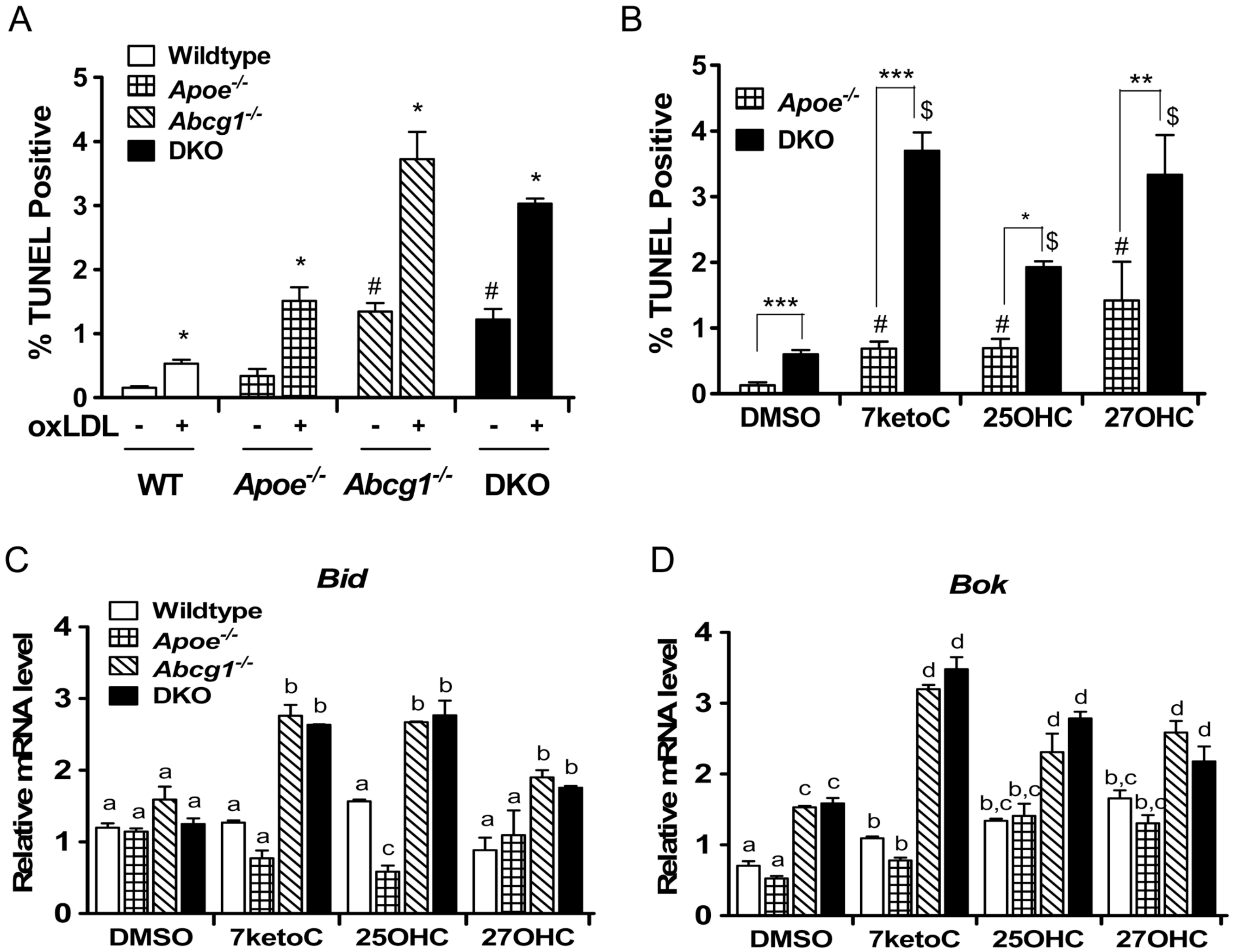

Figure 4.

Macrophages lacking ABCG1 exhibit increased apoptosis in response to oxLDL or oxysterols and enhanced induction of pro-apoptotic genes. BMDMs in quadruplicate were differentiated in L929-conditioned media containing 10% FBS. After 7 days the media was replaced with media containing 0.2% BSA media ± oxidized LDL (50 μg/ml) (A) or the indicated oxysterol (10 μM) (B–D). After 8 h the number of TUNEL-positive apoptotic cells (total cells >1000 cells/field; 6 fields/genotype) (A, B) or the mRNA levels of *Bid* and *Bok* (C, D) were determined. Data are expressed as mean ±SEM and are representative of two experiments. (A) * $p<0.001$, significantly different than (−) oxLDL; [#] $p<0.001$, significantly different than WT and *Apoe*$^{-/-}$. (B) #, $p<0.001$ significantly different than *Apoe*$^{-/-}$ DMSO-treated; $, $p<0.001$ significantly different than DKO DMSO-treated. (C, D) [a,b,c,d] bars with different letters are significantly different from one another at the level of $p<0.001$.

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Table 1

Macrophages and brain sterol levels are given as μg or ng sterol/mg protein (macrophages) or /mg wet weight (brain), respectively. Macrophages were isolated from 5 mice/genotype and combined for sterol analysis. Brain sterols were determined for individual brains (8 brains/genotype) and the mean and SE provided.

Genotype	Cholesterol (μg/mg)	24(*S*)-OH-cholesterol (ng/mg)	25-OH-cholesterol (ng/mg)	27-OH-cholesterol (ng/mg)
Macrophages: WT	21.57	0.1	0.2	0.6
Abcg1$^{-/-}$	115.95	4.7	62.1	17.9
Apoe$^{-/-}$	48.78	3.6	41.9	4.1
Abcg1$^{-/-}$*Apoe*$^{-/-}$	93.95	4.1	52.6	19.8
Brain: *Apoe*$^{-/-}$	16.43 ± 0.3	42.03 ± 1.7	0.03 ± 0.003	0.31 ± 0.008
Abcg1$^{-/-}$*Apoe*$^{-/-}$	17.17 ± 0.6	50.06 ± 4.3	0.07* ± 0.009	0.47** ± 0.02

* $p<0.005$;

** $p<0.00005$