Supplemental Material

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

Supplemental Methods

Plasma Lipid and Lipoprotein Analysis

Plasma cholesterol and triglyceride, and high-density lipoprotein cholesterol (HDL-C) were determined enzymatically for individual mice as described.[1] The cholesterol lipoprotein profile was obtained after combining plasma from 6-8 or 13-16 mice/group (Supplemental Fig. 1A or 1B, respectively)and analyzing 100-200 μl on FPLC.[2]

Quantification of Atherosclerotic Lesions and Calcium Deposits

Briefly, the heart and proximal aorta were excised, washed and the apex and lower half of the ventricles removed. The remainder was embedded and frozen on dry ice in Tissue-Tek (Miles). Serial cryosections were taken through the ventricle until the appearance of the aortic valves. Serial 10 μm sections (50-60 sections per mouse), obtained from the appearance to the disappearance of the aortic valves, were collected on poly-D-lysine coated slides. Alternate sections (25-30/mouse; n=6 mice/group in Fig. 1 and n=10-13 mice/group in Fig.2) were then stained with Oil Red O and counterstained with haematoxylin and fast green prior to examination by light microscopy.[3] Atherosclerotic lesions per section were scored by determining the total intimal area using an ocular μm^2 grid.[3] The average atherosclerotic lesion area was quantified and normalized to 50 sections.

To identify mineral calcium deposition, sections consecutive to those analyzed for atherosclerotic lesions were stained by von Kossa stain.[4, 5] Positive calcium salt deposits are identified by brown-black deposits. Calcium deposits

identified by von Kossa staining were also easily identified after staining adjacent tissue sections with hematoxylin and counter staining with fast green; under these latter conditions calcium deposits give an intense purple staining.[5] Vascular calcification in the aortic lesion was identified as intense purple staining and scored semi-quantitatively as the number of positive stained sections per mouse, by an observer who was unaware of the genotypes of the mice (n=6-13 mice/group as indicated in the legends). Photomicrographs taken at high magnification are presented in supplemental Fig. 4 demonstrate that the calcium deposition is within the lesion adjacent to the medial layer.

Quantification of the lesion size/coverage in the entire descending aorta by *en face* analysis was performed as described (n=8 mice/group in Fig.1 and n=13-16 mice/group in Fig. 2).[6] Each aorta was stained with Sudan IV and three low power photomicrographs taken that correspond to the aortic arch (from the aortic root to the first intercostal), the thoracic segment (from the first intercostal to the mesenteric artery) and the abdominal segment ending at the iliac artery bifurcation. The total area and the area corresponding to the lesion (staining positive with Sudan IV) were determined in the each of the three sections and the values routinely added together to determine the percent lesion coverage. In supplemental Fig. 5 the lesion coverage in each individual segment is indicated separately.

LacZ Expression

β-galactosidase staining of the frozen sections of the aortic root was performed as previously described.[7] Briefly, hearts, lungs or spleens were removed and placed in ice-cold 4% paraformaldehyde for 4 hours. After rinsing with PBS, tissues were cryoprotected by immersing in 20% sucrose in PBS at 4^0C overnight. The next day hearts were snap frozen in Optimal Cutting Temperature™ (OCT) embedding medium on dry ice and stored at -80^0C. Ten micrometer sections of the aortic root were collected on glass slides (Fisher super frost plus) and stored at -80^0C for future use. β-galactosidase enzymatic activity was detected by incubating slides containing sections in buffer containing

X-gal (5-bromo-4-chloro-3-indolylβ-Dgalactopyranoside) for 12-16 hours at room temperature.[7] Slides were washed in PBS/0.1% Tween-20 three times, counterstained with nuclear fast red, briefly washed with water, dehydrated and mounted using organic based media.

Immunohistochemistry

Immunohistochemical detection of cleaved caspase-3 and macrophages was carried out on 10 μM frozen sections. Sections were blocked in 5% normal goat serum in 1xPBS/0.3% Triton X-100 (blocking buffer) for 30 min. Primary antibodies were diluted (1:500) in blocking buffer and sections were incubated at 4°C overnight in a humidified chamber. Slides were rinsed 3x in 1xPBS. Cleaved caspase-3 and mac-3 were detected using species-specific rabbit polyclonal antibodies conjugated to AlexaFluor® fluorochromes (Molecular Probes), also diluted (1:500) in blocking buffer and incubated at room temperature for 1 hour. Cleaved caspase-3 was detected using goat anti-rabbit AlexaFluor® 488 (green) and mac-3 was detected using goat anti-rat AlexaFluor® 594 (red). Slides were rinsed 3x in 1xPBS before mounting on cover slips with Prolong® Gold Anti-fade (Molecular Probes).

RNA Isolation and Analysis

RNA was extracted from bone marrow derived macrophages using the Trizol/Chloroform method (Invitrogen). cDNA synthesis was performed using 1μg of DNase1-treated RNA using DNA-free kit (Ambion). Real-time quantitative PCR was performed using LightCycler 480 SYBR Green Master mix (Roche) on a Light Cycler 480 II detection system (Roche). Primer sequences are available upon request. Values were normalized to levels of the ribosomal protein 36B4 and calculated using the absolute quantification 2nd derivative max method (Roche).

TUNEL-staining

The presence of apoptotic cells was assessed by terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay of paraffin-embedded tissue sections or cultured cells as previously described.[8] Five random fields from at least 3 sections of the aortic root from each of the *Apoe*$^{-/-}$ recipients receiving *Apoe*$^{-/-}$ or DKO bone marrow or from *Apoe*$^{-/-}$ or DKO mice were analyzed for apoptotic TUNEL-positive and total (DAPI-stained) cells (average n=2547)(4 mice per genotype).

Statistical Analysis

Aortic root and *en face* atherosclerotic lesion data from bone marrow transplant studies was analyzed by unpaired Student *t* test. Atherosclerotic lesion coverage in the proximal, mid and distal thirds of the descending aortas was analyzed by two-way ANOVA, with genotype of the bone marrow donor as one factor and aortic section as another. Lesion calcification and apoptosis in the atherosclerotic lesions and freshly isolated macrophages was analyzed by unpaired Student *t* test. Apoptosis in macrophages treated with oxLDL was analyzed by two-way ANOVA, with genotype of the bone marrow donor as one factor and treatment with ox-LDL as another. Results are expressed as means ±SEM. Where there was an effect of either genotype or treatment with no apparent interaction, data were further analyzed by *post hoc* Bonferroni test to determine differential effects.

Supplemental Figure Legends

Supplemental Figure I. *Apoe*$^{-/-}$ or DKO mice (A) or *Apoe*$^{-/-}$ mice transplanted with indicated bone marrow (B) were fed a western diet for 12 or 16 weeks respectively and fasted overnight. Plasma was combined, (A) 8 or (B) 13-16 mice/genotype, and analyzed by FPLC as described.[2]

Supplemental Figure II. DKO mice accumulate neutral lipid in the lung and spleen. Frozen tissue sections from *Apoe*$^{-/-}$ or DKO mice were stained for β-galactosidase (LacZ) activity, or with Oil Red O, as indicated.

Supplemental Figure III. LacZ positive cells accumulate in atherosclerotic lesions of DKO mice.

Supplemental Figure IV. Calcium deposition in the atherosclerotic lesions. *Apoe*$^{-/-}$ mice, or *Apoe*$^{-/-}$ mice transplanted with *Apoe*$^{-/-}$ marrow were fed a high fat diet for 12-16 weeks. Shown are photomicrographs of von Kossa-stained sections of the aortic root from an *Apoe*$^{-/-}$ mouse (A) and from an *Apoe*$^{-/-}$ mouse transplanted with *Apoe*$^{-/-}$ bone marrow (B). A higher magnification of the calcium deposits are shown for each, demonstrating that the calcium deposits are within the lesions and adjacent to the media.

Supplemental Figure V. Atherosclerotic lesions are decreased in the thoracic and abdominal aorta in bone marrow transplanted mice. *Apoe*$^{-/-}$ mice received bone marrow from *Apoe*$^{-/-}$ or DKO mice prior to administration of a western diet for 12 weeks. The descending aorta was stained with Sudan IV and lesions quantified in the proximal, thoracic and abdominal sections as described in Methods (n=13-16 mice/genotype).

Supplemental Figure VI. Induction of LXR target genes following treatment of macrophages with oxidized LDL. Bone marrow derived macrophages were isolated from wild type, *Apoe*$^{-/-}$, *Abcg1*$^{-/-}$ or *Abcg1*$^{-/-}$*Apoe*$^{-/-}$ mice and cultured for 8 days before addition of oxidized LDL (oxLDL) (50 μg/ml) for 8 h. RNA was isolated for quadruplicate dishes prior to RT-qPCR analysis of the indicated mRNAs. (A) * $p<0.001$, significantly different from (-) oxLDL; $^{\#}$ $p<0.05$, significantly from WT, *Apoe*$^{-/-}$ and DKO oxLDL-treated. (B) * $p<0.001$, significantly different from (-) oxLDL; $^{\#}$ $p<0.05$, significantly different from WT, *Apoe*$^{-/-}$, *Abcg1*$^{-/-}$ oxLDL-treated.

References

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Supplemental Table I Plasma Lipid Levels of *Apoe*$^{-/-}$ and *Abcg1*$^{-/-}$*Apoe*$^{-/-}$ Mice

Genotype	Triglyceride (mg/dL)	Total Cholesterol (mg/dL)	HDL (mg/dL)	Unesterified Cholesterol (mg/dL)	Free Fatty Acids (mg/dL)
***Apoe*$^{-/-}$**	51.50 ± 1.91	1064.50 ± 86.56	17.00 ± 1.27	357.00 ± 31.51	33.50 ± 2.18
***Abcg1*$^{-/-}$ *Apoe*$^{-/-}$**	70.00 ± 9.36	935.33 ± 39.87	15.17 ± 2.04	315.33 ± 12.79	27.83 ± 1.66

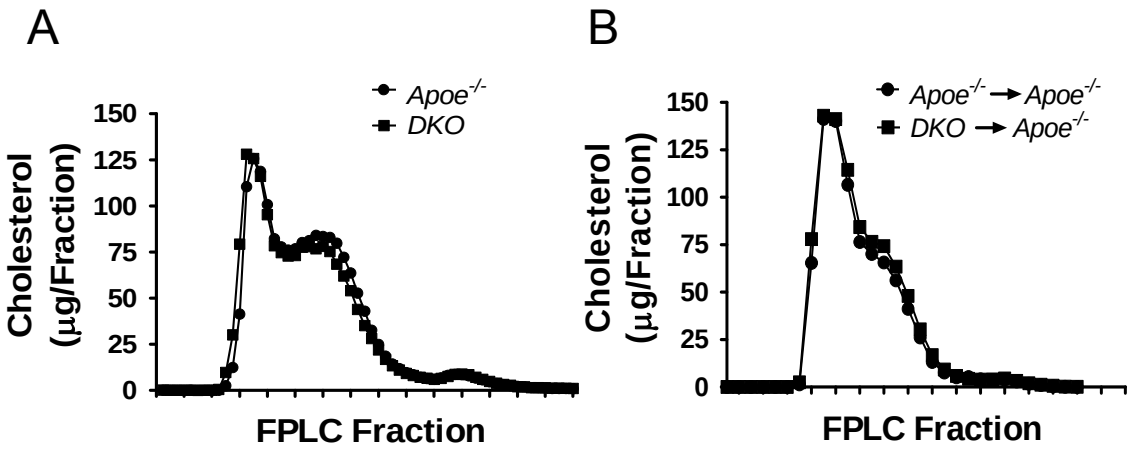
Eight week old single or DKO mice (n=8 mice/group) were fed a western diet and plasma lipids determined 16 weeks later.

Supplemental Table II Plasma Lipid Levels of *Apoe*$^{-/-}$ Mice following Bone Marrow Transplantation

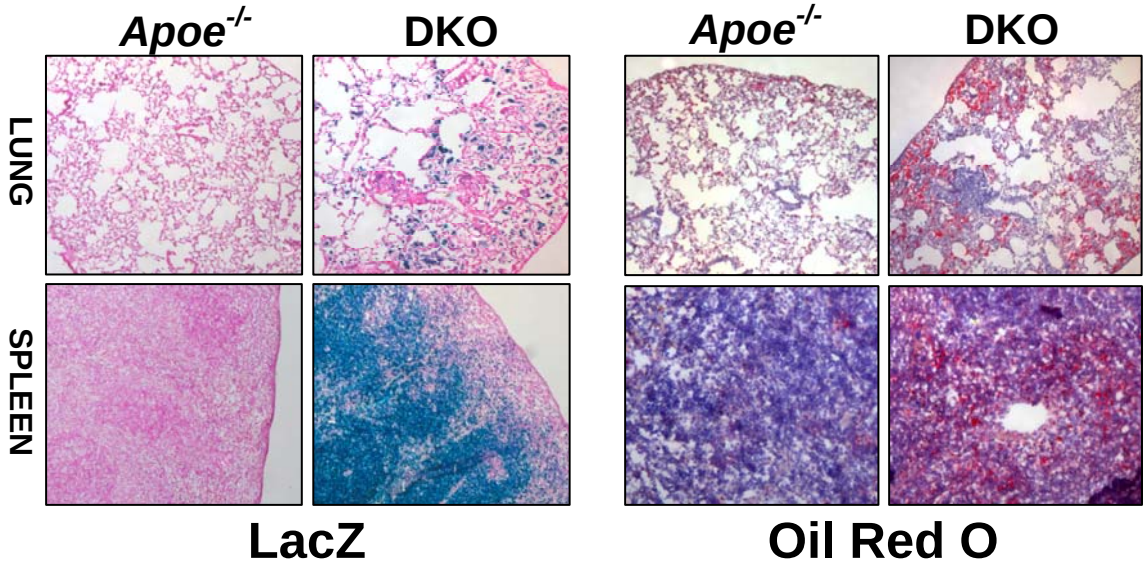
Donor Cell Genotype	Triglyceride (mg/dL)	Total Cholesterol (mg/dL)	HDL (mg/dL)	Unesterified Cholesterol (mg/dL)	Free Fatty Acids (mg/dL)
***Apoe*$^{-/-}$**	28.13 ± 2.98	1104.75 ± 64.80	14.50 ± 0.99	324.56 ± 17.83	34.31 ± 0.80
***Abcg1*$^{-/-}$ *Apoe*$^{-/-}$**	29.77 ± 4.24	1131.62 ± 53.29	14.23 ± 0.90	328.62 ± 18.99	36.08 ± 1.18

Eight week old *Apoe*$^{-/-}$ mice (13-16 mice/group) were transplanted with the indicated donor bone marrow. Four weeks later they were fed a western diet and plasma lipid levels were determined after 12 weeks on the diet.

Supplemental Fig.I

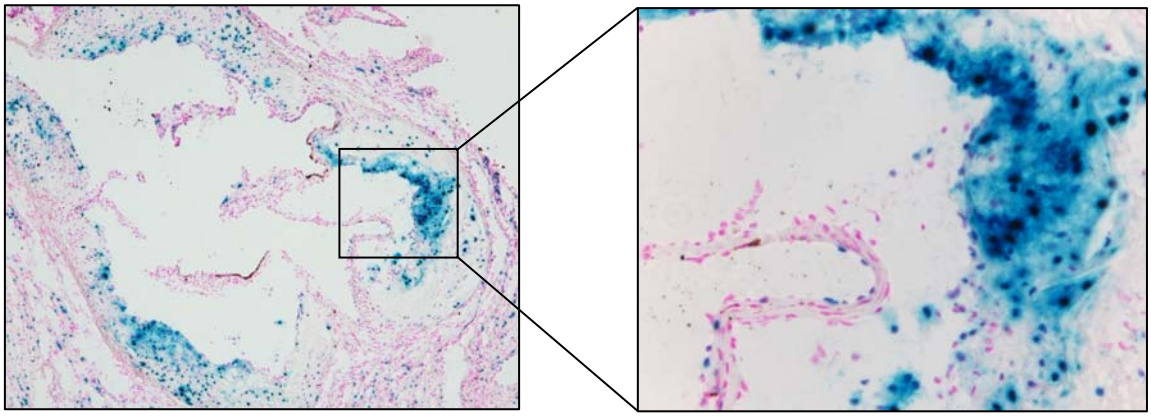


Supplemental Fig. II

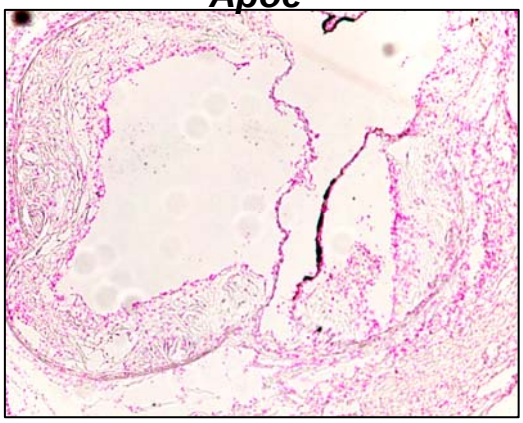


Supplemental Fig. IV

DKO

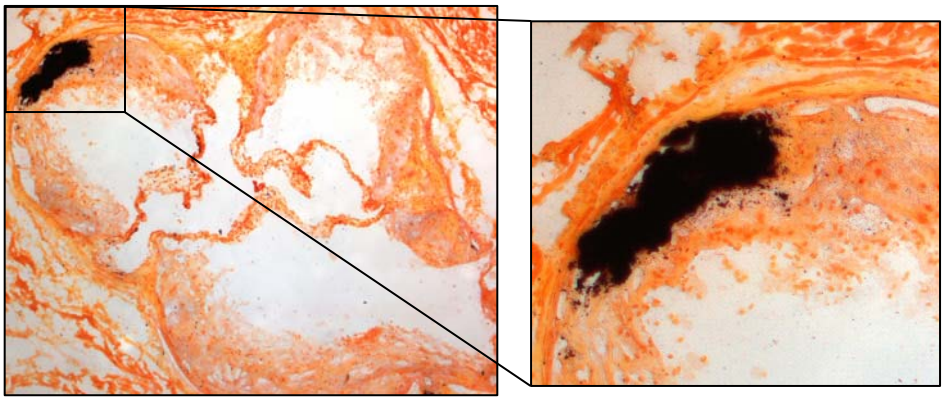


***Apoe*$^{-/-}$**



Supplemental Fig. IV

A



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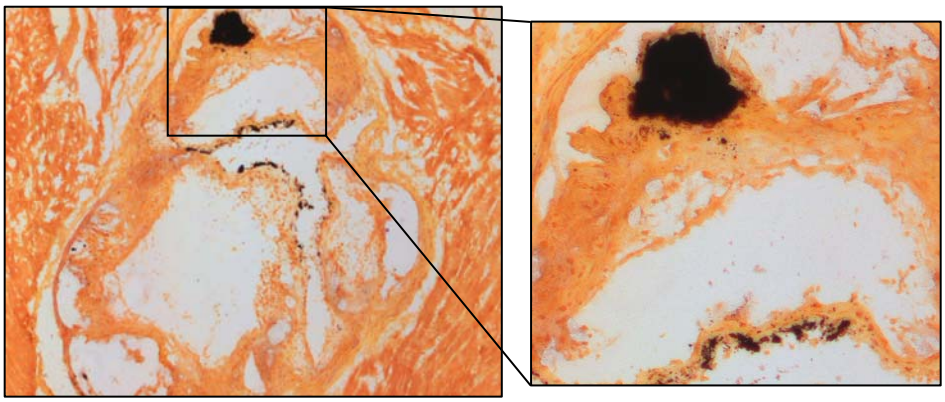

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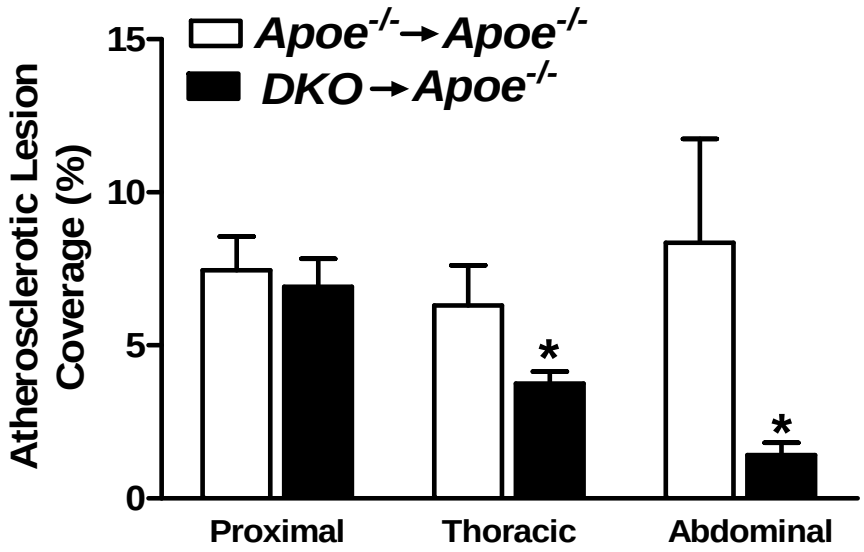

Supplemental Fig. VI