Severely altered cholesterol homeostasis in macrophages lacking apoE and SR-BI

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Abstract **Mice deficient in scavenger receptor class B type I (SR-BI) and apolipoprotein E (apoE) [double knockout (DKO) mice] develop dyslipidemia, accelerated atherosclerosis, and myocardial infarction, and die prematurely. We examined effects of apoE and SR-BI deficiency on macrophage cholesterol homeostasis. DKO macrophages had increased total cholesterol (TC) stores (220–380 μg/mg protein) compared with apoE$^{-/-}$ cells (40 μg/mg), showed significant lysosomal lipid engorgement, and increased their TC by 34% after exposure to HDL. DKO macrophages from apoE$^{-/-}$ mice reconstituted with DKO bone marrow showed less cholesterol accumulation (89 μg/mg), suggesting that the dyslipidemia of DKO mice explains part of the cellular cholesterol defect. However, analyses of DKO and apoE$^{-/-}$ macrophages from transplanted apoE$^{-/-}$ mice revealed a role for macrophage SR-BI, inasmuch as the TC in DKO macrophages increased by 10% in the presence of HDL, whereas apoE$^{-/-}$ macrophage TC decreased by 33%. After incubation with HDL, the free cholesterol (FC) increased by 29% in DKO macrophages, and decreased by 8% in apoE$^{-/-}$ cells, and only DKO cells had FC in large peri-nuclear pools. Similar trends were observed with apoA-I as an acceptor. Thus, the abnormal cholesterol homeostasis of DKO macrophages is due to the plasma lipid environment of DKO mice and to altered trafficking of macrophage cholesterol. Both factors are likely to contribute to the accelerated atherosclerosis in DKO mice.—** Yancey, P. G., W. G. Jerome, H. Yu, E. E. Griffin, B. E. Cox, V. R. Babaev, S. Fazio, and M. F. Linton. **Severely altered cholesterol homeostasis in macrophages lacking apoE and SR-BI.** ***J. Lipid Res.*** **2007.** 48: **1140–1149.**

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Apolipoprotein E (apoE) and scavenger receptor class B type I (SR-BI) are essential for maintenance of normal cholesterol homeostasis. ApoE regulates plasma cholesterol levels by facilitating the uptake of remnant lipoproteins (1), and hepatic SR-BI functions in reverse cholesterol transport by mediating the uptake of HDL cholesterol (2, 3). In addition to these important functions, apoE and SR-BI may be relevant in peripheral cell cholesterol homeostasis as facilitators of cholesterol efflux to a number of acceptors, including HDL (4, 5) and apoA-I (6). Numerous studies have demonstrated that both SR-BI and apoE are important in minimizing atherosclerotic lesion development. Single-gene deletion of either apoE (7, 8) or SR-BI (9) in mice accelerates atherosclerotic lesion development, whereas low-level transgenic expression of either SR-BI (3) or apoE (10, 11) reduces the extent of atherosclerosis. Furthermore, the combined deficiency of SR-BI and apoE produces the only mouse model of accelerated and fatal atherosclerosis. SR-BI and apoE double knockout (DKO) mice display a toxic dyslipidemia, develop early occlusive atherosclerotic coronary artery disease, and die prematurely at 6–8 weeks of age with evidence of myocardial infarction, mimicking features of human atherosclerosis (12).

SR-BI (13) and apoE (14) are expressed in macrophages. Macrophage apoE has an antiatherogenic effect independent of its effect on plasma lipids (15, 16). Studies utilizing bone marrow (BM) transplants have substantiated the protective roles of macrophage SR-BI and apoE in atherosclerotic lesion development. Introduction of BM with low expression levels of apoE reduces atherosclerosis development in apoE-deficient mice (17). A study by Covey and colleagues (18) demonstrated that transplantation of SR-BI-deficient BM into LDL receptor-deficient mice accelerates atherosclerosis development. Similarly, we have recently shown that transplantation of DKO marrow into apoE-null mice enhances atherosclerotic lesion formation, indicating that macrophage SR-BI expression is antiatherogenic (19).

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Abbreviations: apoE, apolipoprotein E; BM, bone marrow; CE, cholesteryl ester; DKO, double knockout; EM, electron microscopy; FC, free cholesterol; SR-BI, scavenger receptor class B type I; TC, total cholesterol.

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Macrophages are critical determinants of atherosclerotic lesion progression through internalization of intimal lipoproteins and transformation into foam cells. The degree of cholesterol accumulation in the macrophage, obviously a function of lipoprotein entry, is finely regulated by genes coordinating cholesterol efflux. In recent years, many efflux mechanisms have been identified that may operate to minimize macrophage foam cell formation. These include ABCA1 (20, 21), ABCG1 (22, 23), SR-BI (24, 25), and apoE (26–28). Given the vast redundancy of the efflux system and the minimal effects of macrophage SR-BI expression levels on cholesterol efflux (18, 19, 28, 29), it is not clear what contribution macrophage SR-BI makes in preventing foam cell formation, particularly in relation to apoE. The goal of the present study is to examine the effect of combined deletion of SR-BI and apoE on cholesterol homeostasis in macrophages. We demonstrate that macrophages deficient in SR-BI and apoE accumulate substantial cholesterol stores in vivo. This cholesterol accumulation is due to a combination of the DKO mouse plasma lipid environment and defective intracellular trafficking of cholesterol in macrophages resulting from the absence of SR-BI. Thus, both toxic dyslipidemia and defective macrophage cholesterol mobilization are likely to contribute to the accelerated atherosclerotic lesion development in mice with combined deficiency of SR-BI and apoE.

EXPERIMENTAL PROCEDURES

Materials

FBS, BSA, Dulbecco's phosphate-buffered saline (DPBS), penicillin, and streptomycin were purchased from Sigma. Tissue culture plasticware was obtained through Falcon (Lincoln, NJ). Lipid-free human apoA-I was obtained from Calbiochem. Human HDL was isolated by sequential ultracentrifugation as previously described. All other reagents and organic solvents were purchased from Fisher.

Mice and genotyping

SR-BI$^{+/-}$ (1:1 mixed C57BL/6×S129 genetic background) and apo E$^{-/-}$ mice on a C57BL/6 background were obtained from The Jackson Laboratory. SR-BI$^{+/-}$apoE$^{-/-}$ mice on a C57BL/6 background were then generated by mating the SR-BI$^{+/-}$ mice with apoE$^{-/-}$ mice and backcrossing the resulting doubly heterozygous offspring with apoE$^{-/-}$ mice for nine generations. SR-BI$^{+/-}$apoE$^{-/-}$ mice were then mated to generate SR-BI$^{-/-}$ apoE$^{-/-}$ mice on the C57BL/6 background (19). ApoE genotypes were determined by using a PCR protocol from The Jackson Laboratory. SR-BI genotypes were determined by PCR analysis of DNA extracted from ear punches (30).

Macrophages and BM transplantations

Mouse peritoneal macrophages were harvested by peritoneal lavage 3 to 4 days after intraperitoneal injection of 3% thioglycollate. To enable comparisons of apoE$^{-/-}$ and SR-BI$^{-/-}$ apoE$^{-/-}$ macrophages that were exposed to the same lipid environment in vivo, cells were harvested from apo E$^{-/-}$ mice reconstituted with either apoE$^{-/-}$ or SR-BI$^{-/-}$apoE$^{-/-}$ BM. Eight-week- old female apo E$^{-/-}$ mice were lethally irradiated with a single dose of 9 Gray using a cobalt-60 γ source. The same day, the recipient mice were injected with 5×10^6 BM cells via the retro-orbital venous complex. After transplantation, the apo E$^{-/-}$-recipient mice were maintained on a chow diet for 4 weeks, and to obtain apo E$^{-/-}$ and SR-BI$^{-/-}$apoE$^{-/-}$ macrophages loaded with cholesterol in vivo, recipient mice were then maintained for 8 weeks on a Western-type diet prior to elicitation of cells with thioglycollate.

Mobilization of macrophage cholesterol stores

ApoE$^{-/-}$ or DKO macrophages were suspended in 5% FBS/DMEM and plated onto 12-well plates at a density of 1×10^6 cells/well. After 4 h, nonadherent cells were removed by washing two times with 1 ml of DMEM containing HEPES (25 mM). For cholesterol mobilization, the cells were incubated for 24 h or 48 h in DMEM alone or containing either lipid-free apoA-I (20 μg/ml) or human HDL (150 μg protein/ml). As a control for testing acceptor efflux capacity in experiments involving mobilization with macrophages isolated from DKO mice, apoE$^{-/-}$ macrophages were cholesterol enriched by incubation for 3 days with apoE$^{-/-}$ remnant lipoproteins (d = 1.006 to 1.019 g/ml, 20 μg protein/ml), and cholesterol mobilization was examined. The cells were then washed two times with DPBS, and the cell monolayer was air dried. Cell lipids were extracted by overnight incubation at room temperature in isopropanol containing cholesteryl methyl ether (2.5 μg/well) as an internal standard. The lipid extract free cholesterol (FC) and total cholesterol (TC) contents were then measured by gas-liquid chromatography following the procedure of Ishikawa et al. (31) as modified by Klansek and colleagues (32). Cell proteins were solubilized by addition of 1 N NaOH to the wells, and the protein content measured using the method of Lowry et al. (33) as modified by Markwell et al. (34).

Fluorescence and electron microscopy

Microscopy was used to visualize macrophage lipids before and after efflux. Fluorescence microscopy was used to visualize cellular FC by filipin staining. Transmission electron microscopy (EM), in combination with acid phosphatase staining, was used to detect lipid-engorged lysosomes. For filipin staining of cell FC, macrophages were plated onto sterile glass coverslips placed in the bottom of 35 mm tissue culture wells. After incubations, the cells were washed, fixed for 15 min (2.5% paraformaldehyde), and stained at room temperature with filipin stain solution (1.25 mg of filipin dissolved in dimethyl sulfoxide and diluted in 25 ml of PBS). Filipin-stained FC was detected with ultraviolet filter excitation and viewed through a 510 nm barrier filter using a Zeiss Axioplan motorized fluorescent microscope (Oberkochen, Germany) equipped with a Photometrics Coolsnap charge-coupled device camera (Nikon Instruments; Melville, NY). Acid phosphatase staining of lysosomes and related organelles was done essentially as described previously (35). Briefly, the cells or arteries were first washed four times in 0.1 M cacodylate buffer (pH 7.4) containing 0.1 M sucrose at 4°C, and then washed two times in the same buffer at room temperature. The cells were then fixed for 2 min at 22°C and 8 min at 4°C in 4% glutaraldehyde in 0.1 M cacodylate/sucrose buffer, washed, and stained to show the presence of acid phosphatase using our standard modification of the Gomori (36) lead precipitation method with β-glycerol phosphate as the substrate. After incubation with the substrate, the cells were postfixed in 1% osmium tetroxide, dehydrated, and embedded in epoxy resin. The sections were viewed without additional staining to verify the enzymatic reaction and were then stained with uranyl acetate. Transmission EM was performed using a Phillips CM-12 operated

at 80 kiloelectron volts to ultrastructurally analyze cell lysosomal lipid volume. Images were processed with Metamorph (Universal Imaging Co.) and Photoshop (Adobe) software.

Quantitation of lipid accumulation

The volume of lipid in foam cells within atherosclerotic lesions was estimated from EM sections of lesions as described previously (37). Briefly, lesions were identified and that area of the artery removed, fixed, and processed for acid phosphatase cytochemistry. Starting at an arbitrary point, the lesion was dissected by cross-sectioning into five equal parts. Five sections were taken from each part, and 20 random fields (50 μm × 50 μm) containing lesions were analyzed from each section. Volume of lysosomal lipids and inclusion of lipid per volume of cell were estimated using standard point count stereology (38). Stereologic volume density determination in isolated macrophages was also done by stereologic point counting. Isolated macrophages were fixed and stained to demonstrate acid phosphatase as described above. Cross sections through 30 individual cells were visualized by EM, and the volume of lipid in lysosomes (acid phosphatase-positive) and cytoplasmic inclusions was estimated using point count stereology as described previously for acid phosphatase-stained cells (35).

Immunoblotting of ABCA1

Protein extracts from macrophages were separated using 3% to 8% Tris-acetate gels (NuPAGE) and transferred to nitrocellulose membranes. Murine ABCA1 was detected with a primary antibody (Novus Biological) and visualized by chemiluminescence.

RESULTS

Cholesterol homeostasis of macrophages isolated from DKO mice

Previous studies by Krieger and colleagues (39–41) have shown that mice deficient in both SR-BI and apoE develop extreme dyslipidemia (worse than that presented by apoE$^{-/-}$ mice), even when maintained on a chow diet. DKO mice have total plasma cholesterol levels of 800–1,000 mg/dl (40, 41) and show abnormally enlarged, FC-enriched HDL particles. We initially examined the effects of this abnormal lipid environment on cholesterol homeostasis in DKO macrophages. Measurement of the FC and cholesteryl ester (CE) content of peritoneal macrophages isolated from either apoE$^{-/-}$ or DKO mice (on chow diet) showed that DKO macrophages become extremely enriched in cholesterol in vivo (**Fig. 1**). DKO cells contained between 220 μg and 380 μg of cholesterol/mg cell protein, with CE representing 67% of the total. In contrast, macrophages from apoE$^{-/-}$ mice did not show any cholesterol enrichment, compared with controls, despite the hypercholesterolemic environment (TC = 475 ± 46 mg/dl). Moreover, the apoE$^{-/-}$ macrophages contained primarily FC, and only 7% of TC was CE. We used filipin staining to identify the location of the massive FC accumulation seen in DKO macrophages. FC in macrophages isolated from DKO mice occurred in large perinuclear pools (**Fig. 2A**) typical of FC-engorged lysosomes (42). The lysosomal location was confirmed by acid phosphatase staining (Fig. 2B). In contrast, FC was not detectable in intracellular vesicles in apoE$^{-/-}$ macrophages exposed to the plasma lipid environment of apoE$^{-/-}$ mice maintained on a chow diet (data not shown).

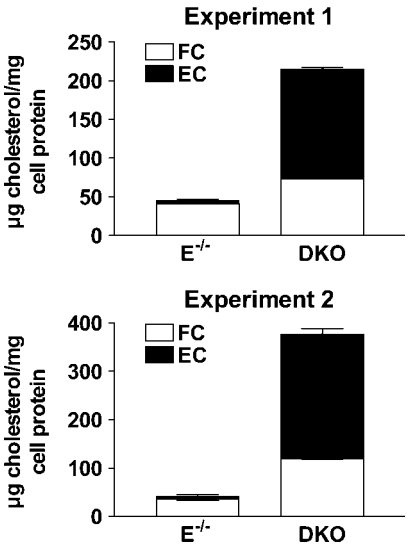

Fig. 1. Cellular cholesterol content of peritoneal macrophages isolated from either apolipoprotein E-null (apoE$^{-/-}$) or double knockout (DKO) mice. Peritoneal macrophages were isolated from either apoE$^{-/-}$or DKO mice consuming a chow diet. After an overnight incubation in 5% FBS/DMEM, the cells were washed two times with Dulbecco's phosphate-buffered saline (DPBS), and the free cholesterol (FC) and esterified cholesterol (EC) contents of the cells were measured as described in Experimental Procedures. Shown are values for two independent experiments. Values are means ± SD of triplicate determinations. Data are representative of four independent experiments.

We next examined whether the massive cholesterol stores of DKO macrophages could be mobilized to cho-

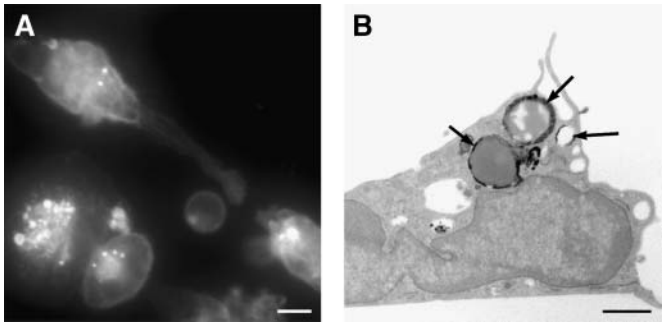

Fig. 2. Fluorescence photomicrographs of filipin-stained cholesterol and electron micrographs of acid phosphatase-stained lysosomes in DKO macrophages. Peritoneal macrophages were isolated from DKO mice consuming a chow diet. After an overnight incubation in 5% FBS/DMEM, the cells were fixed and stained with filipin (A) or acid phosphatase (B) as described in Experimental Procedures. A: In addition to plasma membrane staining, bright fluorescence is seen in large perinuclear pools indicative of FC in lysosomes. Magnification, ×1,500; bar = 3 μm. B: DKO macrophages contain significant lipid within cytoplasmic droplets, but there is also major engorgement of lysosomes with lipid as evidenced by the acid phosphatase activity associated with lipid (arrow). Magnification, ×7,000; bar = 1 μm.

lesterol acceptors (**Fig. 3**). Interestingly, incubation with human HDL did not induce a decrease in the cholesterol content of DKO macrophages (Fig. 3A). Indeed, by the end of 48 h, the FC and TC content of the cells increased by 82 ± 5% and 34 ± 2%, respectively. As a control, apoE$^{-/-}$ macrophages were cholesterol enriched by incubation with remnant lipoproteins and subsequently incubated with the same preparation of HDL. When incubated with apoE$^{-/-}$ mouse remnant lipoproteins in vitro, the cholesterol content of control apoE$^{-/-}$ macrophages reached 233 μg TC/mg cell protein (22 ± 1% of TC is FC) prior to initiation of efflux. In contrast to the DKO macrophages, the same preparation of HDL reduced the cholesterol content of control apoE$^{-/-}$ macrophages by 42 ± 1%, demonstrating the capacity of this HDL preparation to promote net efflux as expected (data not shown). Incubation of the DKO macrophages with apoA-I induced a major reduction (64 ± 5%) in the cellular CE content (Fig. 3B). However, much of the FC derived from hydrolysis of CE was not mobilized out of the cells to the apoA-I, inasmuch as the FC content increased by 43 ± 1% and the TC only decreased by 22 ± 1%. In contrast, incubation of the control apoE$^{-/-}$ macrophages with the same preparation of apoA-I promoted a 44 ± 2% reduction in TC (data not shown). Taken together, these results indicate that macrophages isolated from DKO mice have a severe derangement in cholesterol homeostasis, characterized by extreme cholesterol enrichment, lysosomal engorgement, and impaired mobilization to cholesterol acceptors.

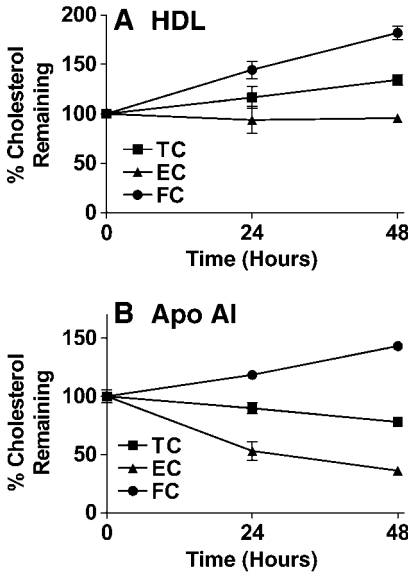

Fig. 3. Effect of HDL or lipid-free apolipoprotein A-I (apoA-I) on the cholesterol content of peritoneal macrophages isolated from DKO mice. Peritoneal macrophages were isolated from DKO mice consuming a chow diet. After a 4 h adherence phase in 5% FBS/DMEM, the macrophages were washed and incubated for up to 48 h in DMEM containing either 150 μg of HDL protein/ml (A) or 20 μg of apoA-I/ml (B). For each time point, the cells were washed two times with DPBS, and the total (TC), free (FC), and esterified (EC) cholesterol contents of the cells were measured as described in Experimental Procedures. Data are expressed as percent of initial cholesterol remaining in the cells, and the values are means ± SD of triplicate determinations. Data are representative of three independent experiments using HDL as an acceptor and two independent experiments using apoA-I as an acceptor.

Cholesterol homeostasis of DKO and apoE$^{-/-}$ mice

Macrophages exposed to the milder plasma lipid environment of apoE$^{-/-}$ mice. We next conducted a series of experiments to differentiate between the contribution of the mouse plasma lipid environment versus the contribution of the cellular phenotype produced by the combined absence of apoE and SR-BI as factors contributing to the abnormal cholesterol homeostasis in DKO macrophages. To achieve this goal, we used macrophages isolated from apoE$^{-/-}$-recipient mice that had been transplanted with either apoE$^{-/-}$ or DKO marrow and fed either normal chow or a Western-type diet. ApoE$^{-/-}$ and DKO macrophages isolated from apoE$^{-/-}$-recipient mice maintained on a chow diet contained the same low level of cholesterol (35 ± 1 μg/mg cell protein). Moreover, in both cells, only a minor portion of the cholesterol was esterified (**Fig. 4A**). After feeding on a Western diet for 8 weeks, the recipients of apoE$^{-/-}$ and DKO BM had similar plasma TC levels (900 ± 41 versus 937 ± 118 mg/dl), and their peritoneal macrophages were cholesterol enriched to a similar degree (88 ± 3 versus 89 ± 1 μg cholesterol/mg protein; Fig. 4B). This demonstrates that the massive cholesterol accumulation in macrophages isolated from DKO mice is in part due to the lipid environment to which the cells are exposed in vivo. However, examination of the proximal

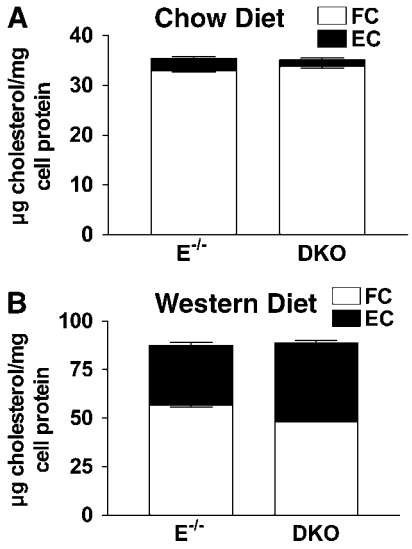

Fig. 4. Cholesterol content of peritoneal macrophages isolated from either apoE$^{-/-}$→apoE$^{-/-}$ or DKO→apoE$^{-/-}$ mice. Peritoneal macrophages were isolated from either apoE$^{-/-}$→apoE$^{-/-}$ or DKO→apoE$^{-/-}$ mice consuming either a chow (A) or Western-type (B) diet for 8 weeks post bone marrow transplant. After a 4 h adherence in 5% FBS/DMEM, the cells were washed two times with DPBS, and the free (FC) and esterified (EC) cholesterol contents of the cells were measured as described in Experimental Procedures. Values are means ± SD of triplicate determinations. Data are representative of three independent experiments.

aorta lesions by EM showed that the DKO arterial macrophages have a severe derangement in cholesterol homeostasis when exposed in vivo to the lipid environment of apoE$^{-/-}$ mice fed a Western diet (**Fig. 5**). Lesions in apoE$^{-/-}$→apoE$^{-/-}$ mice on the Western diet were small and consisted of only a few macrophage foam cells, usually no more than one foam cell deep (Fig. 5A). Only 60% of the cytoplasm of apoE$^{-/-}$ foam cells was occupied by lipid. In contrast, the lesions in the proximal aorta of DKO→apoE$^{-/-}$ mice fed the Western diet for 8 weeks were dramatically larger than those in apoE$^{-/-}$→apoE$^{-/-}$ mice (Fig. 5B). Furthermore, the macrophage-derived foam cells in lesions of DKO→apoE$^{-/-}$ mice showed excessive lipid engorgement (Fig. 5B), with an average of 97% of the cell volume occupied by lipid. This clearly demonstrates that exposure to the same lipid environment in vivo affects cholesterol homeostasis more in DKO macrophages than in apoE$^{-/-}$ macrophages.

The observation of extreme lipid engorgement in DKO arterial macrophages compared with apoE$^{-/-}$ arterial cells warranted comparisons of lysosomal lipid engorgement and cholesterol mobilization in the two cell types after exposure to the same plasma lipid environment in vivo. In this way, we could directly examine the role of macrophage SR-BI in maintaining normal cholesterol homeostasis. Acid phosphatase staining of macrophages isolated from apoE$^{-/-}$-recipient mice on the Western diet showed lysosomal lipid accumulation in both cell types (**Fig. 6A, B**) but severe lysosomal lipid engorgement only in DKO macrophages. Indeed, quantitation of the cell lipid volume (Fig. 6E) in lysosomes as detected by the acid phosphatase staining showed that DKO macrophages have 3.1-fold higher lysosomal lipid volume than do apoE$^{-/-}$ macrophages. DKO macrophages also have substantial cytoplasmic lipid. Filipin fluorescence shows the presence of FC in lysosomes of both cell types, but dramatically more in DKO macrophages (Fig. 6C, D), demonstrating

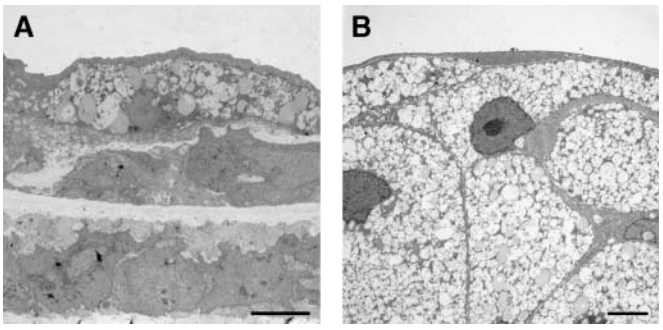

Fig. 5. Electron micrographs of foam cells in aorta from either apoE$^{-/-}$→apoE$^{-/-}$ or DKO→apoE$^{-/-}$ mice. Aortas were fixed by perfusion with 2% cacodylate-buffered glutaraldehyde and isolated from either apoE$^{-/-}$→apoE$^{-/-}$ (A) or DKO→apoE$^{-/-}$ (B) mice consuming a Western diet for 8 weeks post bone marrow transplant. A: Lesion is only a single layer of foam cells. The lumen of the artery is at the top and the first elastic lamina is in the middle. Magnification, ×2,000; bar = 4 μm. B: The lumen of the artery is at the top. There was another 72 μm of lesion below before reaching the first elastic lamina. Magnification, ×1,500; bar = 4 μm.

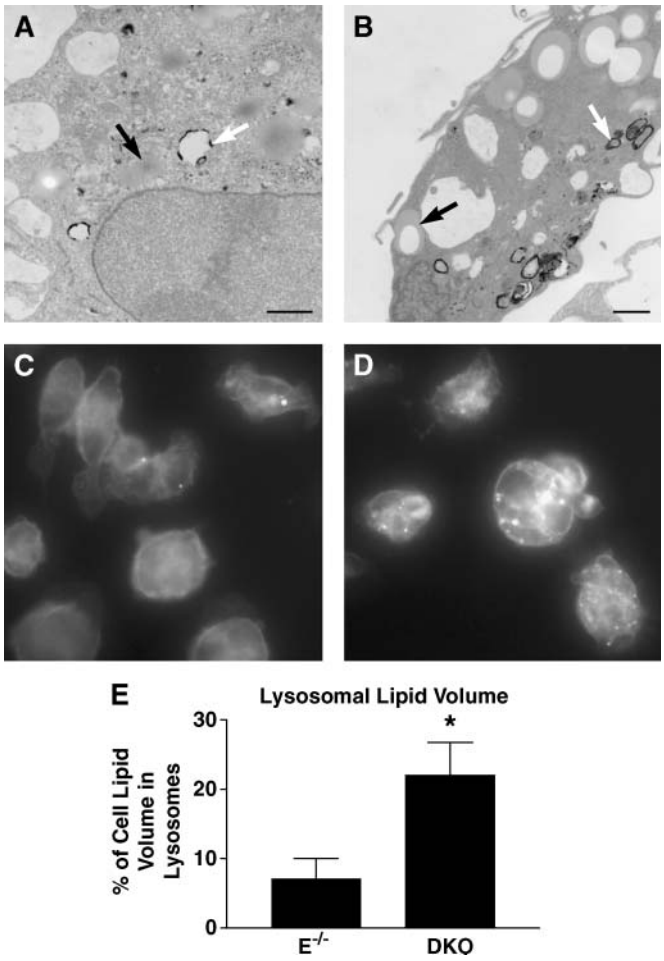

Fig. 6. Electron micrographs of acid phosphatase-stained lysosomes and fluorescence photomicrographs of filipin-stained cholesterol in macrophages from either apoE$^{-/-}$→apoE$^{-/-}$ or DKO→apoE$^{-/-}$ mice. Peritoneal macrophages were isolated from either apoE$^{-/-}$→apoE$^{-/-}$ (A, C) or DKO→apoE$^{-/-}$ (B, D) mice consuming a Western-type diet for 8 weeks post bone marrow transplant. After 4 h incubation in 5% FBS/DMEM, the cells were fixed and stained with acid phosphatase (A, B) or filipin (C, D) as described in Experimental Procedures. A: ApoE$^{-/-}$ macrophages contain cytoplasmic lipid droplets (closed arrow) and have some lipid accumulation in lysosomes (open arrow). Magnification, ×13,000; bar = 0.5 μm. B: Compared with apoE$^{-/-}$ cells, DKO cells have much more accumulation of lipid in lysosomes (open arrow) and significant accumulation of cytoplasmic lipid droplets, which are amorphous (closed arrrow). Magnification, ×4,500; bar = 0.5 μm. C, D: Consistent with significantly more lipid being associated with acid phosphatase-stained lysosomes in DKO cells versus apoE$^{-/-}$ cells (A, B), DKO macrophages (D) also have much more bright fluorescence in large perinuclear pools, which is indicative of more FC in lysosomes of DKO cells compared with apoE$^{-/-}$ cells (C). Magnification, ×600. E: Quantitation of the cell lipid volume in lysosomes as detected by acid phosphatase staining demonstrates that compared with apoE$^{-/-}$ cells, the DKO macrophages have a 3.1-fold higher lysosomal lipid volume. The asterisk indicates that the difference in the means is statistically significant ($P < 0.001$) using an unpaired t-test.

that regardless of the lipid environment in vivo, the DKO macrophages accumulate lipid in lysosomes. Addition of HDL to apoE$^{-/-}$ or DKO macrophages isolated from apoE$^{-/-}$- recipient mice gave clear indications that the

abnormal cholesterol homeostasis of DKO macrophages is due to inefficient mobilization of cholesterol. By the end of 48 h incubation with HDL (100 μg protein/ml), the DKO macrophages had only hydrolyzed 37 ± 5% of their CE, compared with 72 ± 15% for the apoE$^{-/-}$ cells (**Fig. 7A, B**). In addition, the DKO macrophages did not mobilize the liberated FC, inasmuch as there actually was a 29 ± 1% increase in FC compared with baseline (Fig. 7A), and the cholesterol content of the cells did not change (Fig. 7B). In contrast, by the end of 48 h, the cholesterol content of apoE$^{-/-}$ cells decreased by 33 ± 1%, and the FC content decreased by 8 ± 1%. Filipin staining analyses (**Fig. 8**) showed a decrease in FC content in apoE$^{-/-}$ cells after incubation with HDL. In contrast, HDL did not induce a reduction in filipin staining of FC of lysosomes in DKO macrophages, suggesting that the FC resulting from hydrolysis of CE is sequestered in compartments not linked to the efflux gates.

Finally, we examined the integrity of the pathway leading to cholesterol mobilization to apoA-I in DKO macrophages. During 48 h incubation of apoE$^{-/-}$ and DKO macrophages with apoA-I (**Fig. 9A, B**), only small differences were observed in hydrolysis of CE. However, the FC content of DKO macrophages increased by 25 ± 2% at 48 h, whereas the FC content of apoE$^{-/-}$ cells decreased by 16 ± 1% (Fig. 9A). As a result, cholesterol content only decreased by 21 ± 1% in the DKO macrophages compared with 41 ± 2% in the apoE$^{-/-}$ cells. These differences in mobilization to apoA-I were not due to differences in ABCA1 levels (**Fig. 10**), because the ABCA1/β-actin ratio of intensity was 1.9 compared with 2.1 for DKO cells versus apoE$^{-/-}$ macrophages.

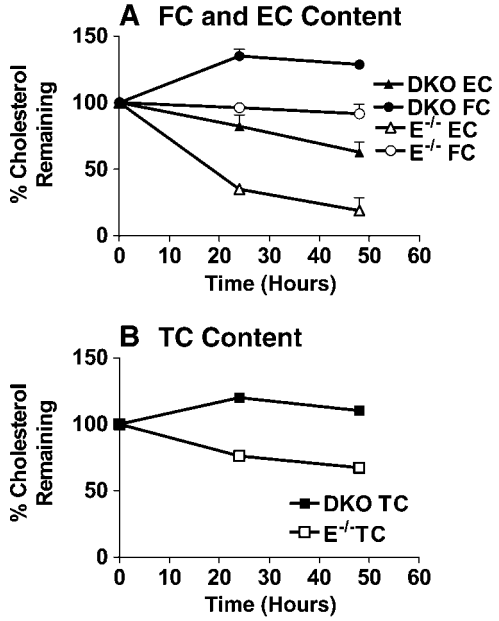

Fig. 7. Effect of HDL on the cholesterol content of macrophages from apoE$^{-/-}$→apoE$^{-/-}$ or DKO→apoE$^{-/-}$ mice. Peritoneal macrophages were isolated from either apoE$^{-/-}$→apoE$^{-/-}$ or DKO→apoE$^{-/-}$ mice consuming a Western-type diet for 8 weeks post bone marrow transplant. After 4 h adherence in 5% FBS/DMEM, the macrophages were washed and incubated for up to 48 h in DMEM containing 150 μg of HDL protein/ml. For each time point, the cells were washed two times with DPBS, and the free (FC) (A), esterified (EC) (A), and total (TC) (B) cholesterol contents of the cells were measured as described in Experimental Procedures. Data are expressed as percent of initial cholesterol remaining in the cells, and the values are means ± SD of triplicate determinations. Data are representative of three independent experiments.

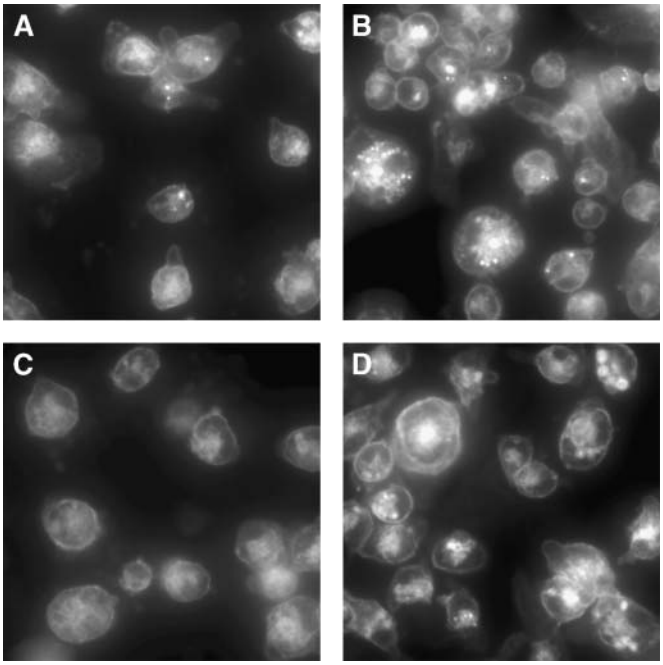

Fig. 8. Fluorescence photomicrographs of filipin-stained cholesterol in apoE$^{-/-}$→apoE$^{-/-}$ or DKO→apoE$^{-/-}$ macrophages before and after incubation with HDL. Peritoneal macrophages were isolated from either apoE$^{-/-}$→apoE$^{-/-}$ (A, C) or DKO→apoE$^{-/-}$ mice (B, D) consuming a Western-type diet for 8 weeks post bone marrow transplant. Macrophage FC was stained with filipin before (A, B) and after (C, D) 48 h incubation with 150 μg HDL protein/ml. A, B: Prior to incubation with HDL, the DKO macrophages, compared with apoE$^{-/-}$ cells, have more filipin staining of FC in large perinuclear pools. Magnification, ×450. C, D: After incubation with HDL, the filipin staining of intracellular FC decreased in apoE$^{-/-}$ macrophages (A versus C). In contrast, the presence of large perinuclear pools of FC did not decrease when DKO macrophages were incubated with HDL (B versus D). Magnification, ×450.

DISCUSSION

Mice with combined deficiency of apoE and SR-BI have extreme dyslipidemia, develop early occlusive atherosclerotic coronary artery disease, and die prematurely at 6–8 weeks of age with evidence of myocardial infarction, mimicking features of human coronary heart disease (39). The current study demonstrates that macrophages with combined deficiency of apoE and SR-BI have a severe derangement in cholesterol homeostasis visible even when the cells are taken out of the abnormal plasma lipid environment of the DKO mice. This is due to altered macrophage trafficking of cholesterol, including lysosomal

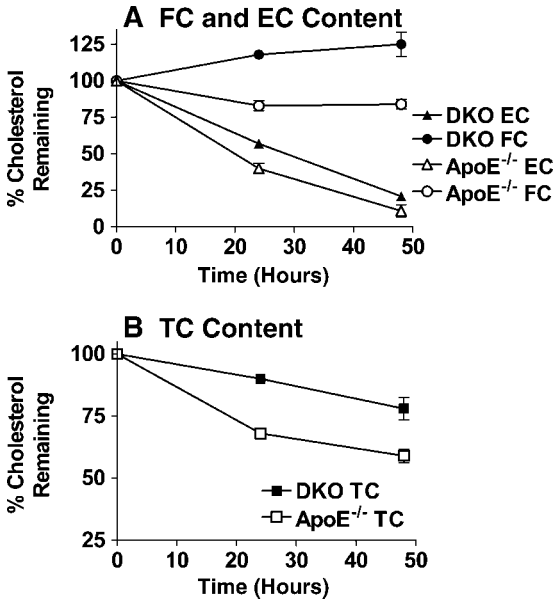

Fig. 9. Effect of apoA-I on the cholesterol content of macrophages from apoE$^{-/-}$→apoE$^{-/-}$ or DKO→apoE$^{-/-}$ mice. Peritoneal macrophages were isolated from either apoE$^{-/-}$→apoE$^{-/-}$ or DKO→apoE$^{-/-}$ mice consuming a Western-type diet for 8 weeks post bone marrow transplant. After 4 h adherence in 5% FBS/DMEM, the macrophages were washed and incubated for up to 48 h in DMEM containing 20 μg of apoA-I/ml. For each time point, the cells were washed two times with DPBS, and the FC (A), EC (A), and TC (B) contents of the cells were measured as described in Experimental Procedures. Data are expressed as percent of initial cholesterol remaining in the cells, and the values are means ± SD of triplicate determinations. Data are representative of three independent experiments.

engorgement and disrupted efflux. It follows that this severe abnormality of cholesterol homoestasis produces accelerated foam cell formation and contributes to the exaggerated atherosclerosis development in DKO mice.

Our study shows that peritoneal macrophages isolated from DKO mice have excessive stores of cholesterol even when the animals are maintained on a chow diet (Fig. 1). Furthermore, a substantial amount of this cholesterol is sequestered intracellularly, as evidenced by the presence

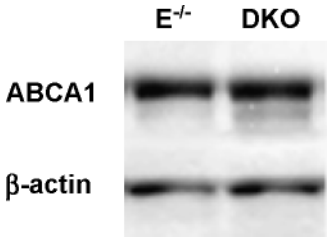

Fig. 10. ABCA1 levels in macrophages from apoE$^{-/-}$→apoE$^{-/-}$ or DKO→apoE$^{-/-}$ mice. Peritoneal macrophages were isolated from either apoE$^{-/-}$→apoE$^{-/-}$ or DKO→apoE$^{-/-}$mice consuming a Western-type diet for 8 weeks post bone marrow transplant. After 4 h adherence in 5% FBS/DMEM, ABCA1 and β-actin were detected by Western blotting as described in Experimental Procedures. Data are representative of two independent experiments.

of lipid-engorged lysosomes (Fig. 2) and by the inefficient clearance of cholesterol to apoA-I and HDL (Fig. 3). This extreme cholesterol accumulation and sequestration is due, in part, to the toxic lipid environment of plasma in DKO mice, inasmuch as the cellular cholesterol stores were much smaller when the DKO macrophages were exposed to the plasma lipid environment of apoE$^{-/-}$ mice consuming a Western diet (Figs. 1, 4). These differences occurred despite similar plasma cholesterol levels (900–1,000 mg/dl) of DKO mice on a chow diet and apoE$^{-/-}$ mice on a Western diet. It is known that the single gene deletion of either apoE or SR-BI results in formation of abnormal lipoproteins. Mice deficient in apoE have low HDL-C (43), resulting from low LCAT activity (44) and increased sphingomyelin content, (45, 46) whereas deficiency of SR-BI results in the formation of enlarged, FC-enriched HDL particles (30). In addition, apoE$^{-/-}$ mice accumulate remnant lipoproteins that are abnormally enriched in sphingomyelin (46). DKO mice have elevated levels of remnant lipoproteins compared with apoE$^{-/-}$ mice and accumulate grossly enlarged HDLs (the size of VLDL and IDL) compared with SR-BI$^{-/-}$ mice (39 –41). In addition, 80% of the total plasma cholesterol in DKO mice is FC (39–41). The atherogenicity of the DKO plasma lipids is substantiated by studies demonstrating that a lipid-lowering and anti-oxidant drug, probucol, improves the lipoprotein abnormalities and rescues the mice from early death (47). There are a number of features of the atherogenic lipoproteins in DKO mice that may contribute to the extreme cholesterol accumulation and lysosomal sequestration in macrophages isolated from DKO mice. First, it is likely that the abnormally enlarged, FC-enriched HDL particles are inefficient at mobilizing cellular cholesterol, because studies have demonstrated that FC-enriched HDL_1 particles have reduced efflux capacity (48). Interestingly, we have found that in contrast to HDL from apoE$^{-/-}$ mice, DKO HDL induces an increase (57 ± 1%) in the cholesterol content of unloaded wild-type macrophages (unpublished observation). Second, elevated levels of sphingomyelin-enriched remnants in DKO mouse plasma could also play a role, because studies have demonstrated that the sphingomyelin-enriched remnants in apoE$^{-/-}$ mice are more susceptible to aggregation via the action of sphingomyelinase and, as a result, are potent inducers of cholesterol accumulation in macrophages (46, 49). Third, the fact that probucol prolongs the life span of DKO mice (47) and that apoE$^{-/-}$ mouse plasma is enriched in oxidized phospholipids (44) suggests that oxidized lipoproteins may also play a role in abnormal DKO macrophage cholesterol homeostasis. The lipid-engorged lysosomes in macrophages exposed to the plasma lipid environment of DKO mice is consistent with a role for aggregated and/or oxidized lipoproteins in DKO mouse plasma in promoting abnormal macrophage cholesterol homeostasis. In this regard, studies have shown that aggregated (50) and oxidized LDL (35, 51) are slowly processed in lysosomes and promote accumulation of lipid-engorged lysosomes in macrophages. It is also worth noting that cholesterol in lipid-engorged lyso-

somes is inefficiently transferred to cholesterol acceptors (42, 52).

The importance of the current study, however, is in the demonstration that the deranged cholesterol homeostasis in DKO macrophages is evident even outside the DKO environment, and is therefore caused by intracellular defects in cholesterol trafficking and mobilization. It is clear from our study that the combined absence of macrophage apoE and SR-BI directly impacts cholesterol homeostasis. This concept is substantiated by the finding that when apoE$^{-/-}$ and DKO macrophages were cholesterol enriched via the same plasma lipid environment, the DKO cells had substantially more lipid-engorged lysosomes than did apoE$^{-/-}$ cells (Fig. 6). In addition, the cholesterol stores in DKO macrophages were less efficiently transferred to either HDL or apoA-I than those in apoE$^{-/-}$ cells (Figs. 7, 8, 9). The decreased mobilization to HDL as an acceptor appears to be contradictory to the results of our previous study (19). In that study, no differences were found in the levels of ^{3}H-FC released to the media (with either HDL or apoA-I discs as acceptors) from apoE$^{-/-}$ versus DKO macrophages. However, it should be realized that when macrophages are preloaded with cholesterol, as was the case for cells isolated from DKO mice (Fig. 1), ^{3}H-FC will only track the cholesterol pools with which it equilibrates, and that cholesterol sequestered in lysosomes and cytoplasmic inclusions will not equilibrate with the ^{3}H-FC (53). Furthermore, measurement of medium ^{3}H-FC in the presence of HDL may not accurately reflect net movement of cellular cholesterol (54). Indeed, we show that DKO macrophages extracted from either DKO (Figs. 2, 3) or apoE$^{-/-}$ (Figs. 6–9) mice have substantial sequestration of cholesterol even in the presence of HDL or apoA-I as acceptors. Macrophage SR-BI deficiency resulted in a more extreme impairment in cholesterol mobilization to HDL than to apoA-I. Indeed, HDL paradoxically increased the FC and TC contents of DKO macrophages, despite abundant initial stores of FC. This observation suggests that cholesterol-enriched DKO macrophages lack a sufficient plasma membrane FC gradient to drive net flux to HDL (55). Consistent with this concept, SR-BI expression in cell lines is associated with formation of plasma membrane cholesterol domains (56, 57). In addition, the present study demonstrates that the DKO cells have significant intracellular stores of FC. The impaired mobilization of cholesterol to apoA-I as an acceptor was not due to changes in ABCA1 levels. Because the effects of macrophage SR-BI/apoE deficiency on cholesterol mobilization to apoA-I were smaller than with HDL as an acceptor, it is likely that SR-BI deficiency results in decreased cholesterol enrichment of the nascent HDL particles formed via ABCA1 (58).

Our study demonstrates that DKO macrophages retain large amounts of FC in both the absence and the presence of cholesterol acceptors, and that some of this FC is sequestered in lysosomes. This observation suggests a profound influence on cholesterol trafficking that maybe due to direct or indirect effects of SR-BI. Consistent with a direct role for SR-BI in cholesterol trafficking are studies showing that SR-BI is localized to endosomes (59) and cytoplasmic droplets (60). In addition, studies suggest that SR-BI functions in intracellular trafficking of HDL particles and sorting of HDL cholesterol in hepatocytes (59). It is also worth noting that HDL can be endocytosed to multivesicular compartments in macrophages and then re-secreted as a more cholesterol-enriched particle (61). A possible indirect effect of SR-BI could be mediated by its stabilization of caveolin-1 (62), a protein implicated in cholesterol trafficking (58, 63, 64). Consistent with this possibility is the enhanced secretion of caveolins to HDL by macrophages (65) in conjunction with studies demonstrating that caveolae-associated FC is released to mature HDL (66) and the nascent particles formed via ABCA1 (58).

In summary, we demonstrate that DKO macrophages have a severe derangement in cholesterol homeostasis. Aspects of this derangement include intracellular FC sequestration, lysosomal lipid engorgement, and inefficient cholesterol mobilization to HDL and apoA-I. It is likely that impaired cholesterol trafficking in DKO macrophages is involved in the severe atherosclerosis and shorter lifespan of mice lacking apoE and SR-BI.

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