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Research Article **Cardiology**

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Combined deficiency of ABCA1 and ABCG1 promotes foam cell accumulation and accelerates atherosclerosis in mice

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Introduction

Plasma HDL levels have a strong inverse relationship to the incidence of heart attack and stroke (1), but the underlying mechanisms are poorly understood. The ability of HDL to promote efflux of cholesterol from peripheral tissues and transport to the liver was proposed long ago as an antiatherogenic mechanism (2). However, HDL also has antiinflammatory, antioxidant, antithrombotic, and vasodilating properties that could all potentially be involved in its antiatherogenic effects (3, 4). In vivo proof of the relevance of these various potential antiatherogenic mechanisms is still lacking.

HDL and its major apolipoprotein, apoA-1, have the ability to promote the efflux of cholesterol from cholesterol-loaded macrophages and smooth muscle cells, the major cellular components of atherosclerotic plaques (5). Several different pathways of cellular cholesterol efflux have been described, such as aqueous diffusion (6), efflux of cholesterol associated with apoE secretion (7, 8), scavenger receptor–BI–mediated (SR-BI–mediated) cholesterol efflux (9), and active cholesterol efflux via the ATP-binding cassette transporters ABCA1 and ABCG1 (10, 11). ABCA1 promotes cholesterol and phospholipid efflux from cells to lipid-poor apoA-1 but not to mature HDL particles, while ABCG1 promotes cholesterol efflux to HDL and other lipoprotein particles but not to lipid-poor apoA-1 (12–14), suggesting that the 2 transporters could have complementary activities in vivo. ABCA1 is the mutant molecule in Tangier disease, characterized by low HDL levels, by macrophage foam cell accumulation in the tonsils and spleen, and probably by modestly increased atherosclerosis (15–18). However, the increased risk of atherosclerosis in patients with Tangier disease does not appear as dramatic as one would expect in individuals with an almost complete absence of HDL (18), and targeted inactivation of ABCA1 did not affect the development of atherosclerosis in *apoE*$^{-/-}$ and *Ldlr*$^{-/-}$ mice, 2 mouse models of atherosclerosis (19). Transplantation of BM from *Abca1*$^{-/-}$ mice into *Ldlr*$^{-/-}$ or *apoE*$^{-/-}$ recipients causes a moderate increase in atherosclerosis (19, 20), but there are conflicting reports on the effects of ABCA1 overexpression in transgenic mouse models of atherosclerosis (21, 22). *Abcg1*$^{-/-}$ mice accumulate macrophage foam cells in various tissues such as the lung (23), but the effects of ABCG1 deficiency on atherosclerosis are controversial and poorly understood. BM transplantation from *Abcg1*$^{-/-}$ mice into *apoE*$^{-/-}$ or *Ldlr*$^{-/-}$ recipients resulted in either a modest increase (24) or decrease in atherosclerosis (25, 26). The decreased atherosclerosis was attributed to increased macrophage apoptosis in response to oxidized LDL (26) or to compensatory upregulation of ABCA1 and increased apoE secretion in *Abcg1*$^{-/-}$ macrophages (25).

In an attempt to resolve these controversies (24–26) and to test the hypothesis that effects of single transporter deficiency are masked by compensatory upregulation of the other transporter, we carried out BM transplantation from mice with single or combined deficiencies of ABCA1 or ABCG1 into *Ldlr*$^{-/-}$ mice. Consistent with our hypothesis, the *Abca1*$^{-/-}$*Abcg1*$^{-/-}$ BM recipients showed increased atherosclerosis and massive myocardial foam cell accumulation. Mechanistic studies showed a major defect in cholesterol efflux in *Abca1*$^{-/-}$*Abcg1*$^{-/-}$ macrophages, decreased apoE secretion, increased apoptosis in response to loading with free cholesterol or oxidized LDL, and increased expression of inflammatory and chemokine genes. Thus, many of the proposed atheroprotective properties of HDL appear to be secondary to macrophage sterol efflux mediated by these 2 transporters.

Nonstandard abbreviations used: LXR, liver X receptor; SR-BI, scavenger receptor–BI.

Conflict of interest: A.R. Tall has received consulting fees from Pfizer, Merck, AstraZeneca, and Roche; lecture fees from Merck; and grant support from Merck and Pfizer.

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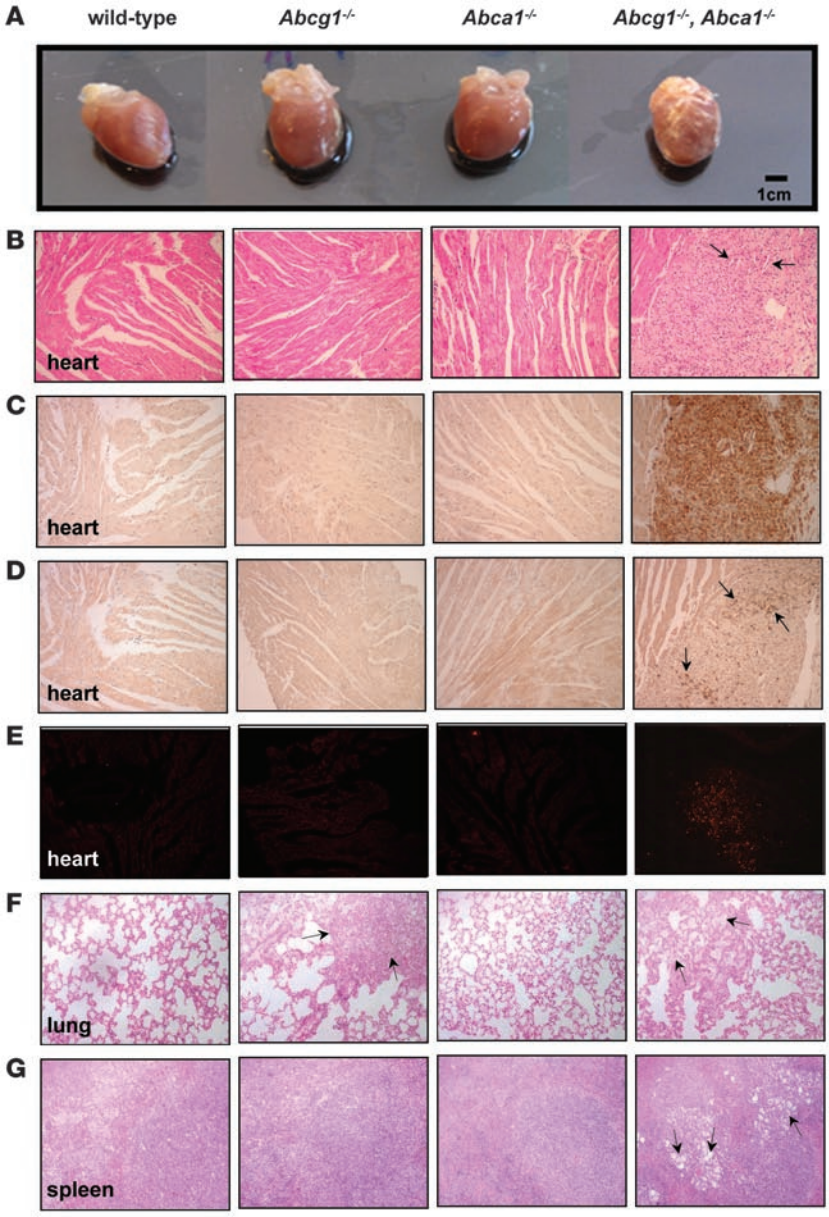


Figure 1
Foam cell infiltration of the myocardium in *Abca1*$^{-/-}$*Abcg1*$^{-/-}$ BM recipients but not WT or single-KO recipients. (**A**) Representative hearts obtained from *Ldlr*$^{+/-}$ mice transplanted with BM derived from WT, *Abcg1*$^{-/-}$, *Abca1*$^{-/-}$, or *Abca1*$^{-/-}$*Abcg1*$^{-/-}$ mice and fed a high-cholesterol diet for 12 weeks. Hearts from all *Abca1*$^{-/-}$*Abcg1*$^{-/-}$ recipients were visibly smaller and pale compared with WT and single-KO controls. Scale bar: 1 cm. (**B**–**G**) Paraffin-embedded serial sections obtained from the myocardium surrounding the proximal aorta, lung, and spleen of mice transplanted with BM of all 4 genotypes. Original magnification, ×200. (**B**) H&E staining revealed an extensive cellular infiltrate in the myocardium of *Abca1*$^{-/-}$*Abcg1*$^{-/-}$ recipients (9 of 9) but not controls (WT, 0 of 12; *Abcg1*$^{-/-}$, 0 of 8; *Abca1*$^{-/-}$, 0 of 16). The infiltrate resembled foam cell lesions of the vessel wall, including apparent cholesterol clefts (arrows). (**C**) Mac-3 immunostaining (dark brown) confirmed that the majority of cells were macrophages. (**D**) MCA771G immunostaining (dark brown) revealed a moderate number of neutrophils (arrows) as well. (**E**) TUNEL staining revealed pockets of apoptotic cells (red) in the infiltrated regions of myocardium from *Abca1*$^{-/-}$*Abcg1*$^{-/-}$ recipients (7 of 9) only. (**F** and **G**) H&E staining revealed an extensive cellular infiltrate in the lungs and spleens of *Abca1*$^{-/-}$*Abcg1*$^{-/-}$ recipients as well as in the lungs of *Abcg1*$^{-/-}$ recipients. Arrows indicate foam cells.

Results

Massive myocardial foam cell accumulation in Abca1$^{-/-}$*Abcg1*$^{-/-}$ *BM recipients*. We bred mice with single or combined KO of *Abca1* and *Abcg1*, transplanted BM from these mice into irradiated *Ldlr*$^{+/-}$ mice, and, after a recovery period, placed the recipients on a high-cholesterol diet for 12 weeks. Upon sacrificing the mice, we noticed a striking phenotype that was seen in all recipients of *Abca1*$^{-/-}$*Abcg1*$^{-/-}$ BM (n = 9) but not in mice receiving WT, *Abca1*$^{-/-}$, or *Abcg1*$^{-/-}$ BM. In the *Abca1*$^{-/-}$*Abcg1*$^{-/-}$ BM recipients, the hearts were smaller and were pale compared with mice of the other genotypes (Figure 1A). Pallor was also observed in the small intestine, and the spleen was enlarged, in *Abca1*$^{-/-}$*Abcg1*$^{-/-}$ recipients. Microscopic examination of the hearts of *Abca1*$^{-/-}$*Abcg1*$^{-/-}$ mice revealed massive infiltration with cells containing foamy cytoplasm that stained positively with Mac-3, indicating that they were macrophage foam cells (Figure 1, B and C). In addition, these infiltrates contained empty spaces of similar appearance to the cholesterol clefts commonly seen in atherosclerotic lesions (Figure 1C). There were also a moderate number of neutrophils in the infiltrate as shown by MCA771G staining (Figure 1D). TUNEL staining revealed clusters of apoptotic cells in the midst of the inflammatory infiltrate and this was observed only in *Abca1*$^{-/-}$*Abcg1*$^{-/-}$ BM recipients (Figure 1E). Previous studies have shown accumulation of foam cells and other inflammatory cells in the lungs of *Abcg1*$^{-/-}$ mice (23). We also found foci of foam cells and other inflammatory cells including neutrophils in the lungs of *Abcg1*$^{-/-}$ BM recipients; interestingly, this phenotype was not increased in *Abca1*$^{-/-}$*Abcg1*$^{-/-}$ recipients beyond what was seen in *Abcg1*$^{-/-}$ recipients (Figure 1F). In *Abca1*$^{-/-}$*Abcg1*$^{-/-}$ recipients, the spleen also showed an increase in foam cells compared with control and single-KO recipients (Figure 1G).

Accelerated atherosclerosis in Abca1$^{-/-}$*Abcg1*$^{-/-}$ *BM recipients*. After 12 weeks on the high-cholesterol diet, control mice receiving WT BM had developed only sparse, foam cell–containing atherosclerotic lesions in their proximal aortas (Figure 2B). Atherosclerotic lesion development was similar to control mice in *Abcg1*$^{-/-}$ BM recipi-

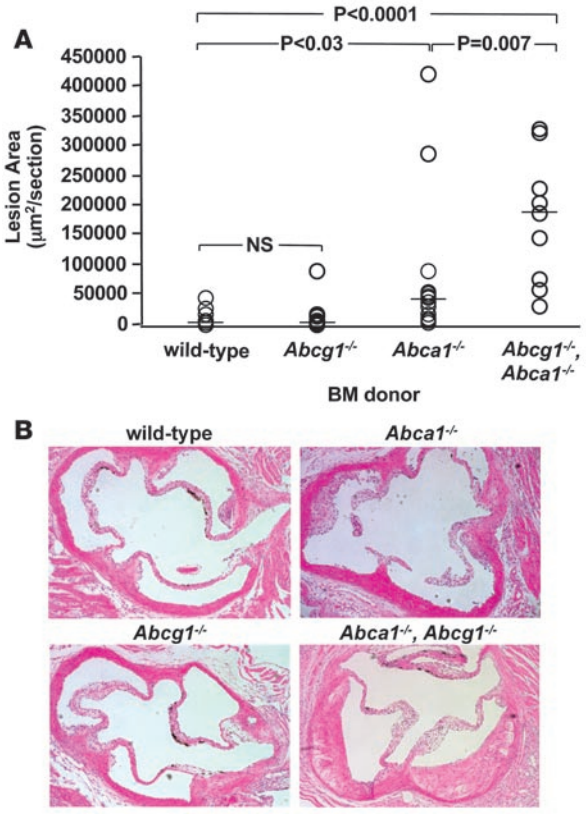


Figure 2
Atherosclerotic lesion development in the proximal aorta. (**A**) Quantification of proximal aortic root lesion area by morphometric analysis of H&E-stained sections in *Ldlr*$^{+/-}$ mice transplanted with WT (n = 12), *Abcg1*$^{-/-}$ (n = 8), *Abca1*$^{-/-}$ (n = 16), or *Abca1*$^{-/-}$*Abcg1*$^{-/-}$ (n = 9) BM after 12 weeks of high-cholesterol diet. Values for individual mice are shown as open circles, representing the average of 5 sections per mouse. Horizontal bars indicate the group medians: control, 3,994 μm²/section; *Abcg1*$^{-/-}$, 3,724 μm²/section; *Abca1*$^{-/-}$, 40,070 μm²/section; *Abca1*$^{-/-}$*Abcg1*$^{-/-}$, 186,182 μm²/section. Mean lesion areas (mean ± SEM) were as follows: control, 9,090 ± 3,872 μm²/section; *Abcg1*$^{-/-}$, 15,412 ± 1,640 μm²/section; *Abca1*$^{-/-}$, 72,675 ± 28,879 μm²/section; *Abca1*$^{-/-}$*Abcg1*$^{-/-}$, 174,958 ± 36,269 μm²/section. ANOVA was performed with square root–transformed data. (**B**) Representative H&E-stained proximal aortas from mice receiving BM of all the 4 genotypes after 12 weeks of high-cholesterol diet. Original magnification, ×100.

ents and was moderately increased in recipients of *Abca1*$^{-/-}$ BM (Figure 2A). In contrast, atherosclerosis was more dramatically increased in mice receiving *Abca1*$^{-/-}$*Abcg1*$^{-/-}$ BM (Figure 2). Further characterization of these atherosclerotic lesions revealed complex plaques containing macrophages, necrotic cores, fibrous caps (Figure 2), and small numbers of neutrophils and TUNEL-positive apoptotic cells (6–8 cells per high-power field; data not shown). TUNEL-positive cells were rarely observed in lesions from mice of the other genotypes.

Plasma lipoprotein profiles. We next carried out studies to elucidate potential mechanisms responsible for increased macrophage foam cell accumulation in arteries, myocardium, and other tissues in mice receiving *Abca1*$^{-/-}$*Abcg1*$^{-/-}$ BM. The atherogenic diet resulted in moderate hyperlipidemia in all groups of mice (Supplemental Table 1; supplemental material available online with this article; doi:10.1172/JCI33372DS1). Plasma lipoprotein analysis by fast protein liquid chromatography revealed slightly increased VLDL and LDL cholesterol levels (less than 30%) in mice receiving *Abca1*$^{-/-}$*Abcg1*$^{-/-}$ BM (Supplemental Figure 1A) and no major difference in the lipoprotein profile of animals receiving *Abca1*$^{-/-}$ or *Abcg1*$^{-/-}$ compared with WT BM (data not shown). Decreased macrophage apoE secretion (see below) could explain the slightly higher VLDL and LDL levels (27, 28) and lower levels of apoE in these fractions in *Abca1*$^{-/-}$*Abcg1*$^{-/-}$ BM recipients (Supplemental Figure 1B). There were no changes in HDL-cholesterol levels, most likely because after 12 weeks on the atherogenic diet, mice showed a redistribution of apoE among plasma lipoproteins and less than 10% associated with the HDL fraction (29). Although the increase in VLDL and LDL could have contributed to increased atherosclerosis, it is unlikely to have been the major causative factor.

Defective cholesterol efflux and foam cell formation in Abca1$^{-/-}$*Abcg1*$^{-/-}$ *macrophages*. To test the hypothesis of a defect in cholesterol efflux as the underlying cause of increased atherosclerosis, we carried out cholesterol efflux studies and measured the cholesterol content of freshly isolated peritoneal macrophages. *Abcg1*$^{-/-}$ cells displayed a decrease in cholesterol efflux to serum, polyethyleneglycol-HDL (PEG-HDL), and human HDL isolated by ultracentrifugation (20%, 30%, and 30% decreases, respectively), while in *Abca1*$^{-/-}$*Abcg1*$^{-/-}$ macrophages there was a more profound decrease (more than 2-fold) in the ability of serum and HDL to promote cholesterol efflux compared with single-KO and control macrophages (Figure 3). Cholesterol efflux to HDL was slightly increased in *Abca1*$^{-/-}$ cells (Figure 3C), likely reflecting a compensatory induction of ABCG1 (see below). Cholesterol efflux studies were also performed using media devoid of acceptors or containing lipid-poor apoA-1 or apoE (Figure 3D). This showed a profound defect in cholesterol efflux under all 3 conditions in *Abca1*$^{-/-}$*Abcg1*$^{-/-}$ macrophages, even below the level observed for *Abca1*$^{-/-}$ cells. Defective cholesterol efflux led to a marked increase in cholesterol content primarily in the form of cholesteryl esters in peritoneal macrophages isolated from *Abca1*$^{-/-}$*Abcg1*$^{-/-}$ mice fed standard chow (Figure 4A) or high-cholesterol diet (Figure 4B). In contrast, single-KO mice did not show increased cholesterol content when fed standard chow and only modestly increased cholesterol content compared with controls when fed high-cholesterol diet (Figure 4, A and B). These studies suggest synergistic roles of ABCA1 and ABCG1 in promoting macrophage cholesterol efflux, consistent with recent in vivo studies of macrophage reverse cholesterol transport showing additive effects of deficiencies of ABCG1 and ABCA1 in macrophages (30). Together these data indicate that a major defect in cholesterol efflux in *Abca1*$^{-/-}$*Abcg1*$^{-/-}$ macrophages is the underlying mechanism responsible for increased atherosclerosis.

Decreased apoE secretion in Abca1$^{-/-}$*Abcg1*$^{-/-}$ *macrophages*. Next we assessed apoE secretion in transporter-deficient macrophages. Previous studies have suggested that ABCA1 deficiency impairs apoE secretion most likely secondary to the role of ABCA1 in adding phospholipids and cholesterol to apoE (25, 31, 32) and have shown increased apoE secretion in *Abcg1*$^{-/-}$ macrophages associated with upregulation of ABCA1 (25). Macrophage apoE accumulation in media was measured in freshly isolated peritoneal macrophages or following loading of macrophages with acLDL plus liver X receptor (LXR) activator (T0901317) to maximally upregulate apoE expression and secretion (33). ABCG1 protein was increased in *Abca1*$^{-/-}$ cells, while ABCA1 protein was increased in *Abcg1*$^{-/-}$ cells (Figure 5), reflecting compensato-

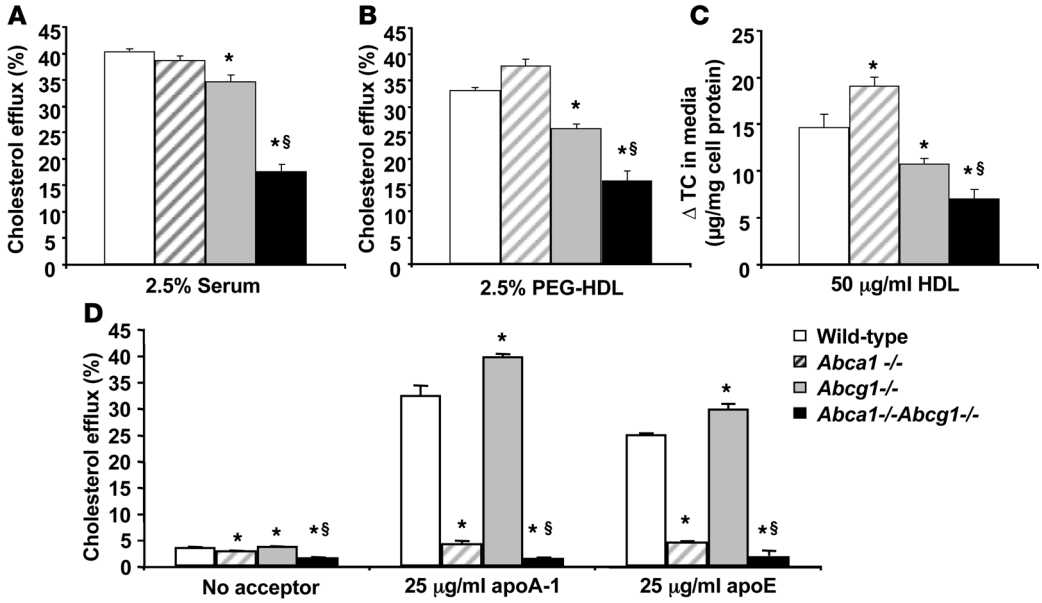
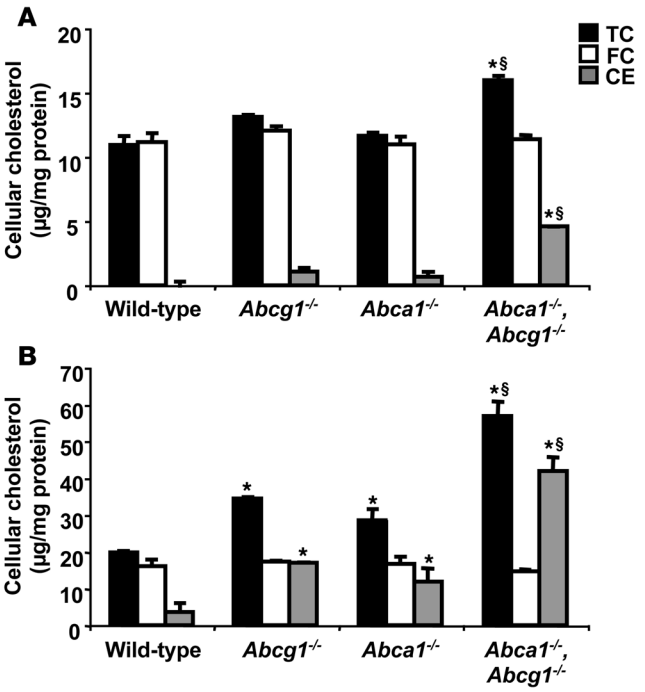


Figure 3
Cholesterol efflux from peritoneal macrophages isolated from WT, *Abca1*$^{-/-}$, *Abcg1*$^{-/-}$, and *Abca1*$^{-/-}$*Abcg1*$^{-/-}$ mice. (**A**, **B**, and **D**) Thioglycollate-elicited macrophages were cultured for 24 h in DMEM plus 0.2% BSA containing 50 μg/ml acLDL, 3 μmol/l T0901317, and 2 μCi/ml [^{3}H]-cholesterol. Then, a pool of human serum (2.5%; **A**), a pool of human serum devoid of apoB-containing particles (2.5%; **B**), and media devoid of acceptors or containing lipid-poor apoA-1 or apoE (**D**) were added as acceptor and incubated for 6 h before the media and cells were collected for analysis. (**C**) Cholesterol efflux was also confirmed by cholesterol mass analysis. Cells were cultured overnight with 50 μg/ml acLDL and 3 μmol/l T0901317 and then incubated for 6 hours with 50 μg/ml human HDL isolated by ultracentrifugation. The HDL-mediated net cholesterol efflux (ΔTC) was calculated as the difference of cholesterol mass of the medium with and without cells. Values are mean ± SEM. *$P < 0.05$ versus WT. §$P < 0.05$ versus single-KOs.

ry induction of their cognate mRNAs (data not shown). There was a small decrease in apoE secretion from *Abca1*$^{-/-}$ cells under basal conditions and an increase in apoE secretion in *Abcg1*$^{-/-}$ cells (Figure 5), as expected (25, 32). ApoE secretion was profoundly decreased in *Abca1*$^{-/-}$*Abcg1*$^{-/-}$ cells compared with control and *Abcg1*$^{-/-}$ cells and was even significantly lower than in *Abca1*$^{-/-}$ cells (Figure 5). These effects were posttranscriptional: apoE mRNA was not changed in *Abcg1*$^{-/-}$ cells and slightly increased in *Abca1*$^{-/-}$ and *Abca1*$^{-/-}$*Abcg1*$^{-/-}$ macrophages compared with WT cells (data not shown). Cell lysates from *Abca1*$^{-/-}$ and *Abca1*$^{-/-}$*Abcg1*$^{-/-}$ macrophages showed amounts of apoE protein similar to those of WT cells, consistent with a defect in secretion rather than intracellular apoE degradation as the underlying cause (Figure 5). These findings suggest that the increase in apoE secretion in *Abcg1*$^{-/-}$ cells is secondary to induction of ABCA1 and indicate a possible role of ABCG1 in supporting residual apoE secretion in *Abca1*$^{-/-}$ cells. The profound defect in apoE secretion in *Abca1*$^{-/-}$*Abcg1*$^{-/-}$ macrophages likely contributes to the increased atherosclerosis in *Abca1*$^{-/-}$*Abcg1*$^{-/-}$ BM recipients.

Figure 4
Cellular cholesterol mass in peritoneal macrophages from WT, *Abcg1*$^{-/-}$, *Abca1*$^{-/-}$, and *Abca1*$^{-/-}$*Abcg1*$^{-/-}$ macrophages. Thioglycollate-elicited macrophages were harvested from WT, *Abcg1*$^{-/-}$, *Abca1*$^{-/-}$, and *Abca1*$^{-/-}$*Abcg1*$^{-/-}$ mice fed standard chow (**A**) or high-cholesterol diet for 2 weeks (**B**). After a 1-hour incubation at 37°C, nonadherent cells were removed and adherent cells consisting of macrophages were directly used to estimate cellular cholesterol and cholesteryl ester mass content by gas-liquid chromatography. TC, total cholesterol; FC, free cholesterol; CE, cholesteryl esters. Values are mean ± SEM. *$P < 0.05$ versus WT. §$P < 0.05$ versus single-KOs.

Increased secretion of inflammatory cytokines and chemokines by Abca1$^{-/-}$*Abcg1*$^{-/-}$ *macrophages.* In order to understand the increased infiltration of inflammatory cells in the tissues of *Abca1*$^{-/-}$*Abcg1*$^{-/-}$ mice, we measured the secretion of inflammatory cytokines and



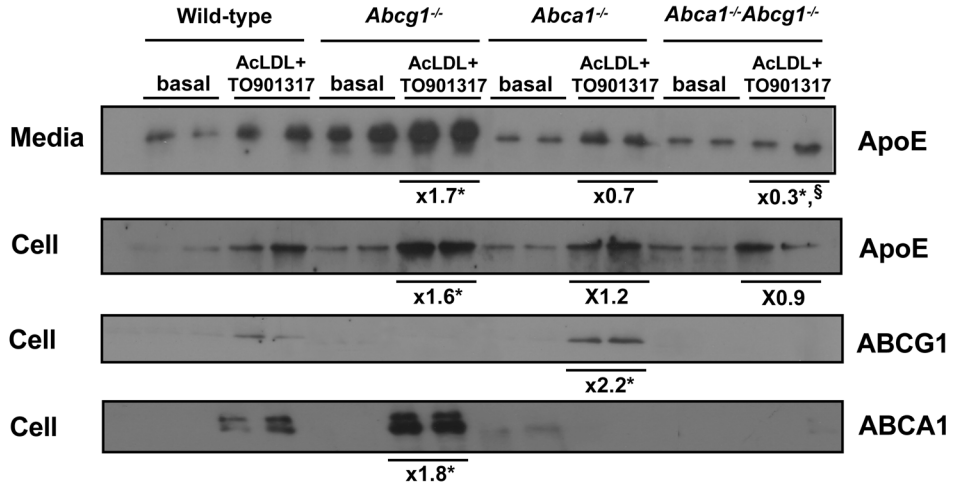


Figure 5
Western blots showing ApoE protein in media and cell lysates of peritoneal macrophages derived from WT, *Abcg1*$^{-/-}$, *Abca1*$^{-/-}$, and *Abca1*$^{-/-}$*Abcg1*$^{-/-}$ mice. Thioglycollate-elicited peritoneal macrophages were loaded with 50 μg/ml acLDL and treated with LXR activator (3 μmol/l T0901317) for 24 h and then incubated for 16 h in DMEM plus 0.2% BSA. Equivalent volumes of media normalized for cellular protein levels and equal amounts of cellular proteins (20 μg) from each sample were used for western blot analysis. Numbers below blots indicate change in KO cells compared with loaded control cells from 3 independent experiments. **P* < 0.05 versus WT. §*P* < 0.05 versus single KOs.

chemokines into media by freshly isolated peritoneal macrophages not treated with exogenous inflammatory stimuli during 6 h in cell culture. This showed increased secretion of a variety of inflammatory cytokines and chemokines in *Abca1*$^{-/-}$*Abcg1*$^{-/-}$ macrophages, notably TNF-α, IL-6, IL-1β, and IL-12 as well as MIP-1α, MIP-2, growth-regulated oncogene α, and, to a lesser extent, MCP-1 (Figure 6). There was also significantly increased secretion of some inflammatory cytokines and chemokines in *Abcg1*$^{-/-}$ cells (i.e., TNF-α, IL-1β, IL-12, MIP-1α, MIP-2, and MCP-1), but, except for a slight increase in MIP-2 and IL-12, no major difference in *Abca1*$^{-/-}$ cells (Figure 6). The increased secretion of inflammatory cytokines and chemokines paralleled mRNA expression changes as determined by real-time PCR (data not shown) and likely explains the increased tissue inflammatory responses in *Abca1*$^{-/-}$*Abcg1*$^{-/-}$ BM recipients.

Increased apoptosis after loading with free cholesterol or oxidized LDL in Abca1$^{-/-}$*Abcg1*$^{-/-}$ *macrophages.* The *Abca1*$^{-/-}$*Abcg1*$^{-/-}$ BM recipients showed increased numbers of apoptotic macrophages in the heart and atherosclerotic lesions. Apoptosis of macrophages in atherosclerotic lesions involves different mechanisms and may be brought about by accumulation of oxysterols derived from oxidized LDL or by accumulation of free cholesterol related to uptake of modified LDL and a failure of ACAT-mediated cholesterol esterification (34, 35). In order to understand the accumulation of apoptotic cells in heart and atherosclerotic lesions, we compared the apoptotic responses of macrophages of the 4 different genotypes using oxidized LDL or a standardized free cholesterol loading procedure (acLDL plus ACAT inhibitor 58035) in the presence or absence of HDL (Figure 7). Free cholesterol–induced apoptosis was dramatically increased in *Abca1*$^{-/-}$*Abcg1*$^{-/-}$ macrophages compared with cells of the other genotypes (Figure 7A). Apoptosis induced by oxidized LDL was also markedly increased in *Abca1*$^{-/-}$*Abcg1*$^{-/-}$ and *Abcg1*$^{-/-}$ cells, but not in cells of the other genotypes (Figure 7B). Treatment with HDL ameliorated apoptotic responses induced by oxidized LDL in WT and *Abca1*$^{-/-}$ cells, but not in *Abcg1*$^{-/-}$ or *Abca1*$^{-/-}$*Abcg1*$^{-/-}$ macrophages. The enhanced susceptibility of *Abca1*$^{-/-}$*Abcg1*$^{-/-}$ macrophages to cholesterol- and oxysterol-induced apoptosis likely explains the in vivo findings of increased apoptotic cells in the inflammatory infiltrate in the heart and atherosclerotic lesions.

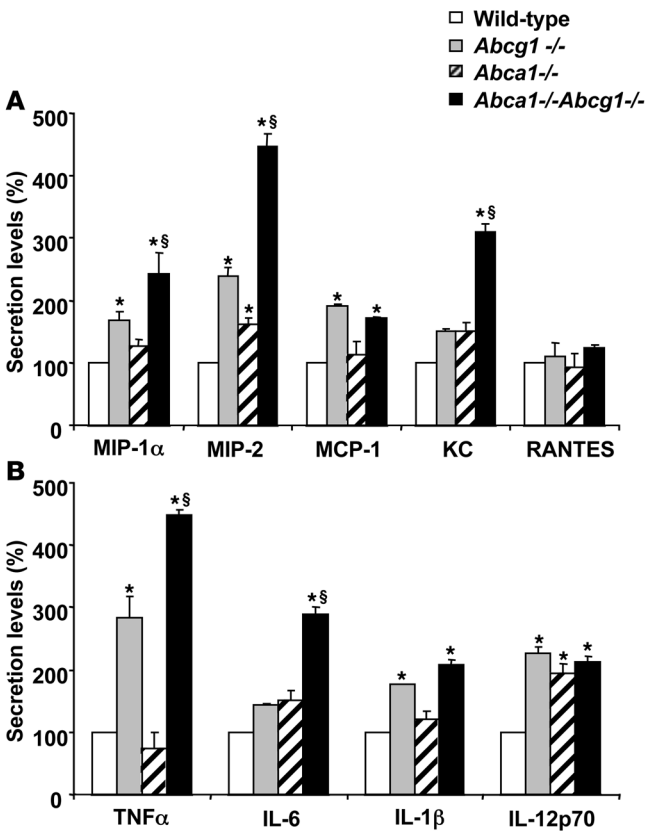


Figure 6
Inflammatory and chemokine gene expression in WT, *Abcg1*$^{-/-}$, *Abca1*$^{-/-}$, and *Abca1*$^{-/-}$*Abcg1*$^{-/-}$ macrophages. Peritoneal macrophages were harvested from WT, *Abcg1*$^{-/-}$, *Abca1*$^{-/-}$, and *Abca1*$^{-/-}$*Abcg1*$^{-/-}$ mice fed high-cholesterol diet for 2 weeks. After a 1-h incubation at 37°C, nonadherent cells were removed and adherent cells consisting of macrophages were incubated in 0.2% BSA DMEM. After 6 h, media were used for secretion analysis. (**A**) Secretion of chemokines MIP-1α, MIP-2, and MCP-1. KC, growth-regulated oncogene α. (**B**) Secretion of inflammatory cytokines IL-6, TNF-α, and IL-1β. Secretion levels were normalized to cellular protein amount and expressed as a percentage of WT. Values are mean ± SEM. **P* < 0.05 versus WT. §*P* < 0.05 versus single-KOs.

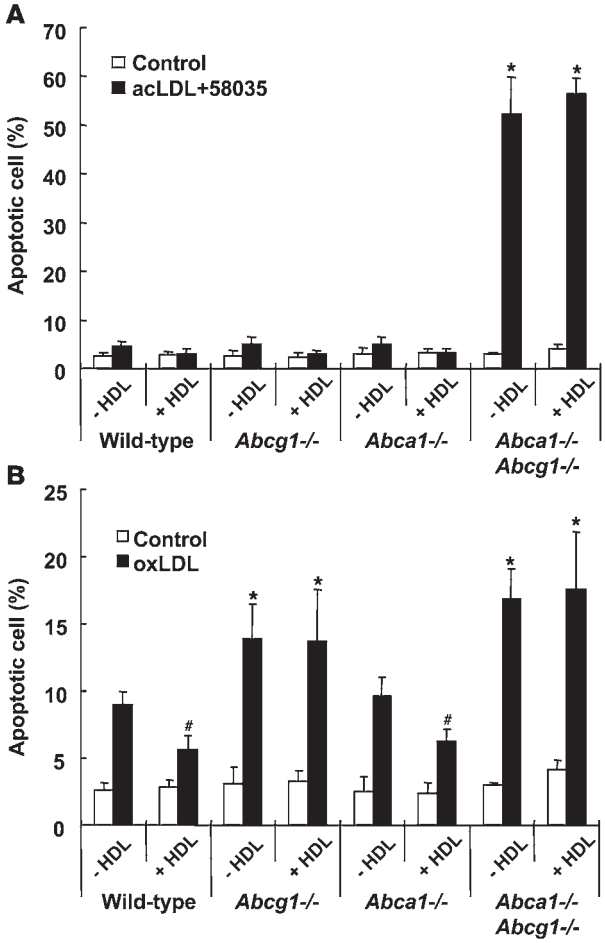


Figure 7

Apoptosis of peritoneal macrophages after loading with free cholesterol loading or oxidized LDL. Peritoneal macrophages were cultured in DMEM plus 0.2% BSA containing 100 μg/ml acLDL plus ACAT inhibitor (compound 58035; **A**) or 100 μg/ml Cu-oxidized LDL (oxLDL; **B**) for 24 h in the presence or absence of 100 μg/ml human HDL. Apoptosis of macrophages was determined by annexin V staining. Results are mean ± SEM. $^{*}P < 0.05$, genotype effect. $^{\#}P < 0.05$, HDL effect.

Discussion

Our studies suggest that the combined activities of ABCA1 and ABCG1 in macrophages are central to the antiatherogenic effects of HDL and also account, at least in part, for some of the associated mechanisms that have been considered as potential atheroprotective properties of HDL. Together these transporters have a synergistic role mediating macrophage cholesterol efflux and protecting against macrophage foam cell formation, inflammatory cell infiltration, apoptosis, and atherosclerosis. Because ABCA1 and ABCG1 are both targets of LXR transcription factors (36, 37), when one transporter is deficient, the other is upregulated as a result of sterol accumulation and LXR activation. The role of the individual transporters in these effects is masked by mutual compensation, likely explaining some of the mixed results of previous studies (19–26) and also why individual deficiencies of ABCA1 and ABCG1 produce relatively modest phenotypes.

The elucidation of ABCA1 as the defective molecule in Tangier disease (15–17), explaining the underlying defect in cholesterol and phospholipid efflux from cells to apoA-1 (12, 13), was a major advance supporting the idea that macrophage cholesterol efflux was central to the antiatherogenic effects of HDL (1, 38). The lack of a dramatic atherosclerosis phenotype in Tangier disease patients and in *Abca1*$^{-/-}$ mice has traditionally been explained by the low levels of VLDL- and LDL-cholesterol that accompany markedly reduced HDL levels. Our findings suggest that another factor is likely to be the compensatory upregulation of ABCG1 in *Abca1*$^{-/-}$ macrophages. Even though *Abca1*$^{-/-}$ mice have very low HDL levels, ABCG1 can support cholesterol efflux to other lipoprotein particles (14, 23), and our present studies revealed a further role of ABCG1 in supporting residual apoE secretion and cholesterol efflux to apoA-1 and apoE in *Abca1*$^{-/-}$ cells.

Tangier disease cells have relatively normal cholesterol efflux to HDL (39), and ABCA1 is almost inactive in mediating cholesterol efflux to plasma HDL particles (13, 14); thus activity of ABCA1 could not readily account for the inverse relationship between HDL levels and atherosclerosis observed in epidemiological studies. The idea that there might be an LXR-activated active transport pathway for cholesterol involving HDL particles (40) led to the discovery that ABCG1 could promote net efflux of cholesterol from macrophages to HDL particles (14, 23). Disappointingly, however, while *Abcg1*$^{-/-}$ mice accumulated foam cells in lungs and other tissues (23), 2 laboratories found that ABCG1 deficiency in BM-derived cells actually protected mice from atherosclerosis (25, 26) and another found that enhanced expression of ABCG1 in *Ldlr*$^{-/-}$ mice increased atherosclerosis (41). The results of the present study confirmed that atheroprotection in the former studies (25, 26) is related to compensatory upregulation of ABCA1 in *Abcg1*$^{-/-}$ BM recipients. Along with recent studies of in vivo macrophage reverse cholesterol transport (30), our findings indicate additive or synergistic effects of ABCA1 and ABCG1 in protecting against macrophage foam cell formation and atherosclerosis. The enhanced sterol accumulation in *Abca1*$^{-/-}$*Abcg1*$^{-/-}$ macrophages led to markedly increased inflammatory and chemokine gene expression and secretion and profoundly increased apoptotic responses, explaining the dramatic in vivo phenotype.

We measured cholesterol efflux from macrophages to isolated HDL or to serum and documented an additive or synergistic role of ABCA1 and ABCG1 in mediating macrophage cholesterol efflux. In the presence of HDL there was still a considerable level of residual mass and isotopic efflux that was not explained by these 2 transporters (Figure 3). A number of other pathways of macrophage cholesterol efflux to HDL particles have been described. These include passive or diffusional cholesterol efflux and SR-BI–mediated cholesterol efflux. These 2 pathways only result in net cholesterol efflux when HDL composition is altered such that there is a relative depletion in unesterified cholesterol relative to phospholipids, for example as may occur with lecithin cholesterol acyltransferase–mediated (LCAT-mediated) cholesterol esterification in HDL. SR-BI is expressed at low levels in murine macrophages, and SR-BI$^{-/-}$ macrophages show no defect in net cholesterol efflux to HDL (42, 43) or in vivo reverse cholesterol transport (30). Moreover, in contrast to synergistic effects on cholesterol efflux resulting from coexpression of ABCA1 and ABCG1 (44, 45), when ABCA1 and SR-BI are coexpressed in the same cell there is inhibition of ABCA1-mediated cholesterol efflux to apoA-1, as a result of a futile cycle of cholesterol efflux to apoA-1, followed by reuptake into the cell via SR-BI (46). These studies speak against a

significant role of SR-BI in macrophage cholesterol efflux. However, a number of studies have shown an increase in atherosclerosis in *apoE*$^{-/-}$ or *Ldlr*$^{-/-}$ mice transplanted with SR-BI–deficient BM, and a potential in vivo role of SR-BI in macrophage cholesterol efflux cannot be definitively excluded.

A role for macrophage ABCA1 in promoting macrophage apoE secretion has been documented in several studies (25, 31, 32), and in the present study we confirm that induction of ABCA1 in *Abcg1*$^{-/-}$ cells is responsible for their increased apoE secretion (25). Moreover, compared with *Abca1*$^{-/-}$ cells, *Abca1*$^{-/-}$*Abcg1*$^{-/-}$ macrophages showed a further decrease in cholesterol efflux to lipid-poor apoA-1 or apoE, in spontaneous cholesterol efflux (Figure 3), and in apoE secretion (Figure 5). These observations provide a hint that ABCG1 has additional cellular activities beyond its ability to promote cholesterol efflux to lipoprotein particles, which might be involved in supporting apoE and cholesterol secretion. The profound decrease in apoE secretion in *Abca1*$^{-/-}$*Abcg1*$^{-/-}$ macrophages is likely an important factor in accelerated atherosclerosis in *Abca1*$^{-/-}$*Abcg1*$^{-/-}$ BM recipients. Macrophage apoE deficiency has marked proatherogenic consequences (29), which are thought to reflect antiinflammatory effects of macrophage apoE secretion as well as the role of macrophage apoE in cholesterol efflux (47).

There was a marked accumulation of apoptotic cells, most likely macrophages, in the heart and atherosclerotic lesions of *Abca1*$^{-/-}$*Abcg1*$^{-/-}$ BM recipients. In an earlier study *Abcg1*$^{-/-}$ macrophages were shown to have increased susceptibility to apoptosis induced by oxidized LDL, and lesions contained increased numbers of TUNEL-positive cells (26). Recently, we have shown that this reflects a specific role of ABCG1 in mediating efflux of 7-ketocholesterol and related oxysterols from macrophages to HDL (48). The decrease in atherosclerosis was attributed to increased apoptosis of macrophages in early lesions (26), because some mutations that increase macrophage apoptosis result in smaller foam cell lesion formation (49). In contrast, in the present study an increase in apoptosis in *Abca1*$^{-/-}$*Abcg1*$^{-/-}$ BM recipients was associated with increased lesion formation. This does not signify that conclusions from the earlier study (26) were necessarily incorrect, because multiple factors such as decreased cholesterol efflux, decreased apoE secretion, and increased inflammatory gene expression could have been causative for increased atherosclerosis in the *Abca1*$^{-/-}$*Abcg1*$^{-/-}$ BM recipients. However, it is important to point out that macrophage apoptosis has been associated with the critical clinical events associated with atherosclerosis, i.e., plaque rupture and thrombus formation. Our findings suggest that ABCA1 and ABCG1 may have an important role in suppressing macrophage apoptosis in advanced atherosclerotic plaques and protecting against critical plaque complications.

A striking finding was the massive accumulation of foam cells and, to a lesser extent, neutrophils in the heart. HDL has been shown to have antiinflammatory properties, and these have been proposed to underlie antiatherogenic effects of HDL (4). We documented increased secretion of a variety of inflammatory cytokines and chemokines in freshly isolated peritoneal macrophages from *Abca1*$^{-/-}$*Abcg1*$^{-/-}$ mice. MIP-1α and MIP-2 belong to the 2 best-understood classes of chemokines, namely the CXC and CC families, and are potent neutrophil and macrophage chemokines (50). Increased TNF-α, IL-6, and IL-1β secretion from *Abca1*$^{-/-}$*Abcg1*$^{-/-}$ foam cells is likely to lead to induction of genes such as VCAM-1 and ICAM-1 in lesional endothelial cells, leading also to increased recruitment of monocytes and possibly neutrophils into lesions. Previous studies have documented increased expression of inflammatory and chemokine genes as a result of loading of unesterified cholesterol or oxysterols in monocytes and macrophages (35, 51). Together with the efflux and sterol accumulation data, our findings suggest that increased secretion of inflammatory cytokines and chemokines is secondary to sterol accumulation in *Abca1*$^{-/-}$*Abcg1*$^{-/-}$ macrophages. Increased foam cell accumulation in the spleen was also found in *Abca1*$^{-/-}$*Abcg1*$^{-/-}$ BM recipients. It is not clear why foam cells accumulate in certain tissues but not others. Interestingly, *Abcg1*$^{-/-}$ BM recipients showed foci of mixed inflammatory cells in the lung, as reported previously (26). However, this phenotype was not increased further in *Abca1*$^{-/-}$*Abcg1*$^{-/-}$ BM recipients. This could be related to the specific role of ABCG1 in promoting efflux of oxysterols from cells (48) and our present finding that oxidized LDL-induced apoptosis occurred at fairly similar levels in *Abcg1*$^{-/-}$ and *Abca1*$^{-/-}$*Abcg1*$^{-/-}$ macrophages. These findings suggest that ABCG1 has a special role in protecting lung cells from damage mediated by oxidative modification of sterols.

Recently, there has been intense interest in the development of novel therapies directed at HDL, such as CETP inhibitors, niacin with flushing inhibitors, infusion of apoA-1 or apoA-1–like peptides, and LXR activators (52, 53). Our studies suggest that the interaction of HDL and its apolipoproteins with ABCA1 and ABCG1 in macrophages is the key to their antiatherogenic properties, involving relatively small changes in sterol pools in specific inflammatory cells, and that such atheroprotective effects probably do not require an overall stimulation of reverse cholesterol transport to the liver. Treatment with niacin or CETP inhibitors leads to increased HDL, apoA-1, and cholesterol levels and is associated with prominent accumulation of large HDL-2 type particles. Importantly, large apoE- and LCAT-rich HDL particles accumulating in genetic CETP deficiency or in subjects treated with CETP inhibitors have increased ability to promote cholesterol efflux from macrophages via ABCG1 (54). While the CETP inhibitor torcetrapib failed in a recent clinical trial, this may have been related to underdosing and off-target toxicity (55). Our results confirmed the role of ABCG1 in protecting against atherosclerosis and were consistent with the idea that enhancing macrophage sterol efflux via ABCG1 could have antiatherogenic consequences. While our studies suggest that the beneficial effects of increasing sterol efflux via ABCG1 could be partly offset by decreased ABCA1 expression, it is interesting to note that ABCG1 appears to have a unique role in protecting against macrophage apoptosis induced by oxidized LDL (48) and in suppressing inflammatory and chemokine gene expression (Figure 6). Thus, increasing HDL by niacin or CETP inhibitors could have antiinflammatory and antiapoptotic effects in advanced atherosclerotic lesions, and perhaps in some inflammatory lung diseases, as a result of promotion of cholesterol and oxysterol efflux via ABCG1. In principle, strategies that increase macrophage sterol efflux by upregulating both ABCA1 and ABCG1, or by increasing overall levels of particles or peptides that increase efflux via both transporters, could ultimately prove to be the most effective therapies for increasing macrophage sterol efflux and decreasing atherosclerosis.

Methods

Mice. Abca1$^{-/-}$ mice in DBA background were obtained from The Jackson Laboratory, and *Abcg1*$^{-/-}$ backcrossed into the C57BL/6 background were originally purchased from DeltaGen. Because we were unable to breed mice with combined deficiency in the C57BL/6 background, we bred them in a mixed C57BL/6 × DBA background and transplanted BM from these animals into the genetically uniform F1 generation obtained by crossing

C57BL/6 *Ldlr*$^{-/-}$ mice with DBA mice (The Jackson Laboratory). The malformation of the placenta in C57BL/6 *Abca1*$^{-/-}$ mice resulting in severe embryo growth retardation, fetal loss, and neonatal death was most likely related to the difficulty of breeding the *Abca1*$^{-/-}$ trait into the C57BL/6 background (56). Animal protocols were approved by the Institutional Animal Care and Use Committee of Columbia University. Animals had ad libitum access to both food and water.

BM transplantation. BM transplantation was performed as previously described (57). The atherosclerosis studies were conducted in moderately hyperlipidemic (Supplemental Table 1) F1 hybrid C57BL/6 × DBA *Ldlr*$^{+/-}$ mice fed high-fat/cholesterol/bile salt diet containing 1.25% cholesterol, 7.5% cocoa butter, and 0.5% cholic acid (TD 88051; Harlan Teklad). A similar approach has been used previously (58–60). Combined results of males and females were shown for each genotype: similar results were obtained with data divided by sex. Mice were allowed to recover for 5 weeks after irradiation and BM transplantation before diet studies were initiated.

Histological analysis of the myocardium and proximal aortas. The myocardium and proximal aortas were serially paraffin sectioned and stained with H&E as previously described (57). Aortic lesion size of each animal was calculated as the mean of lesion areas in 5 sections from the same mouse. Mac-3 and MCA771G antibodies (BD Biosciences — Pharmingen) were used to stain macrophages and neutrophils in paraffin sections, respectively. Apoptotic cells were detected using TUNEL assays (57).

Isolation of HDL from plasma. HDLs (density, 1.063–1.21 g/ml) were isolated by preparative ultracentrifugation from normolipidemic human plasma and stored in PBS containing 1 mM EDTA. ApoB-containing particles was precipitated from serum by adding 100 μl serum to 40 μl 20% polyethyleneglycol (PEG; P-2139 in 200 mM glycine, pH 10; Sigma-Aldrich) solution. This mixture was incubated at room temperature for 15 min. After this incubation, the solution was centrifuged at 1,900 *g* for 20 min. The supernatant, containing HDL fractions, was removed and used for experiments.

Isolation of mouse peritoneal macrophages. Fresh peritoneal macrophage cells were harvested from WT, *Abcg1*$^{-/-}$, *Abca1*$^{-/-}$, and *Abca1*$^{-/-}$*Abcg1*$^{-/-}$ mice 3 days after receiving an i.p. injection of thioglycollate and plated in 10% FBS in DMEM as previously described (25). After a 1-h incubation at 37°C, nonadherent cells were removed and adherent cells consisting of macrophages were used for the experiment as described in the Figure 6 legend.

Isotopic cholesterol efflux assay. Thioglycollate-elicited macrophages were cultured for 24 h in DMEM plus 0.2% BSA containing 50 μg/ml acLDL, 3 μmol/l T0901317, and 2 μCi/ml of [^{3}H]-cholesterol. Cholesterol efflux was performed for 6 h in 0.2% BSA DMEM containing different acceptors as described in the Figure 3 legend. The cholesterol efflux was expressed as the percentage of the radioactivity released from the cells in the medium relative to the total radioactivity in cells plus medium (14).

Cholesterol mass analysis. Thioglycollate-elicited macrophages were first incubated in 0.2% BSA DMEM with LXR agonist (T0901317, 3 μmol/l) and with 50 μg/ml acLDL for 24 h. Then, cholesterol efflux was performed for 6 h in DMEM containing 0.2% BSA in presence or absence of HDL (50 μg protein/ml). After incubation with HDL, the lipid fractions were extracted from the collected media with hexane or from cells using hexane/isopropanol (3:2, vol/vol) in presence of stigmasterol (5 μg per sample) added as the internal standard. Total cholesterol was determined after saponification by gas-liquid chromatography. The HDL-mediated net cholesterol efflux was calculated as the difference of cholesterol mass of the medium cultured with and without cells (14). The cholesterol mass in cells was determined from briefly cultured macrophages harvested from mice fed standard chow or high-cholesterol diet for 2 weeks.

Western blot analysis. The expression of ABCG1, ABCA1, and apoE were measured in peritoneal macrophage cells by Western blot analysis. Primary antibodies for ABCG1, ABCA1, and apoE were purchased from Santa Cruz Biotechnology Inc., Abcam, and Biodesign, respectively.

Secretion analysis. Mouse peritoneal macrophages were harvested and cultured for 6 h in DMEM containing 0.2% BSA. Cytokines and chemokines were measured by Luminex assay (Cytokine Core Laboratory).

Apoptosis analysis. Macrophages were incubated in 0.2% BSA supplemented with acLDL (100 μg/ml) plus ACAT inhibitor (compound 58035) or Cu-oxidized LDL (100 μg/ml) for 24 h with or without HDL (100 μg/ml). Apoptosis of macrophages was determined by annexin V staining as previously described (57). Free cholesterol–induced apoptosis was not prominent in WT macrophages because we used thioglycollate-elicited macrophages in these experiments (I. Tabas, personal communication).

Statistics. Data are shown as mean ± SEM. Comparison of mean values between groups was evaluated by 2-tailed Student's *t* test or ANOVA with square root–transformed data. A *P* value less than 0.05 was considered significant.

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