Impaired Development of Atherosclerosis in Hyperlipidemic \textit{Ldlr}^{-/-} and \textit{ApoE}^{-/-} Mice Transplanted With \textit{Abcg1}^{-/-} Bone Marrow

Ángel Baldán, Liming Pei, Richard Lee, Paul Tarr, Rajendra K. Tangirala, Michael M. Weinstein, Joy Frank, Andrew C. Li, Peter Tontonoz, Peter A. Edwards

\textbf{Objective}—The lungs of \textit{Abcg1}^{-/-} mice accumulate macrophage foam cells that contain high levels of unesterified and esterified cholesterol, consistent with a role for ABCG1 in facilitating the efflux of cholesterol from macrophages to high-density lipoprotein (HDL) and other exogenous sterol acceptors. Based on these observations, we investigated whether loss of ABCG1 affects foam cell deposition in the artery wall and the development of atherosclerosis.

\textbf{Methods and Results}—Bone marrow from wild-type or \textit{Abcg1}^{-/-} mice was transplanted into \textit{Ldlr}^{-/-} or \textit{ApoE}^{-/-} mice. After administration of a high-fat/high-cholesterol diet, plasma and tissue lipid levels and atherosclerotic lesion size were quantified and compared. Surprisingly, transplantation of \textit{Abcg1}^{-/-} bone marrow cells resulted in a significant reduction in lesion size in both mouse models, despite the fact that lipid levels increased in the lung, spleen, and kidney. Lesions of \textit{Ldlr}^{-/-} mice transplanted with \textit{Abcg1}^{-/-} cells contained increased numbers of apoptotic cells. Consistent with this observation, in vitro studies demonstrated that \textit{Abcg1}^{-/-} macrophages were more susceptible to oxidized low-density lipoprotein (ox-LDL)-dependent apoptosis than \textit{Abcg1}^{+/+} cells.

\textbf{Conclusions}—Diet-induced atherosclerosis is impaired when atherosclerotic-susceptible mice are transplanted with \textit{Abcg1}^{-/-} bone marrow. The demonstration that \textit{Abcg1}^{-/-} macrophages undergo accelerated apoptosis provides a mechanism to explain the decrease in the atherosclerotic lesions. (\textit{Arterioscler Thromb Vasc Biol}. 2006;26:2301-2307.)

\textbf{Key Words:} ABCG1 ■ atherosclerosis ■ cholesterol ■ LXR ■ macrophage

Elevated blood levels of low-density lipoprotein (LDL) result in enhanced entry of LDL into the arterial subendothelial space, where it can become trapped and oxidized to generate oxidized LDL (ox-LDL) and aggregated ox-LDL.\textsuperscript{1-3} Ox-LDL contains bioactive lipids that stimulate endothelial cells and macrophages to secrete numerous cytokines that promote the entry of circulating monocytes into the subendothelial space where they differentiate into macrophages and take up oxidized and/or aggregated LDL.\textsuperscript{1-3} These lipido-loaded “foam cells,” containing multiple cytoplasmic cholesterol ester lipid droplets, are found in both early fatty streaks and more advanced atherosclerotic lesions.\textsuperscript{1-3} The latter often contain necrotic cores, extracellular lipids that include cholesterol crystals, and even calcified tissue.\textsuperscript{1-3}

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Numerous studies have led to the concept that the liver-X-receptor/retinoid-X-receptor (LXR/RXR) heterodimeric nuclear receptor complex functions as a “cellular sterol sensor.”\textsuperscript{4-6} In addition, oxysterols present in macrophage foam cells have been shown to activate LXR and alter gene expression. LXR target genes that are induced affect many pathways including apoptosis (AIM\textsuperscript{2}L) and lipid homeostasis (SREBP-1c, FAS, ApoE, Apo-CII, CETP, LPL, ABCA1, and ABCG1).\textsuperscript{4-6} ABCG1 and ABCA1, two LXR target genes that are highly induced as macrophages convert to foam cells,\textsuperscript{8-10} are members of the ATP-binding cassette (ABC) superfamily of transmembrane transporters.\textsuperscript{11} Both transporters promote the efflux of lipids to specific exogenous acceptors; in vitro studies that used cells that either overexpressed ABCG1\textsuperscript{12-14} or lacked ABCG1,\textsuperscript{15} led to the proposal that ABCG1 stimulates the efflux of cellular cholesterol to high-density lipoprotein (HDL), phospholipid vesicles, or phospholipid/apo-AI complexes, whereas ABCA1 mediates the efflux of cellular phospholipids and cholesterol to lipid-poor apoproteins (apo-AI, apo-E, apo-CII). Whether these two transporters function in independent pathways or whether they function in concert to mediate cholesterol efflux is currently unknown. Nonetheless, the cumulative evidence suggests that both ABCA1 and ABCG1 play essential roles in macrophage lipid metabolism.

Interestingly, atherosclerotic lesions were not observed when \textit{Abca1}^{-/-} mice were fed chow or an atherogenic diet for
many months. In addition, compared with the control mice, lesion size was not increased in ApoE−/−/Abacl−/− or Ldlr−/−/Abacl−/− mice. Nonetheless, support for a role of ABCA1 in the development of atherosclerosis has come from bone marrow transplantation studies; transplantation of Abca1−/− or ApoE−/−/Abca1−/− cells into Ldlr−/−, or ApoE−/− mice, respectively, followed by administration of a high-fat diet resulted in a 50% to 60% increase in atherosclerotic lesions.

In contrast to ABCA1, the physiological importance and function of ABCG1 is not well understood and its role in atherosclerosis has not been reported. Studies with both Abcg1−/−/LacZ knock-in mice and human ABCG1 transgenic mice revealed the critical importance of ABCG1 in controlling intracellular lipid homeostasis. These studies demonstrated that administration of a high-fat/high-cholesterol diet to Abcg1−/− mice resulted in the accumulation of both unesterified and esterified cholesterol in the artery wall and increased atherosclerosis. We report here studies that were performed to test this hypothesis.

Methods

Animals and Bone Marrow Transplants

Female C57Bl/6 ApoE−/− and Ldlr−/− mice were obtained from Jackson Laboratories (Bar Harbor, Me). Abcg1−/−/LacZ knock-in mice, on a C57Bl/6 background, were generated and maintained as described. Abcg1−/− mice and their wild-type littermates were fed the development of atherosclerosis, we performed bone marrow transplantation studies; transplantation of Abca1−/− or ApoE−/−/Abca1−/− cells into Ldlr−/−, or ApoE−/− mice, respectively, followed by administration of a high-fat diet resulted in a 50% to 60% increase in atherosclerotic lesions.

ABCG1. Pulmonary macrophages were particularly sensitive to loss of ABCG1. Based on these previous studies with Abcg1−/− mice, we hypothesized that deletion of Abcg1 would result in the accumulation of macrophage foam cells in the artery wall and increased atherosclerosis. We report here studies that were performed to test this hypothesis.

Results

Inactivation of the Mouse Abcg1 Gene Does Not Affect the Development of Atherosclerosis

The finding that cholesterol-loaded macrophage foam cells accumulate in the lungs of Abcg1−/− mice suggested that loss of ABCG1 expression might also result in both the accumulation of foam cells in the artery wall and accelerated atherosclerosis. To test this hypothesis, Abcg1−/−/LacZ knock-in mice and their wild-type C57Bl/6 litter mates were fed an atherosclerotic diet (21% fat, 1.25% cholesterol, 0.5% cholate) for 19 weeks. All mice exhibited similar increases in plasma lipid levels and in lipid deposition in the liver (data not shown). However, because none of the mice developed significant atherosclerotic lesions in the aortic arch (data not shown), we concluded that loss of ABCG1 per se is not sufficient to promote atherosclerosis.

As an alternative approach to assess the role of ABCG1 in the development of atherosclerosis, we performed bone marrow transplant into genetically engineered mice (Ldlr−/− and ApoE−/−) that display altered lipoprotein profiles and accelerated atherosclerosis in response to a high-fat high-cholesterol (HF/HC) diet.

Bone Marrow Transplants into Ldlr−/− Mice

Irradiated Ldlr−/− mice were transfused with bone marrow cells derived from either wild-type or Abcg1−/− mice. After a 4-week recovery period, mice were fed a HF/HC diet for 16 weeks. At the conclusion of the experiment, no significant differences in body weights were observed between mice transplanted with wild-type or Abcg1−/− bone marrow cells (Table 1). As expected, the HF/HC diet resulted in severe hypercholesterolemia and hypertriglyceridemia (Table 1). However, although total plasma lipid levels in Ldlr−/− mice were independent of the genotype of the transplanted bone marrow (Table 1), HDL cholesterol levels were decreased by 20% in mice that had received Abcg1−/− cells (Table 1). The reduced HDL levels were unexpected as previous studies have shown that plasma HDL levels do not differ between wild-type and Abcg1−/− bone marrow cells. The mechanism by which Abcg1−/− bone marrow cells promote a decrease in plasma HDL is unknown at this time.

Necropsy of the HF/HC-fed recipient Ldlr−/− mice revealed that, as compared with wild-type→Ldlr−/−, the lungs,
spleen and, to a lesser extent, liver and kidney of the Abcg1<sup>−/−</sup>→Ldlr<sup>−/−</sup> mice were paler in appearance and/or contained pale foci (Figure 1). Quantification of tissue lipid levels revealed that unesterified and/or esterified cholesterol accumulated to higher levels in the lungs, spleens, and kidneys of Ldlr<sup>−/−</sup> mice receiving Abcg1<sup>−/−</sup>, as compared with wild-type bone marrow (Table 2). The lungs of the mice transplanted with Abcg1<sup>−/−</sup> cells also exhibited an altered architecture, subpleural macrophage accumulation, the presence of numerous multinucleated giant cells, lymphocytic infiltration, and oil red O-positive lipid droplets (Figure 2F versus 2E; 2H versus 2G). The extrafollicular regions of the spleen and secretory tubules of the kidney also stained positive with oil red O after Abcg1<sup>−/−</sup>→Ldlr<sup>−/−</sup> transplantation (Figure 2L versus 2K; 2P versus 2O). Analysis of hematoxylin and eosin (H&E) stained spleen and kidney sections indicated that these tissues are histologically normal (Figure 2I versus 2J; 2M versus 2N). In contrast, the livers of all Ldlr<sup>−/−</sup> mice showed signs of steatosis, as judged both by staining with H&E (Figure 2A and 2B) or oil red O (Figure 2C and 2D), consistent with the elevated levels of cholesterol esters and triglycerides (Table 2). We conclude that lipid homeostasis of many tissues in hyperlipidemic mice is dependent on ABCG1 expression in bone marrow-derived cells.

To demonstrate that Abcg1<sup>−/−</sup>/LacZ bone marrow donor cells repopulate multiple tissues, we stained frozen tissue sections for β-galactosidase activity; LacZ-positive (and thus, Abcg1<sup>−/−</sup>) cells were identified in the liver (Kupffer cells), lung (macrophages and lymphocytes), spleen (macrophages and lymphocytes) and kidney (macrophages in glomeruli and distal collecting tubules) of Ldlr<sup>−/−</sup> recipient mice (supplemental Figure I, available online at http://atvb.ahajournals.org). As expected, LacZ-positive cells were not observed in tissues obtained from mice transplanted with wild-type bone marrow (supplemental Figure I).

The increased lipid content of several tissues of the Ldlr<sup>−/−</sup> mice (Table 2; Figures 1 and 2) prompted us to examine the pattern of expression of genes involved in lipid metabolism; supplemental Figure II shows that mRNA levels encoding Srebp-1c, Fas, Scd-1, and Hmgcr are decreased in the lungs of Abcg1<sup>−/−</sup>→Ldlr<sup>−/−</sup> mice, as compared with those mice that received wild-type cells. Srebp-1c and Hmgcr levels were also decreased in the livers of the Abcg1<sup>−/−</sup>→Ldlr<sup>−/−</sup> animals (supplemental Figure II). Expression of these same genes in the spleen and kidneys of the recipient Ldlr<sup>−/−</sup> mice were not affected by the genotype of the donor cells (data not shown). Thus, the results of Figures 1 and 2 and supplemental Figure II demonstrate that ABCG1 expression in bone marrow-derived cells is critical for the maintenance of lipid homeostasis in a number of tissues in mice that exhibit hyperlipidemia.

Finally, we quantified atherosclerotic lesions in Ldlr<sup>−/−</sup> mice. The extent of atherosclerosis was determined using oil red O-stained tissue sections of the aortic root, and en face analysis along the length of the aorta. Surprisingly, atherosclerotic lesions were reduced significantly in Ldlr<sup>−/−</sup> mice transplanted with Abcg1<sup>−/−</sup> bone marrow cells; lesion size in the aortic root and in the descending aorta of Abcg1<sup>−/−</sup>→Ldlr<sup>−/−</sup> mice were decreased 40% and 35%, respectively (Figure 3A through 3D). The reduction in lesions in Abcg1<sup>−/−</sup>→Ldlr<sup>−/−</sup> mice is unlikely a consequence of a decreased macrophage migration into the subendothelial space of the blood vessels because the aortic roots of Ldlr<sup>−/−</sup> mice infused with Abcg1<sup>−/−</sup> cells contained large and intensely oil red O-stained, LacZ-positive (Abcg1<sup>−/−</sup>) macrophage foam cells (Figure 3D, compare panels d versus c; h versus g).

**Bone Marrow Transplants into ApoE<sup>−/−</sup> Mice**

Previous studies have shown that both the size of atherosclerotic lesions and the level of hyperlipidemia in ApoE<sup>−/−</sup> mice are significantly attenuated when these mice are recipients of

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**TABLE 1. Plasma Lipid Levels in Ldlr<sup>−/−</sup> and ApoE<sup>−/−</sup> Mice Transplanted With Wild-Type or Abcg1<sup>−/−</sup> Bone Marrow**

<table>
<thead>
<tr>
<th>Donors</th>
<th>Wild-Type</th>
<th>Abcg1&lt;sup&gt;−/−&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Ldlr&lt;sup&gt;−/−&lt;/sup&gt; recipients</strong></td>
<td>(n=12)</td>
<td>(n=10)</td>
</tr>
<tr>
<td>Weight, g</td>
<td>23.5±1.1</td>
<td>22.5±0.9</td>
</tr>
<tr>
<td>Plasma lipids, mg/dL</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Triglycerides</td>
<td>152.5±29.0</td>
<td>164.4±29.1</td>
</tr>
<tr>
<td>Total cholesterol</td>
<td>1082.6±66.1</td>
<td>1042.6±75.0</td>
</tr>
<tr>
<td>HDL cholesterol</td>
<td>52.8±3.2</td>
<td>41.0±4.6*</td>
</tr>
<tr>
<td>Unsterified cholesterol</td>
<td>331.3±28.3</td>
<td>356.9±23.0</td>
</tr>
<tr>
<td>FFA</td>
<td>36.0±2.7</td>
<td>51.2±3.0*</td>
</tr>
<tr>
<td><strong>ApoE&lt;sup&gt;−/−&lt;/sup&gt; recipients</strong></td>
<td>(n=17)</td>
<td>(n=18)</td>
</tr>
<tr>
<td>Weight, g</td>
<td>18.8±0.2</td>
<td>17.9±0.2</td>
</tr>
<tr>
<td>Plasma lipids, mg/dL</td>
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<td></td>
</tr>
<tr>
<td>Triglycerides</td>
<td>27.4±2.8</td>
<td>21.6±2.4</td>
</tr>
<tr>
<td>Total cholesterol</td>
<td>129.1±8.8</td>
<td>122.2±7.6</td>
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<tr>
<td>HDL cholesterol</td>
<td>44.2±6.1</td>
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<td>35.2±2.4</td>
<td>33.1±2.0</td>
</tr>
<tr>
<td>FFA</td>
<td>24.9±0.9</td>
<td>24.9±1.0</td>
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* P<0.01.
Data are expressed as mean±SEM.
FFA indicates free fatty acid.

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**Figure 1. Severe lipid deposition in tissues from Abcg1<sup>−/−</sup>→Ldlr<sup>−/−</sup> mice.** Representative pictures of the livers, lungs, spleens, and kidneys of Ldlr<sup>−/−</sup> transplanted with either wild-type or Abcg1<sup>−/−</sup> bone marrow after 16 weeks on a HF/HC diet. These tissues from animals receiving Abcg1<sup>−/−</sup> cells were paler and/or showed pale foci (arrowheads) indicative of lipid accumulation.
Abcg1 expression of other LXR target genes such as unknown. These latter data suggested that loss of ABCG1 per transplanted (data not shown). The level of hyperlipidemia in profiles, as determined by fast protein liquid, were superim-posable (data not shown). The level of hyperlipidemia in the liver, lung and spleen of mice receiving (Table 2). As expected, LacZ-positive cells were observed in the liver, lung and spleen of mice receiving Abcg1−/− cells (data not shown).

Lipid deposition and/or histological architectural changes in the lungs and spleens of ApoE−/− mice were also observed after transplantation with Abcg1−/− mice; the lungs contained oil red O-positive macrophages, giant cells, and lymphocytic infiltrates, consistent with elevated lipid levels (Table 2 and data not shown). No such changes were observed in the lungs of mice transfused with wild-type cells (data not shown). In contrast, the livers of all HF/HC-fed transplanted ApoE−/− mice accumulated lipid (Table 2).

As expected, the atherosclerotic lesions in both the aortic root and descending aorta of ApoE−/− mice were much smaller than those present in Ldlr−/− mice (Figure 3E, and 3F versus 3A and 3B). This result is likely a consequence of the much lower plasma cholesterol levels in the ApoE−/−, as compared with Ldlr−/− mice (Table 1; ~125 versus ~1050 mg/dL of cholesterol). Nonetheless, en face analysis of the descending aorta demonstrated that lesions were reduced significantly, by ~60%, when ApoE−/− mice received Abcg1−/−, as compared with Abcg1+/+ bone marrow (Figure 3F; P<0.001). Although we also noted a decrease in lesion size in the aortic root of mice receiving Abcg1−/− as compared with wild-type cells, the values did not reach statistical significance (Figure 3E).

Taken together, the data from both Ldlr−/− and ApoE−/− mice demonstrate that Abcg1−/− monocytes retain the ability

<table>
<thead>
<tr>
<th>TABLE 2. Tissue Lipid Levels in Ldlr−/− and ApoE−/− Mice Transplanted With Wild-Type or Abcg1−/− Bone Marrow</th>
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<tbody>
<tr>
<td>Total Cholesterol</td>
</tr>
<tr>
<td>-------------------</td>
</tr>
<tr>
<td><strong>Ldlr−/− recipients</strong></td>
</tr>
<tr>
<td>Lung</td>
</tr>
<tr>
<td>Wild-type</td>
</tr>
<tr>
<td>Abcg1−/−</td>
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<tr>
<td>Liver</td>
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<tr>
<td>Wild-type</td>
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<tr>
<td>Abcg1−/−</td>
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<tr>
<td>Spleen</td>
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<tr>
<td>Wild-type</td>
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<tr>
<td>Abcg1−/−</td>
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<tr>
<td>Kidney</td>
</tr>
<tr>
<td>Wild-type</td>
</tr>
<tr>
<td>Abcg1−/−</td>
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<tr>
<td><strong>ApoE−/− recipients</strong></td>
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<tr>
<td>Lung</td>
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<td>Abcg1−/−</td>
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<td>Wild-type</td>
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<tr>
<td>Abcg1−/−</td>
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<tr>
<td>Spleen</td>
</tr>
<tr>
<td>Wild-type</td>
</tr>
<tr>
<td>Abcg1−/−</td>
</tr>
</tbody>
</table>

*P<0.01.
ND indicates not detectable.
Data (mg/g of tissue) are expressed as mean±SEM (n=4).

ApoE−/− bone marrow transplantation.23–25 Nonetheless, infusion of ApoE+/−/LxrαB−/− double knockout cells into ApoE−/− mice increased atherosclerosis ~2-fold.19 Whether this increase in lesion size was caused by low levels of ApoE expression in the LxrαB−/− macrophages or to reduced expression of other LXR target genes such as Abcg119 is unknown. These latter data suggested that loss of ABCG1 per se might increase atherosclerosis in ApoE−/− mice. To test this hypothesis, we infused ApoE−/− mice with either wild-type or Abcg1−/−-derived bone marrow cells and quantitated lesion development.

The data of Table 1 indicate that body weight and plasma lipid levels were similar in ApoE−/− mice receiving either wild-type or Abcg1−/− bone marrow. Plasma lipoprotein profiles, as determined by fast protein liquid, were superimposable (data not shown). The level of hyperlipidemia in transplanted ApoE−/− mice after 12 weeks on a HF/HC diet was relatively mild (Table 1), likely a result of a functional ApoE gene in the donor cells. Nonetheless, cholesterol and cholesterol ester levels were increased significantly in the lungs and spleens of ApoE−/− mice that had been infused with Abcg1−/− bone marrow, as compared with Abcg1+/+ cells (Table 2). As expected, LacZ-positive cells were observed in the liver, lung and spleen of mice receiving Abcg1−/− cells (data not shown).
to both enter the subendothelial space and differentiate into macrophage foam cells. However, the decreased lesions of Abcg1<sup>−/−</sup>→<i>Ldlr</i><sup>−/−</sup> and <i>Abcg1</i><sup>−/−</sup>→<i>ApoE</i><sup>−/−</sup> mice suggest that <i>Abcg1</i><sup>−/−</sup> macrophages may either exit the lesion or undergo some form of cell death.

**Abcg1<sup>−/−</sup>** Macrophages Undergo Increased Apoptosis In Vivo and In Vitro

The presence of enlarged oil red O-positive <i>Abcg1</i><sup>−/−</sup> macrophages in atherosclerotic lesions (Figure 3C and 3D) is consistent with cholesterol ester accumulation, noted previously in pulmonary <i>Abcg1</i><sup>−/−</sup> macrophages. We hypothesized that the decrease in atherosclerotic lesions in mice infused with <i>Abcg1</i><sup>−/−</sup> bone marrow may result from increased apoptosis of the macrophages in response to altered lipid metabolism.

To test this hypothesis, paraffin-embedded sections taken from atherosclerotic lesions from four individual wild-type→<i>Ldlr</i><sup>−/−</sup> and <i>Abcg1</i><sup>−/−</sup>→<i>Ldlr</i><sup>−/−</sup> mice were stained for TUNEL activity. The data of Figure 4A and 4B demonstrate unequivocally that lesions in the aortic roots of <i>Ldlr</i><sup>−/−</sup> mice receiving <i>Abcg1</i><sup>−/−</sup> bone marrow contained a 3.9-fold increase in the TUNEL-positive cells as compared with mice receiving <i>Abcg1</i><sup>+/+</sup> cells. In contrast, the smooth muscle cell rich media lacks LacZ-positive (<i>Abcg1</i><sup>−/−</sup>) (Figure 3D) and apoptotic cells (Figure 4A).

To address the important question of whether the increase in apoptotic cells observed in <i>Abcg1</i><sup>−/−</sup>→<i>Ldlr</i><sup>−/−</sup> lesions was a macrophage-autonomous response, we isolated peritoneal macrophages from wild-type and <i>Abcg1</i><sup>−/−</sup> mice (n=4/genotype) were stained for the presence of apoptotic cells (green cells marked with arrows). Apoptotic cells were not observed in the media (smooth muscle) (arrowheads). For comparative purposes, sections with similar lesion sizes are shown. B, Quantification of apoptotic cells in multiple sections of the aortic roots are shown. C, Thioglycollate-elicited peritoneal macrophages were cultured in the presence of ox-LDL (50 μg/mL of protein) for 24 hours, and cell apoptosis was measured by TUNEL staining. Ten random fields from wild-type and <i>Abcg1</i><sup>−/−</sup> macrophages were analyzed for apoptotic-positive and total cells (n=2391 or 2393). Data are expressed as mean±SEM.
Based on the data of both in vivo and in vitro studies (Figure 4A through 4C), we conclude that Abcg1−/− macrophages are more susceptible than wild-type macrophages to apoptosis after exposure to lipid/cholesterol-rich lipoproteins.

**Discussion**

We recently reported that administration of a HF/HC diet to Abcg1−/− mice resulted in the accumulation of cholesterol and cholesteryl esters in pulmonary macrophages, hepatocytes, and Kupffer cells. In addition, in vitro studies have showed that Abcg1−/− mice might exhibit accelerated atherosclerosis as a result of the accumulation of cholesterol-loaded foam cells in the aorta and/or aortic root. The results of experiments to test this hypothesis were quite unexpected.

First, administration of a high-fat/high-cholesterol/cholic acid diet to Abcg1−/− and wild-type mice resulted in similar levels of hyperlipidemia but to no difference in atherosclerotic lesions. Second, bone marrow transplant studies showed that Abcg1−/− donor cells resulted in a significant decrease in lesion size in both Ldlr−/− and ApoE−/− hyperlipidemic mice (Figure 3). These latter results were unexpected because the Abcg1−/− donor bone marrow cells repopulated many tissues, including the aortic wall, aortic root as well as lung, liver, and spleen, and transplanted Abcg1−/− macrophages in the atherosclerotic lesions stained with oil red O, consistent with the accumulation of neutral lipid (Figure 3). Together, these data indicate that Abcg1−/− monocytes retain the capacity to enter multiple tissues and convert to lipid-engorged macrophages.

The finding that lesions of Ldlr−/− mice transplanted with Abcg1−/− cells contained increased numbers of apoptotic cells and that Abcg1−/− macrophages are more susceptible to apoptosis in vitro following incubation with ox-LDL (Figure 4) provides a mechanism to account for the decrease in atherosclerotic lesions. Key evidence for the importance of apoptosis in lesion development was recently provided by Arai et al. on studies of the anti-apoptotic gene AIM. Expression of AIM is largely restricted to macrophages. Importantly, AIM−/−/Ldlr−/− double-knock-out mice showed a >80% decrease in atherosclerotic lesions as a result of enhanced apoptosis of AIM−/− macrophages in the lesions. In other studies, Tabas et al have shown that macrophages undergo apoptosis when they are treated in vitro with ox-LDL or acetylated-LDL together with an inhibitor of acyl-coenzyme A:cholesterol acyltransferase (ACAT). The ACAT inhibitor is critical as it prevents cholesterol esterification and thus results in an increase in intracellular levels of unesterified cholesterol which in turn activates the unfolded protein response in the endoplasmic reticulum, p38 and JNK, resulting in increased apoptosis. Tabas has proposed that this process may account for the presence of apoptotic cells in atherosclerotic lesions. The current study provides in vivo support for this model. Importantly, we have shown that increased apoptosis (Figure 4) and deposition of unesterified cholesterol crystals in Abcg1−/− macrophages does not require ACAT inhibitors, but is dependent on loss of ABCG1 function. We hypothesize that macrophages in the aortic root/aorta also accumulate unesterified cholesterol and that this ultimately results in apoptosis and decreased lesion development. However, several pro-apoptotic factors, that include oxyxolins, tumor necrosis factor (TNF)-α and nitric oxide, have been shown to be generated after uptake of ox-LDL by macrophages. In addition, scavenger receptors have been linked to macrophage apoptosis. Consequently, additional studies will be needed to determine how loss of ABCG1 affects all these pro- and anti-apoptotic pathways and whether such changes are restricted to lesions or are also present in other tissues.

It is formally possible that the presence of LDL receptor, or ApoE, in the transplanted Abcg1−/− bone marrow cells influences lesion development. However, based on previous transplantation studies into Ldlr−/− mice and the finding that lesions in the aortic roots of hyperlipidemic Ldlr−/− mice fed a high-fat diet were similar after transplantation with either Ldlr−/− or Ldlr−/− bone marrow, we suggest that this is unlikely. Nonetheless, in a different model system, Linton et al and Herijgers et al reported that lesion size was affected by the presence or absence of Ldlr in the donor bone marrow cells. However, the hyperlipidemia was mild, the lesions small, the recipients were C57BL/6 mice, and the diet contained cholic acid, which is known to promote inflammation and to drastically affect bile acid and cholesterol metabolism. Thus, the relevance of the latter studies to those presented here and elsewhere is unclear. Nonetheless, additional studies that use transplantation of either Ldlr−/−/Abcg1−/−, or ApoE−/−/Abcg1−/− donor cells into either Ldlr−/− or ApoE−/− mice, respectively, should provide additional insights into the role of ABCG1 in mice that lack LDLR or ApoE in macrophages.

We have previously demonstrated that liver and lungs of transgenic mice, that express both human and mouse ABCG1, are protected from lipid deposition that follows administration of a HF/HC diet. Consequently, we hypothesize that transplantation of bone marrow from ABCG1 transgenic mice into atherosclerosis-prone animals will be atheroprotective. Additional studies will be required to test this hypothesis and to also determine whether the antiatherogenic effects of LXR agonists result from altered expression of ABCG1.

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**Disclosures**

None.
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Supplementary Figures

Figure S1. Abcg1<sup>−/−</sup> bone marrow-derived cells repopulate multiple tissues in Ldlr<sup>−/−</sup> mice. Frozen sections of livers (A, B), lungs (C, D), spleens (E, F) and kidneys (G, H) were stained for LacZ activity as described in Methods. KC, Kupffer cell; M, macrophage; L, lymphocyte; G, glomerulus.

Figure S2. Altered gene expression in livers and lungs of Abcg1<sup>−/−</sup>→Ldlr<sup>−/−</sup> mice. The expression of key genes involved in lipid homeostasis was determined using RT-qPCR and RNA from the livers and lungs of Ldlr<sup>−/−</sup> mice transplanted with either wild-type (open bar) or Abcg1<sup>−/−</sup> (closed bar) bone marrow and fed a HF/HC diet (n = 4/genotype). Data are expressed as mean ± s.e.m. * p ≤ 0.05; ** p ≤ 0.01
Figure S1

Wild-type → Ldlr

Abcg1−/− → Ldlr

Liver  Lung  Spleen  Kidney

KC  L  M  G

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Figure S2

Srebp-1c  Srebp-2  Fas  Scd-1  Hmgcr

**relative mRNA expression**

Liver

**relative mRNA expression**

Lung

wild-type → Ldr−/−

Abcg1−/− → Ldr−/−