Deficiency of ATP-Binding Cassette Transporters A1 and G1 in Macrophages Increases Inflammation and Accelerates Atherosclerosis in Mice

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Rationale: Plasma high-density lipoprotein levels are inversely correlated with atherosclerosis. Although it is widely assumed that this is attributable to the ability of high-density lipoprotein to promote cholesterol efflux from macrophage foam cells, direct experimental support for this hypothesis is lacking.

Objective: To assess the role of macrophage cholesterol efflux pathways in atherogenesis.

Methods and Results: We developed mice with efficient deletion of the ATP-binding cassette transporters A1 and G1 (ABCA1 and ABCG1) in macrophages (MAC-ABC<K/O> mice) but not in hematopoietic stem or progenitor populations. MAC-ABC<K/O> bone marrow (BM) was transplanted into Ldlr<−/−> recipients. On the chow diet, these mice had similar plasma cholesterol and blood monocyte levels but increased atherosclerosis compared with controls. On the Western-type diet, MAC-ABC<K/O> BM–transplanted Ldlr<−/−> mice had disproportionate atherosclerosis, considering they also had lower very low-density lipoprotein/low-density lipoprotein cholesterol levels than controls. ABCA1/G1-deficient macrophages in lesions showed increased inflammatory gene expression. Unexpectedly, Western-type diet–fed MAC-ABC<K/O> BM–transplanted Ldlr<−/−> mice displayed monocytes and neutrophilia in the absence of hematopoietic stem and multipotential progenitor cells proliferation. Mechanistic studies revealed increased expressions of macrophage colony stimulating factor and granulocyte colony stimulating factor in splenic macrophage foam cells, driving BM monocyte and neutrophil production.

Conclusions: These studies show that macrophage deficiency of ABCA1/G1 is proatherogenic likely by promoting plaque inflammation and uncover a novel positive feedback loop in which cholesterol-laden splenic macrophages signal BM progenitors to produce monocytes, with suppression by macrophage cholesterol efflux pathways.

Key Words: atherosclerosis ■ high-density lipoprotein ■ inflammation ■ macrophages ■ monocytes

Plasma high-density lipoprotein (HDL) levels are inversely correlated with the incidence of cardiovascular disease in humans.1 Although several different approaches to raising HDL are being actively pursued in clinical trials, recent failures in this arena have highlighted the need for a deeper understanding of the relationship between HDL and atheroprotection.2,3 Infusion or increased production of HDL consistently leads to reduced atherosclerosis in animal models and humans, but the underlying mechanisms of protection remain unclear.4,5 A major theory to explain the atheroprotective role of HDL is that it mediates cholesterol efflux from cells in the artery wall, including macrophage and smooth muscle foam cells and arterial endothelial cells, and thus modulates inflammatory and other properties of these cells, especially in a hypercholesterolemic environment.6 Anti-inflammatory effects mediated by mechanisms independent of cholesterol efflux have also been proposed.7,8

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The cholesterol transporters ATP-binding cassette A1 and G1 (ABCA1 and ABCG1) mediate active cholesterol efflux to apolipoprotein A1 (apoA1)9,10 and HDL,11,12 respectively. ABCA1 is the gene that is mutated in Tangier Disease, a...
condition associated with low HDL levels, tissue macrophage foam cell accumulation, and, probably with premature atherosclerosis; however, the relationship to cardiovascular disease is controversial. Transplantation of Abca1−/− bone marrow (BM) into hypercholesterolemic Ldlr−/− recipients resulted in moderately accelerated atherosclerosis, a result which has been widely interpreted as indicating that macrophage ABCA1 deficiency causes accelerated atherosclerosis. However, a direct test of the role of macrophage ABCA1 deficiency in atherosclerosis using LysmCreAbca1fl/flLdlr−/− mice failed to show any difference compared with control Abca1fl/flLdlr−/− mice. One possible explanation for this unexpected result was compensation by alternative cholesterol efflux pathways, notably that mediated by ABCG1. Indeed transplantation of Abca1−/−Abcg1−/− BM into Ldlr−/− recipients resulted in markedly accelerated atherosclerosis, compared with Ldlr−/− mice transplanted with Abca1−/−, Abcg1−/−, or wild-type BM. However, further studies revealed an underlying expansion of hematopoietic stem and multipotential progenitor cells (HSPCs) in Abca1−/−Abcg1−/− BM–transplanted Ldlr−/− mice, leading to increased production of monocytes and neutrophils. Thus, the antiatherogenic role of cholesterol efflux pathways mediated by transporters in macrophages could not be clearly deduced from the results of this experiment in which Abca1 and Abcg1 were deleted in all BM cells.

To more directly evaluate the role of ABCA1 and ABCG1 in macrophages, we have developed Abcg1flox/flox mice and crossed them with LysmCreAbca1flox/flox mice to generate LysmCreAbca1flox/floxAbcg1+/− mice. The goals of our study were to assess effects of macrophage ABCA1 and ABCG1 transporter deficiency on atherosclerosis and plaque inflammation in Ldlr−/− mice, and to compare the effects of macrophage knockout of ABCA1/G1 transporters with general bone marrow deficiency of ABCA1/G1. While demonstrating a role of macrophage ABCA1/G1 in atherogenesis and plaque inflammation, our findings also uncovered an unexpected role of macrophage foam cells in the spleen in driving monocyte production, with suppression of this process by ABCA1/G1 and high levels of HDL.

Methods
An expanded version of the methods can be found in the online Data Supplement.

Results

ABCA1 and ABCG1 Expression in MAC-ABCDKO Mice
We assessed the efficiency of Abca1 and Abcg1 deletion in MAC-ABCDKO macrophages and in BM progenitor populations. ABCA1 and ABCG1 protein expressions were reduced by >95% in thiocholate-elicited peritoneal macrophages (both P<0.05) (Figure 1A and 1B). MAC-ABCDKO BM monocytes showed a >80% reduction in Abca1 and Abcg1 mRNA expression (P<0.05; Figure 1C and 1D). In MAC-ABCDKO neutrophils, there was >50% (P<0.05) reduction of Abca1, while the decrease in Abcg1 was not significant. Importantly, Abca1 and Abcg1 expressions were unchanged in the HSPCs, common myeloid progenitors, and granulocyte macrophage progenitors (GMPs; Figure 1C and 1D). In addition, MAC-ABCDKO mice showed decreased ABCA1 and ABCG1 expressions in macrophage-rich tissues, such as spleen and lung, and decreased ABCG1 expression in the liver, whereas liver ABCA1 expression was not affected (Online Figure II). This is consistent with observations that hepatic ABCG1 is expressed primarily in Kupffer cells, whereas ABCA1 is expressed primarily in hepatocytes. Findings in mice fed the WTD were similar to the chow diet, except that there was also partial reduction of Abca1 and Abcg1 expressions in the GMP population and a >90% reduction of Abca1 and Abcg1 in neutrophils (Online Figure IIIB and IIIC). The more widespread deletion on the WTD may reflect increased liver X receptor–induced expression of Abca1 and Abcg1. These findings are consistent with previous reports on the activity of the LysmCre promoter in different cell types. Thus, the LysmCre promoter caused efficient deletion of Abca1 and Abcg1 in macrophages, partial deletion in monocytes, variable deletion in neutrophils and GMPs depending on diet, and importantly, no deletion in BM stem cells. We refer to the LysmCreAbca1flox/−Abcg1flox mice as MAC-ABCDKO mice, with the caveat that deletion in other cell types may have also contributed to phenotypes.

MAC-ABCDKO Mice Characterization—Chow Diet
Cholesterol efflux to apoA1 and HDL was decreased by 83% and 68% in MAC-ABCDKO thiocholate-elicited macrophages, respectively (both P<0.0001; Online Figure IIIA), similar
to previous data in Abca1−/−Abcg1−/− macrophages.18 Chow-fed MAC-ABC DKO mice showed markedly increased lipid accumulation in thioglycollate-elicited peritoneal macrophages compared with controls (Online Figure IIIB and IIIC). Also chow-fed MAC-ABC DKO BM and MAC-ABC DKO spleens showed prominent lipid accumulation, which was absent in controls (Online Figure IIID and IIIE), and spleens were enlarged (Online Figure IIIF). After lipopolysaccharide treatment, MAC-ABCDKO BM–derived macrophages showed increased expression of IL-6, monocyte chemoattractant protein 1 (Mcp-1), macrophage inflammatory protein 1α (Mip-1α), granulocyte colony stimulating factor (G-csf), and macrophage colony stimulating factor (M-csf) (all \( P \lt 0.05 \); Online Figure IIIG), similar to previous studies in Abca1−/−Abcg1−/− macrophages.23

Atherosclerosis Is Accelerated After Transplantation of MAC-ABC DKO BM Into Ldlr−/− Mice on Chow Diet: Comparison With Transplantation of Abca1−/−Abcg1−/− BM

Previous studies in the B6 background addressing the role of BM ABCA1 and ABCG1 in atherosclerosis have shown that very low-density lipoprotein/low-density lipoprotein (VLDL/LDL) cholesterol levels were decreased by 75% in Ldlr−/− mice transplanted with Abca1−/−Abcg1−/− BM on the WTD.24 As a consequence, the increase in atherosclerosis in these mice was not significant.24 In an attempt to exclude a confounding factor of decreased VLDL/LDL cholesterol levels to atherosclerosis, we used the chow diet to assess the role of ABCA1/G1 deficiency in macrophages and total BM in atherosclerosis. Ldlr−/− mice were transplanted with MAC-ABCDKO, Abca1−/−Abcg1−/−, or wild-type BM. Five weeks after BM transplantation (BMT), the reconstitution of the BM was >95% (results not shown). Cholesterol levels were in a similar range in all groups of mice (Table). There were relatively minor differences in the VLDL/LDL fraction (16% decrease in MAC-ABC DKO BM versus control and 18% in Abca1−/−Abcg1−/− BM versus wild-type; Online Figure IVA and IVB). In MAC-ABC DKO BM–transplanted Ldlr−/− mice, monocyte levels were similar to controls, whereas neutrophil levels were increased by 50% (\( P \lt 0.05 \); Figure 2A). Notably, BM HSPCs were not affected as compared with controls (Online Figure IVC). The neutrophilia was likely caused by 50% (\( P \lt 0.05 \)) increased levels of G-csf mRNA in plasma and 20% (\( P \lt 0.05 \)) increased G-csf mRNA expression in spleen. We observed no differences in plasma M-CSF levels or splenic M-csf mRNA expression.
Macrophage ABCA1 and ABCG1 Deficiency Leads to Monocytosis and Neutrophilia in Ldlr−/− Mice on the WTD, With Reversal by Increased HDL

We next investigated the role of macrophage ABCA1 and ABCG1 in monocytopoiesis and atherosclerosis under more hypercholesterolemic conditions and also assessed the effects of increased HDL levels. Ldlr−/− and Ldlr−/−APOA1TG mice were transplanted with MAC-ABC\textsuperscript{DKO} or control BM and fed the WTD. Because Abca1/g1 expression was not reduced in HSPCs (Online Figure IB and IIC), we anticipated no major increase in blood leukocytes. However, MAC-ABC\textsuperscript{DKO} BM–transplanted Ldlr−/− mice showed increased blood monocyte (2.4-fold) and neutrophil (2.1-fold) levels (both P<0.001), with increases in both Ly6-C\textsuperscript{α} and Ly6-C\textsuperscript{ε} subsets (Figure 3). Expression of the APOA1TG in MAC-ABC\textsuperscript{DKO} BM–transplanted Ldlr−/− decreased leucocytes (monocytes, 43%; neutrophils, 30%; both P<0.001). The APOA1TG did not decrease leucocyte levels in control BM–transplanted Ldlr−/− mice (Figure 3). Plasma levels of human apoA1 were similar (251±9 mg/dL, control BM; and 201±23 mg/dL, MAC-ABC\textsuperscript{DKO} BM) in both APOA1TG groups. Thus, feeding the WTD caused MAC-ABC\textsuperscript{DKO} BM–transplanted Ldlr−/− mice to develop monocytopoiesis and exaggerated the neutrophilia seen on the chow diet. Increased HDL levels achieved by expression of the human APOA1TG led to a suppression of these effects.

Increased Monocyte Proliferation in MAC-ABC\textsuperscript{DKO} BM–Transplanted Ldlr−/− Mice on the WTD

We undertook studies to elucidate the mechanisms underlying the unexpected monocytosis observed in MAC-ABC\textsuperscript{DKO} BM–transplanted Ldlr−/− mice on the WTD. 4',6-diamidino-2-phenylindole (DAPI) staining showed a 48% increase (P<0.01) in BM monocytes and a 10% increase in GMPs (P<0.05) in the G2M phase of the cell cycle, indicating increased BM monocyte and GMP proliferation in MAC-ABC\textsuperscript{DKO} BM–transplanted Ldlr−/− mice (Online Figure VA). HSPC and common myeloid progenitor proliferation were unchanged (Online Figure VA). The increased proliferation was associated with a 40% increase in GMPs in MAC-ABC\textsuperscript{DKO} BM (P<0.05), whereas BM monocyte levels were similar (Online Figure VB and VC). This suggests that monocytosis was attributable to increased production and more rapid release of monocytes into the circulation.

We have shown that ABCA1/G1 deficiency in HSPCs increased their proliferation, associated with increased membrane cholesterol accumulation, and increased cell surface expression of the IL-3 receptor common β subunit (IL-3Rβ).\textsuperscript{19} MAC-ABC\textsuperscript{DKO} BM monocytes showed increased staining of cholera toxin B (≈21%; P<0.05), suggesting increased membrane cholesterol accumulation (results not shown). Surface expression of the IL-3Rβ subunit was modestly increased in MAC-ABC\textsuperscript{DKO} BM monocytes (37%; P<0.01) and GMPs (≈10%; P<0.05; results not shown). Blood MAC-ABC\textsuperscript{DKO} monocytes showed 50% increased lipid accumulation (Online Figure VF and VE), and the surface expression of the IL-3Rβ was increased by 30% (P<0.05; results not shown), but monocytosis proliferation was not increased. We did not find increased monocyte activation in our model as assessed by measuring CD11a, CD11b, CD11c, and VLA4 on monocytes (results not shown).

### Table. Plasma Cholesterol Levels in Ldlr−/− Mice Transplanted With MAC-ABC\textsuperscript{DKO} or Abca1−/−Abcg1−/− Bone Marrow (BM) on a Chow Diet

<table>
<thead>
<tr>
<th>BM Genotype</th>
<th>Plasma Cholesterol Level, mg/dL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>251±7</td>
</tr>
<tr>
<td>MAC-ABC\textsuperscript{DKO}</td>
<td>211±5*[12]</td>
</tr>
<tr>
<td>Wild-type</td>
<td>234±14</td>
</tr>
<tr>
<td>Abca1−/−Abcg1−/−</td>
<td>192±23</td>
</tr>
</tbody>
</table>

Plasma was drawn and cholesterol levels were determined using an enzymatic kit.

*P<0.05.

(results not shown). MCP-1 plasma levels were increased by 57% (P<0.001) in MAC-ABC\textsuperscript{DKO} BM Ldlr−/− mice, whereas splenic Mcp-1 mRNA was unchanged (results not shown), suggesting that macrophage-rich tissues other than spleen contributed to the increased MCP-1 levels in plasma. In contrast to MAC-ABC\textsuperscript{DKO} BM–transplanted Ldlr−/− mice, mice transplanted with BM completely deficient in ABCA1/G1, that is, Abca1−/−Abcg1−/− BM–transplanted Ldlr−/− mice showed increased monocyte levels (>52%; P<0.01) with similar increases in both Ly6-C\textsuperscript{α} and Ly6-C\textsuperscript{ε} monocyte subsets, and neutrophil levels were increased by 2.3-fold (P<0.001; Figure 2B). This coincided with a 2.9-fold BM HSPC expansion (P<0.05; Online Figure IVD), similar to our previous findings.\textsuperscript{19}

Twenty weeks after BMT, mice were euthanized and atherogenesis was assessed in the aortic root. Deficiency of ABCA1 and ABCG1 in macrophages increased atherosclerotic lesion area by ≈73% (P<0.05; Figure 2C). Strikingly, BM ABCA1/G1 deficiency increased atherosclerotic lesion area by 2.7-fold as compared with the control group (P<0.001; Figure 2C), and by ≈54% compared with macrophage ABCA1/G1 deficiency (P<0.05; Figure 2C). For further characterization, lesions were classified as macrophage foam cell rich lesions, complex lesions with fibrous caps, and advanced lesions with necrotic cores and cholesterol clefts. Although controls with detectable atherogenesis mainly showed macrophage foam cell rich lesions, MAC-ABC\textsuperscript{DKO} BM–transplanted Ldlr−/− mice showed an increased number of complex lesions with fibrous caps compared with controls (P<0.01), and Abca1−/−Abcg1−/− BM–transplanted Ldlr−/− mice showed more complex and advanced lesions than controls and MAC-ABC\textsuperscript{DKO} BM–transplanted Ldlr−/− mice (both P<0.001; Figure 2D). Although both MAC-ABC\textsuperscript{DKO} and Abca1−/−Abcg1−/− BM–transplanted Ldlr−/− mice showed neutrophilia, we observed only very low numbers of neutrophils in the lesions (1 or 2 per lesion) and no difference between the genotypes (results not shown).

Importantly, the findings provide direct evidence that defective monocyte/macrophage cholesterol efflux leads to accelerated atherosclerosis. Because monocytosis is greater and lesions are larger and more advanced in Abca1−/−Abcg1−/− BM–transplanted mice compared with MAC-ABC\textsuperscript{DKO} BM–transplanted mice, these results also suggest a major role of HSPC expansion and monocytosis in accelerating lesions development in mice with complete BM deficiency of ABCA1/G1.
Monocytosis and Neutrophilia in MAC-ABC\textsuperscript{DKO} BM–Transplanted Ldlr\textsuperscript{−/−} Mice on the WTD Are Mediated Through a Cell-Extrinsic Mechanism

These observations suggested that monocyte proliferation could be the consequence of ABCA1/G1 deficiency in monocytes, leading to lipid accumulation and increased cell surface IL-3R\textbeta. This would represent a cell autonomous proliferative effect. To assess this, we performed a competitive BMT. Ldlr\textsuperscript{−/−} mice were transplanted with a 1:1 mix of CD45.1 wild-type plus CD45.2 MAC-ABC\textsuperscript{DKO} BM or a 1:1 mix of CD45.1 wild-type plus CD45.2 control BM. Mice were fed WTD, and monocyte levels were assessed. In line with our previous observations, WTD feeding led to increased blood monocyte levels in CD45.1 wild-type plus CD45.2 MAC-ABC\textsuperscript{DKO} BM–transplanted Ldlr\textsuperscript{−/−} mice, compared with their controls (≈50%; \(P<0.001\); Figure 4A). Surprisingly, however, the ratio of CD45.2:CD45.1 blood monocytes was unchanged (Figure 4B). Therefore, although we observed a cell autonomous increase in BM CD45.2 MAC-ABC\textsuperscript{DKO} monocyte proliferation, this did not lead to an increase in this population in the blood. One
A possible explanation would be that there was increased turnover of MAC-ABCDKO monocytes attributable to enhanced apoptosis as shown previously for Abca1−/−Abcg1−/− macrophages.\(^{25}\) However, annexin V staining of blood monocytes showed no difference between MAC-ABCDKO and control monocytes (results not shown). We therefore concluded that a cell-extrinsic mechanism primarily contributed to the monocytosis in WTD-fed MAC-ABCDKO BM–transplanted Ldlr−/− mice. Similar data were found in neutrophils (Figure 4C and 4D). Thus, neutrophilia was also regulated by cell-extrinsic factors.

**Increased Expressions of M-CSF and G-CSF in Spleen and Plasma of MAC-ABCDKO BM–Transplanted Ldlr−/− Mice on the WTD**

We next investigated the nature of the cell-extrinsic factors contributing to monocytosis and neutrophilia in MAC-ABCDKO BM–transplanted Ldlr−/− mice on the WTD. We found that spleens of MAC-ABCDKO mice on the WTD were enlarged (50%; \(P<0.001\)) and showed prominent lipid accumulation (Online Figure VI). G-csf mRNA was 40% increased in MAC-ABCDKO spleens (\(P<0.05\); Figure 5A).
found a 2.5-fold increase (P<0.001) in Mcp-1 mRNA expression and a 40% increase in M-csf mRNA in the spleens of MAC-ABC^{DKO} mice (P<0.05; Figure 5A). Isolation of splenic cells showed this was attributable to increased expressions of Mcp-1 and M-csf mRNA in monocytes and macrophages, respectively (P<0.05 and P<0.01; Figure 5B), together with an increase in these cell populations (P<0.01, monocytes; and P<0.05, macrophages; Figure 5C). Furthermore, plasma M-CSF levels were increased by 40% (P<0.001) and plasma MCP-1 levels by 95% (P<0.01), and this was reversed by human APOA1TG expression (Figure 5D and 5E). Thus, increased Mcp-1 and M-csf expression in the spleen and increased plasma MCP-1 and M-CSF could account for the monocytosis in WTD-fed MAC-ABC^{DKO} BM–transplanted Ldlr^{−/−} mice. G-CSF levels were 2-fold increased in plasma of WTD-fed MAC-ABC^{DKO} BM–transplanted Ldlr^{−/−} mice and partially reversed by the APOA1TG (Figure 5F). Thus the neutrophilia was likely accounted for by increased G-CSF levels.

WTD-fed MAC-ABC^{DKO} BM–transplanted Ldlr^{−/−} mice develop atherosclerosis at low cholesterol levels and show increased inflammatory and chemokine gene expression in atherosclerotic plaques.

Cholesterol levels in MAC-ABC^{DKO} BM–transplanted Ldlr^{−/−} mice were 534±23 mg/dL, which was 54% decreased (P<0.001) compared with cholesterol levels in control BM–transplanted Ldlr^{−/−} mice (1162±68 mg/dL). The decrease was found in the VLDL/LDL fraction (Online Figure VII). After 7.5 weeks of Western-type diet, we found similar atherosclerotic lesion areas in Ldlr^{−/−} mice transplanted with control or MAC-ABC^{DKO} BM (n=6 to 12 mice per group). For A and B, RNA was extracted, and expressions of Mcp-1, M-csf, and G-csf were assessed and corrected for the housekeeping gene m36B4. *P<0.05, **P<0.01, ***P<0.001.

We then performed laser capture microscopy and assessed the mRNA expression of proatherogenic chemokines in the macrophage population. Interestingly, the cells in lesions from MAC-ABC^{DKO} BM–transplanted Ldlr^{−/−} mice showed increased mRNA expression of Mip-1α (P<0.05; Figure 6C) and a trend toward increased Mcp-1 mRNA. Both of these chemokines are involved in monocyte recruitment and have been reported to accelerate atherogenesis.26,27 The expression

Figure 5. Characterization of spleen and plasma of control and MAC-ABC^{DKO} bone marrow (BM)–transplanted Ldlr^{−/−} mice on the Western-type diet (6 weeks). A, Mcp-1, M-csf, and G-csf mRNA expressions in the spleen. B, Mcp-1 and M-csf mRNA expressions in fluorescence-activated cell–sorted splenic monocytes and macrophages. Mono and macro denote monocytes and macrophages, respectively. C, Monocytes and macrophages as percentage of total spleen cells assessed by flow cytometry. D–F, Plasma levels of monocyte chemotactrant protein 1 (MCP-1; D), macrophage colony stimulating factor (M-CSF; E), and granulocyte colony stimulating factor (G-CSF; F) assessed by ELISA in Ldlr^{−/−} and Ldlr^{−/−}APOA1TG mice transplanted with control or MAC-ABC^{DKO} BM (n=6 to 12 mice per group). For A and B, RNA was extracted, and expressions of Mcp-1, M-csf, and G-csf were assessed and corrected for the housekeeping gene m36B4. *P<0.05, **P<0.01, ***P<0.001.
level of other inflammatory cytokines was also assessed but no significant increases were found (results not shown), possibly because of the decreased plasma cholesterol levels. As expected, the cells in lesions from MAC-ABCDKO BM–transplanted Ldlr−/− mice completely lacked Abca1 and Abcg1 mRNA expressions (P<0.001 and P<0.05, respectively; Figure 6C). We also performed laser capture microscopy analysis of samples collected in an earlier study in which Ldlr+/- mice had been transplanted with Abca1−/−Abcg1−/− BM and had been fed the Paigen diet for 10 weeks. This analysis of lesional macrophages deficient in ABCA1/G1 also showed increased Mcp-1 and Mip-1α mRNA levels (Figure 6D), as well as increased expression of several other inflammatory genes (Il-6, Il-1; results not shown).

Discussion

This study has uncovered several distinct antiatherogenic functions of cholesterol efflux pathways mediated by ABCA1 and ABCG1 (Online Figure VIII). Our findings suggest that the role of ABCA1/G1 in controlling HSPC proliferation has antiatherogenic consequences (step 1), extending previous observations. The study has also unveiled a positive feedback loop between lipid-laden macrophages in the spleen and BM monocyte and granulocyte production and has shown that this is suppressed by cholesterol efflux via ABC transporters (step 2). Finally, we provide the first direct demonstration of an antiatherogenic effect of the cholesterol efflux pathways in macrophages, acting in lesional macrophages to suppress expressions of chemokines and inflammatory genes, and thus recruitment of monocytes into lesions (step 3).

A large body of indirect evidence supports the concept that the antiatherogenic role of HDL is tied to its ability to promote cholesterol efflux from macrophage foam cells. Importantly, measurements of macrophage reverse cholesterol transport, a composite measure of macrophage cholesterol efflux, transport in the bloodstream and excretion into the feces, have shown that a variety of genetic or pharmacological interventions exert parallel effects on macrophage reverse cholesterol transport and atherosclerosis.28,29 Moreover, a recent study in humans showed that the capacity of HDL to induce cholesterol efflux from macrophages is a strong inverse predictor of atherosclerotic plaque burden in the coronary or carotid arteries.30 We now provide direct evidence that macrophage cholesterol efflux pathways mediated by ABCA1 and ABCG1 are antiatherogenic in mice. This was indicated by significantly accelerated atherosclerosis in MAC-ABCΔΔKO BM–transplanted Ldlr−/− mice on a chow diet,
when MAC-ABC^{DKO} and control BM–transplanted Ldlr^{−/−} mice had similar moderate elevations of blood cholesterol levels. Even though VLDL/LDL cholesterol levels were reduced by ≈54% in MAC-ABC^{DKO} BM–transplanted Ldlr^{−/−} mice on the WTD, atherosclerosis was similar in both groups, indicating that the expected reduction in lesions resulting from the lower cholesterol levels was counterbalanced by a proatherogenic role of decreased macrophage ABCA1/G1 (Figure 6C). For example, lower levels of blood cholesterol may have led to decreased endothelial expression of cell adhesion molecules that recruit monocytes into lesions, offsetting the effects of increased blood monocyte levels. Because LysmCre also deletes floxed genes in neutrophils and monocytes and to some extent in GMPs (Figure 1C and 1D and Online Figure IIB and IIC), we cannot completely exclude a role of deficiency of cholesterol efflux pathways in these cell types in our results. However, mononcytosis and neutrophilia seemed to be driven by cell-extrinsic factors, notably various chemokines, produced by macrophages. Thus, monocytosis and neutrophilia were explained by macrophage transporter deficiency, indicating that defects in macrophage cholesterol efflux pathways were ultimately responsible for disproportionate atherosclerosis in the MAC-ABC^{DKO} group.

In contrast to the chow diet, WTD-fed MAC-ABC^{DKO} BM–transplanted Ldlr^{−/−} mice displayed significant monocytosis. Initially, we hypothesized that this unexpected monocytosis was caused by a cell-intrinsic effect in monocytes, reflecting lipid accumulation and increased monocyte proliferation (Online Figure VA, VD, and VE), similar to findings in ABCA1/G1-deficient HSPCs. However, this was disproved by the competitive BMT. Further studies revealed increased chemokine and cytokine expressions in macrophages (Online Figure VIII – step 2). Thus, M-csf and Mcp-1 mRNA expressions were increased in MAC-ABC^{DKO} peritoneal macrophages and in splenic monocytes/macrophages of WTD-fed MAC-ABC^{DKO} BM–transplanted Ldlr^{−/−} mice (Figure 5B). These mice also showed increased plasma MCP-1 and M-CSF (Figure 5D and 5E). MCP-1 is a ligand for chemokine receptor 2 that mediates the emigration of monocytes out of the BM, whereas M-CSF stimulates monocyte production by GMPs.

Findings in the BM showing increased monocyte production and release into the blood are consistent with effects of increased M-CSF and MCP-1. These findings suggest that increased MCP-1 and M-CSF expressions in cholesterol-laden macrophages in the spleen led to monocytosis in MAC-ABC^{DKO} BM–transplanted Ldlr^{−/−} mice on the WTD.

Our studies also suggest a major role of cholesterol efflux pathways in HSPCs in controlling monocytosis and neutrophilia and thus atherosclerosis (Figure 2C; Online Figure VIII – step 1). This conclusion was predicted but not proven by earlier studies and is strongly supported by our finding that monocytosis and atherosclerosis are increased in moderately hypercholesterolemic chow-fed mice with deficiency of ABCA1 and ABCG1 in all BM cells, compared with mice with macrophage deficiency of ABCA1/G1 that did not display monocytosis. These findings are also consistent with a recent study in which we used mixed BMT to show that in Apoe^{−/−} mice cell autonomous proliferation and expansion of Apoe^{−/−} HSPCs led to increased monocyte levels and increased entry of Apoe^{−/−} monocytes into atherosclerotic lesions.

Overall, these studies show an atheroprotective role for macrophage cholesterol efflux pathways that operate to suppress production of inflammatory cells and inflammatory cytokines and chemokines in lesions. In macrophages, HDL-induced cholesterol efflux decreases foam cell formation and macrophage inflammation, and consequently also the expression of cytokines that instruct the BM to produce monocytes and neutrophils and stimulate monocyte infiltration into atherosclerotic plaques. Consistent with previous studies, our findings indicate that even in the absence of macrophage ABCA1 and ABCG1, marked 2-fold increases in HDL levels derived from a human APOA1 transgene can lead to almost complete suppression of MCP-1, M-CSF, G-CSF, monocytosis, and neutrophilia. Most likely, this reflects the activity of residual cholesterol efflux pathways (Online Figure IIIA). This suggests that pharmacological interventions that greatly increase HDL production, such as infusions of apoA1 or reconstituted HDL, have strong potential to decrease macrophage inflammatory responses, and suggest that measurements of plasma cytokines, such as MCP-1, M-CSF, G-CSF, or monocyte or neutrophil responses could be a way to monitor the effectiveness of these treatments in humans.

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Disclosures

A.R. Tall is a consultant to Merck, Roche, Amgen, Arisaph, and CSL. J.S. Parks is a consultant to Merck and GlaxoSmithKline. The other authors report no conflicts.

References


Novelty and Significance

What Is Known?

- Bone marrow expression of the ATP-binding cassette transporters A1 and G1 (ABCA1 and ABCG1) decreases atherosclerosis in mice.
- The antatherogenic properties of high-density lipoprotein (HDL) are thought to involve its role in mediating cholesterol efflux via ABCA1 and ABCG1 in macrophages, but direct evidence is lacking.

What New Information Does This Article Contribute?

- Deletion of fixed Abca1/g1 genes in macrophages increases inflammatory gene expression in plaques and accelerates atherosclerosis.
- Splenic macrophage ABCA1 and ABCG1 suppress inflammatory chemokine secretion, and thus bone marrow production of proatherogenic monocytes and neutrophils.
- These findings suggest a role of Abca1/g1 in macrophages and monocytes and monocytc accumulation in atherosclerotic lesions.

Pharmacological interventions to increase HDL are being pursued in clinical trials. However, ambiguous outcomes of these trials highlight the lack of a clear understanding of the relationship between HDL and its atheroprotective effects. A large body of indirect evidence supports the concept that the antatherogenic role of HDL is tied to its ability to promote cholesterol efflux from macrophages mediated, in part, by ABCA1 and ABCG1. We evaluated this hypothesis by investigating the contribution of macrophage ABCA1 and ABCG1 to atherogenesis in mice. We found that macrophage ABCA1 and ABCG1 deficiency increased macrophage lipid accumulation, atherosclerosis, and inflammation in atherosclerotic lesions. We also discovered that splenic macrophage ABCA1 and ABCG1 deficiency increased the secretion of proinflammatory chemokines in plasma that instruct the bone marrow to produce proatherogenic monocytes and neutrophils. This phenotype was reversed by increased HDL levels. Our study provides direct evidence showing that cholesterol efflux pathways mediated by ABCA1 and ABCG1 in macrophages are antatherogenic. These findings suggest that interventions that increase HDL production have strong potential to decrease macrophage inflammatory responses, and that measurements of plasma cytokines and chemokines or monocyte or neutrophil responses could be a way to monitor the effectiveness of these treatments in humans.
Deficiency of ATP-Binding Cassette Transporters A1 and G1 in Macrophages Increases Inflammation and Accelerates Atherosclerosis in Mice

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Supplemental Material

Animals and Diets

Ldlr\(^{-/-}\), APOA1TG, and CD45.1 mice were purchased from Jackson Laboratories (Bar Harbor, ME). Ldlr\(^{-/-}\) and APOA1TG were intercrossed to obtain Ldlr\(^{-/-}\) and Ldlr\(^{-/-}\)APOA1TG littermates. Abca1\(^{-/-}\), Abcg1\(^{-/-}\) were generated as described previously and backcrossed into the C57BL/6J background for at least 10 generations. Abca1\(^{flox/flox}\) mice\(^2\) had been backcrossed into the C57BL/6J background for at least 10 generations. LysmCre mice\(^3\) were kindly provided by Dr. Tabas. We generated Abcg1\(^{flox/flox}\) mice directly into the C57BL/6J background (see below). Abca1\(^{flox/flox}\) and Abcg1\(^{flox/flox}\) mice were intercrossed to generate Abca1\(^{flox/flox}\)Abcg1\(^{flox/flox}\) (control) mice, which were crossbred with LysmCre mice to generate LysmCreAbca1\(^{flox/flox}\)Abcg1\(^{flox/flox}\) (MAC-ABC\(^{DKO}\)) mice. Mice were fed a Western-type diet (21% milk fat, 0.2% cholesterol; TD88137, Harlan Teklad) or chow diet (Purina Mills diet 5053). All protocols were approved by the Institutional Animal Care and Use Committee of Columbia University. The Abca1\(^{flox/flox}\)Abcg1\(^{flox/flox}\) mice will be available at the Jackson Laboratory Repository with the JAX Stock No. 021067 and can be found at http://jaxmice.jax.org/query.

Generation of Abcg1\(^{flox/flox}\) mice

Three loxP sites and one neomycin resistance gene were placed around exon 3, the exon that is deleted in general Abcg1\(^{-/-}\) mice obtained from DeltaGen.\(^4\) The ABCG1 BAC construct was purchased from CHORI. The Frt_LoxP (FL) cassette (146 bp) was inserted at position 41200 and the Lox_Neo_Lox (LNL) cassette at position 41950. A 2 kb short arm downstream of the LNL cassette (ended at position 43950 before exon 4) was retrieved into a plasmid with the DTA cassette as the negative selection marker. A NotI site was engineered in front of the 5 kb long arm for the linearization of the targeting vector. The targeting construct is shown in supplementary Figure I. The targeting construct was electroporated into C2J (albino C57BL/6J) embryonic stem cells. The C2J albino mutation at the mouse tyrosinase locus arose spontaneously in the C57BL/6J inbred strain and causes complete absence of melanin synthesis.\(^5\) The cells were then subjected to positive and negative selection. Of the 48 clones screened, 14 targeted ES clones with all 3 loxP sites in the ABCG1 locus were found. These ES cells were expanded and injected into B6 mouse blastocysts and implanted into pseudopregnant B6CBAF1 female mice. Male chimeras were crossbred with female C2J (albino C57BL/6J) mice on the B6 background to check for germline transmission. When the pups were albino, this indicated that germline transmission was successful. In the albino pups, we tested for the expression of the ABCG1 construct by Southern blot and we found that indeed these mice all expressed it. We thus immediately obtained Abcg1\(^{flox/flox}\) mice on the C57BL/6J background.

ABCA1 and ABCG1 expression in macrophages and tissues

Thioglycollate-elicited macrophages were harvested from the peritoneum of wild-type, control mice or MAC-ABC\(^{DKO}\) mice and cultured in DMEM supplemented with 10% FCS and 1% pen strep. After stimulation with the liver X receptor (LXR) ligand TO901317 (3 μM, 18 h), cells were lysed in RIPA buffer. Also, spleen, lung, and liver were isolated from control or MAC-ABC\(^{DKO}\) mice and lysed. ABCA1 and ABCG1 levels were measured by Western blot using antibodies from Novus Biologicals. β-Actin was measured using an antibody from Sigma. Protein bands were quantified using Image J.

Macrophage cholesterol efflux studies

Thioglycollate-elicited peritoneal macrophages were collected from control or MAC-ABC\(^{DKO}\) mice and cultured in DMEM supplemented with 10% FCS, 1% pen strep. Cells were then incubated with TO901317 (3 μM), 2 μCi/ml \(^{3}H\)-cholesterol, and AcLDL (100 μg/ml) for 24 h in DMEM supplemented with 0.2% FAF-BSA and 1% pen strep to induce cholesterol accumulation. Subsequently, HDL (50 μg/ml) or apoAI (25 μg/ml) were added as cholesterol acceptors. After 6 hours, cells were washed and lysed and \(^{3}H\)-activity was counted in the cell lysate and in the medium. Cholesterol efflux is expressed as \((^{3}H\text{-activity-medium})/(^{3}H\text{-activity-cells+medium})\).
Macrophage inflammation

Bone marrow (BM) cells were isolated from the femur and the tibias of control or MAC-ABC\textsuperscript{DKO} mice and cultured in DMEM supplemented with 10% FCS, 1% pen strep, and 20% L-cell conditioned medium to differentiate into macrophages for a period of 7 days. Cells were washed and cultured o/n in DMEM supplemented with 10% FCS and 1% pen strep. Lipopolysaccharide (50 ng/ml) was added, and after 4 h, cells were washed and lysed. RNA was then extracted using a RNeasy Mini Kit (Qiagen) and cDNA was synthesized using SuperScriptase III (Invitrogen). Levels of \textit{Il-6}, \textit{Mcp-1}, \textit{Mip-1}α, \textit{M-csf}, and \textit{G-csf} mRNA were assessed using qPCR on a Stratagene Mx3000P (Agilent Technologies), and initial differences in RNA quantity were corrected for using the housekeeping gene m36B4.

mRNA Expression Levels in Bone Marrow Cell Subpopulations and Spleen Cells

BM haematopoietic stem and multipotential progenitor cells (HSPC), common myeloid progenitors (CMP), granulocyte macrophage progenitors (GMP), monocytes, and neutrophils were isolated using flow cytometry and for subsequent RNA extraction sorted directly into RNeasy lysis buffer (Qiagen) using the FACS­Aria, running FACSDiVa software. The detailed procedure is described below.

BM was harvested from the femurs and tibias. Tubes were kept at 4°C for the whole procedure unless stated otherwise. Red blood cells (RBCs) were lysed (BD Pharm Lyse, BD Bioscience) and BM cells were centrifuged, washed, and resuspended in HBSS (0.1% BSA, 5 mM EDTA). Cells were incubated with a cocktail of antibodies to lineage committed cells (CD45R, CD19, CD11b, CD3e, TER-119, CD2, CD8, CD4, Ly-6G: all FITC, eBioscience), Sca1-Pacific Blue, ckit-APC Cy7. Where further identification of progenitor cell subsets was required antibodies to CD16/CD32 (FcγRII/III) and CD34 to separate CMP (lin\textsuperscript{-}, Sca1\textsuperscript{+}, ckit\textsuperscript{+}, CD34\textsuperscript{int}, FcγRII/III\textsuperscript{int}) and GMP (lin\textsuperscript{-}, Sca1\textsuperscript{+}, ckit\textsuperscript{+}, CD34\textsuperscript{int}, FcγRII/III\textsuperscript{hi}) were used. HSPCs were identified as lin\textsuperscript{-}, Sca1\textsuperscript{+}, and ckit\textsuperscript{+}. For isolation of bone marrow monocytes and neutrophils, cells were stained with a cocktail of antibodies against CD45-APC-Cy7, Ly6-C/G-PerCP-Cy5.5 (BD Pharmingen), and CD115-APC (eBioscience). Monocytes were identified as CD45\textsuperscript{hi}CD115\textsuperscript{hi} and further separated into Ly6-C\textsuperscript{hi} and Ly6-C\textsuperscript{lo} subsets, and neutrophils were identified as CD45\textsuperscript{hi}CD115\textsuperscript{lo}Ly6-C\textsuperscript{hi} (Gr-1). For the isolation of spleen monocytes and macrophages, spleens were meshed, RBCs lysed as described above and Ly6-C\textsuperscript{hi} monocytes were isolated similar to BM monocytes. For the isolation of spleen macrophages, an additional staining with F4/80-PE-Cy7 (eBioscience) was performed and spleen macrophages were identified as CD45\textsuperscript{hi}CD115\textsuperscript{hi}Ly6-C\textsuperscript{lo}F4/80\textsuperscript{hi}. All BM and spleen cells were sorted directly into RLT buffer. RNA was then extracted using a RNeasy Micro Kit (Qiagen) and cDNA synthesized using SuperScript VILO (Invitrogen). \textit{Abca1}, \textit{Abcg1}, \textit{Mcp-1}, \textit{M-csf}, and \textit{G-csf} mRNA levels were assessed using qPCR on a Stratagene Mx3000P (Agilent Technologies), and initial differences in RNA quantity were corrected for using the housekeeping gene m36B4.

Bone Marrow Transplantation

At 8 weeks of age, \textit{Ldlr}\textsuperscript{-/-} mice were transplanted with \textit{Abca1}\textsuperscript{-/-} \textit{Abcg1}\textsuperscript{-/-} or wild-type BM, and MAC-ABC\textsuperscript{DKO} or control BM. \textit{Ldlr}\textsuperscript{-/-} and \textit{Ldlr}\textsuperscript{-/-}\textit{APOA1TG} mice were transplanted with MAC-ABC\textsuperscript{DKO} or control BM. For competition experiments, \textit{Ldlr}\textsuperscript{-/-} mice were transplanted with a 1:1 mix of \textit{CD45.1} wild-type + \textit{CD45.2} MAC-ABC\textsuperscript{DKO} or \textit{CD45.1} wild-type + \textit{CD45.2} control BM. BM transplantation was performed as described previously. Mice were allowed to recover for 5 weeks after BM transplantation before Western-type diet feeding for 7-8 weeks or chow diet feeding of 15 weeks. After the recovery period, peripheral blood was collected and DNA isolated to determine the efficiency of BM reconstitution by quantification of wild-type \textit{Abcg1} and \textit{Abca1} DNA compared with actin (for the \textit{Abca1}\textsuperscript{-/-} \textit{Abcg1}\textsuperscript{-/-} and wild-type bone marrow transplantation) or wild-type \textit{Ldlr} DNA compared with actin (for the MAC-ABC\textsuperscript{DKO} and control BM transplantation).
Plasma Cholesterol and Lipoprotein Analysis

Blood samples were collected by tail bleeding into heparin-coated tubes. Plasma was separated by centrifugation. To assess lipoprotein cholesterol distribution by fast performance liquid chromatography (FPLC), pooled plasma (n=6-17 per pool) was injected onto a Superose 6 10/300 GL column (Amersham Biosciences) and eluted at a constant flow rate of 500 μl/min in a buffer containing 100 mM Tris and 0.04% NaN₃, pH 7.5. Fractions were assayed for cholesterol using an enzymatic kit from Wako (Cholesterol E), which was also used to measure total plasma cholesterol.

White Blood Counts and Flow Cytometry

Blood samples were collected by tail bleeding into EDTA coated tubes and immediately put on ice. Total white blood counts (WBCs) were quantified using a FORCYTE Veterinary Analyzer (Oxford Science, Inc.).

For analysis of blood leukocyte subsets, tubes were kept at 4°C for the whole procedure unless stated otherwise. Red blood cells (RBCs) were lysed (BD Pharm Lyse, BD Bioscience) and WBCs were centrifuged, washed, and resuspended in HBSS (0.1% BSA, 5 mM EDTA). Staining for monocytes and neutrophils was performed as described above. To assess the levels of BM monocytes, neutrophils, HSPCs, CMPs, and GMPs, stainings were performed as described above. To assess IL-3 receptor common β subunit (IL-3Rβ) expression and neutral lipid, cells were stained with PE-IL-3Rβ antibody (BD Pharmingen) and bodipy 493/503 (Invitrogen), respectively, for 30 min during the same procedure. To measure lipid rafts, after leukocyte subset staining, cells were stained with FITC-choleratoxin B antibody (Sigma) for 1 h at RT. To assess cell cycle, after staining for cell types, cells were fixed and permeabilized using IC fixation buffer (eBiosciences) supplemented with 0.01% saponin (Fluka) and 1% FCS for 30 min. Cells were then washed and stained for DAPI (Invitrogen). All samples were analyzed on an LSRII (BD Biosciences), running FACSDiVa software. To assess lipid accumulation in monocytes, monocytes were sorted directly into HBSS (0.1% BSA, 5 mM EDTA) using the FACSaria running FACSDiVa software. Monocytes were then transferred into wells coated with L-Lysine and allowed to adhere for 1 h at 37°C, fixed (4% paraformaldehyde) and stained with Oil Red O (Sigma). To assess lipid accumulation in macrophages, mice were injected with thioglycollate, and macrophages were isolated at 4 days after injection. They were allowed to adhere for 1 h at 37°C, fixed (4% paraformaldehyde) and stained with Oil Red O (Sigma). Pictures were taken using an Olympus IX-70 microscope equipped with a mercury 100-W lamp (CHIU Technical Corp.), an Olympus LCPlanF1 ×100 objective, DP Manager Basic imaging software (version 3.1; Olympus), and an Olympus DP71 CCD camera.

Oil Red O staining in spleen and bone marrow

Mice were sacrificed after 30 weeks of chow diet or 7.5 weeks of WTD feeding, and spleens and bones were dissected. Spleens were immediately embedded into OCT compound on dry ice. Bones were fixed for 1 h and subsequently decalcified for 30 min using rapid decalcifier buffer (electron microscopy sciences, Hatfield PA), and subsequently embedded into OCT compound on dry ice. Subsequently, 5 µm (frozen) cross-sections were made, fixed and stained for Oil Red O to assess lipid accumulation. Slides were counterstained with haematoxylin, and pictures were taken as described above.

Human apoAI, G-CSF, MCP-1 and M-CSF Analysis in Plasma

Blood samples were collected by tail bleeding into heparin-coated capillaries. Plasma was separated by centrifugation. Human apoAI levels in APOAITG mice were determined by Western blot using an antibody from Biodesign, which was specific for human apoAI and did not measure mouse apoAI. To calculate the apoAI plasma concentration, different concentrations of pure apoAI were used as reference values and protein bands were quantified using Image J. G-CSF, MCP-1 and M-CSF levels were measured using ELISA-kits (RayBio and R&D Systems, respectively) according to the manufacturer’s instructions.
Lesion analysis

After the indicated period of time on chow diet or WTD, mice were sacrificed and the heart was isolated and fixed in phosphate-buffered formalin. Hearts were dehydrated and embedded in paraffin, and were cross-sectioned throughout the aortic root area. Haematoxylin-eosin staining was performed on the sections and the average from 6 sections for each animal was used to determine lesion size. Lesion size was quantified by morphometric analysis using Image-Pro Plus software (Media Cybernetics, USA). Atherosclerotic lesions were classified for severity, according to the American Heart System for humans, which has been adapted for mice. Three classifications were discerned: early fatty streak lesions containing macrophage foam cells, complex lesions containing fibrous caps, and advanced lesions showing cholesterol clefts and necrotic cores.

Laser Capture Microscopy

Hearts were isolated and embedded in OCT, and immediately put on dry-ice. Serial sections (6 µm) were made using a cryostat. Using the H&E staining as a guide, macrophage rich sections were selectively subjected to laser capture using a PALM laser capture microdissection (LCM) machine. RNA was isolated using the RNeasy Micro Kit (Qiagen). Linear amplification and cDNA synthesis was performed using the Ovation Pico WTA System V2 (NuGEN). Subsequently, the expression of the indicated genes was assessed using qPCR on a Stratagene Mx3000P (Agilent Technologies), and initial differences in RNA quantity were corrected for using the housekeeping gene m36B4.

Statistics

All data are presented as means ± SEM. The t-test was used to define differences between 2 datasets, except for atherosclerosis studies where the Mann-Whitney nonparametric test was used. For differences in classification of atherosclerotic lesions, the χ² test was used. To define differences between 4 datasets, One-way Analysis of Variance (ANOVA) was used with a Bonferroni multiple comparison post-test. To test for correlation, the Spearman nonparametric test was used. The criterion for significance was set at P<0.05. Statistical analyses were performed using GraphPad Prism version 5.01 (San Diego, CA).

References

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induced apoptosis and necrotic core formation in advanced atherosclerotic lesions. *Cell Metab.* 2006;3:257-266


**Supplemental Figure I**

**Figure I.** $Abcg1^{flox/flox}$ construct. Exon 3 is indicated by the black box. The Frt_LoxP cassette (grey box) and Lox_Neo_Lox cassette (white box) are inserted in the floxed $Abcg1$ gene. Arrowheads indicate the loxP sites. The long arm and the short arm are indicated.
Supplemental Figure II

**A**

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**B and C**

**Figure II. ABCA1 and ABCG1 expression in MAC-ABCDKO mice.**

A. ABCA1 and ABCG1 expression in tissues of MAC-ABCDKO mice on a chow diet. Spleen, lung, and liver were lysed and Western blot for ABCA1, ABCG1, and β-actin was performed.

B. and C. Abca1 mRNA (B) and Abcg1 mRNA (C) expression in BM cells of Ldlr-/- transplanted with MAC-ABCDKO or control BM fed the WTD for 7.5 weeks. BM cells were sorted into RLT-buffer by FACS sorting and the mRNA expression of Abca1 (B) and Abcg1 (C) were assessed and corrected for the housekeeping gene m36B4. (n=8). **P<0.01, ***P<0.001.
Supplemental Figure III

A

![Bar chart showing cholesterol efflux (%).](image)

- **Control**
- **MAC-ABCDKO**

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B

![Bar chart showing % Oil Red O positive peritoneal monocytes/macrophages.](image)

- **Control**
- **MAC-ABCDKO**

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C

![Images of control and MAC-ABCDKO samples.](image)

D

![Images of control and MAC-ABCDKO samples.](image)
Supplemental Figure III

**Figure III. Characterization of MAC-ABCDKO mice on chow diet.**

**A.** Cholesterol efflux in MAC-ABCDKO macrophages to apoA1 or HDL. Thioglycollate-elicited peritoneal macrophages were loaded with Ac-LDL (100 µg/mL) and [³H]cholesterol (2 µCi/mL) in the presence of the LXR activator TO901317 (3 µM) for 24 h. Cells were washed and then incubated with or without (control) apoA1 (25 µg/mL) or HDL (50 µg/mL) for 6 h and cholesterol efflux was measured (n=4).

**B.** and **C.** Lipid accumulation in MAC-ABCDKO macrophages. Thioglycollate-elicited peritoneal macrophages were stained for Oil Red O immediately after isolation. Arrowheads depict monocytes and macrophages in C. Oil Red O positive monocytes and macrophages were quantified (n=4) (B).

**D** and **E.** Lipid accumulation in bone marrow (D) and spleen (E). Bone and spleen were isolated, sectioned, and stained for Oil Red O. Oil Red O positive cells are depicted by arrowheads. **F.** Spleen mass. **G.** Inflammatory gene expression in MAC-ABCDKO BM derived macrophages. Macrophages were incubated with or without LPS (50 ng/mL) for 4 h and lysed. mRNA levels of the indicated inflammatory genes were assessed and corrected for the housekeeping gene m36B4.

*P<0.05, ***P<0.001.
Figure IV. Lipoprotein cholesterol distribution and BM HSPC levels and atherosclerotic lesion severity in Abca1⁻/⁻Abcg1⁻/⁻ and MAC-ABCΔKO BM transplanted Ldlr⁻/⁻ mice on a chow diet. A and B. Lipoprotein cholesterol distribution. Plasma was pooled (n=7-12 per pool), lipoproteins were size-fractionated by fast performance liquid chromatography and the individual fractions were assayed for cholesterol. C and D. Twenty weeks after BM transplantation, BM was isolated from control and MAC-ABCΔKO BM transplanted Ldlr⁻/⁻ mice (C) or wild-type and Abca1⁻/⁻Abcg1⁻/⁻ BM transplanted Ldlr⁻/⁻ mice (D). BM was stained for HSPCs and analyzed by flow cytometry. *P<0.05.
Figure V. Characterization of MAC-ABC^{DKO} BM transplanted Ldlr⁻/⁻ mice on the Western-type diet (8 weeks) (n=8 per group). All measurements were performed by flow cytometry. Mfi denotes mean fluorescent intensity. A. Cell cycle of BM cells was assessed by DAPI staining and the % of cells in the S/G2M phase was measured. B and C. Total BM HSPC, CMP, GMP (B), monocytes and neutrophils (C) were measured. D. Blood monocytes were sorted by FACS sorting, and stained for Oil Red O. Arrowheads indicate Oil Red O positive monocytes. E. Blood monocytes were stained for BODIPY for neutral lipid and BODIPY positive monocytes were measured by flow cytometry. *P<0.05, **P<0.01.
Figure VI. Lipid accumulation in the spleen of MAC-ABC\textsuperscript{DKO} BMT \textit{Ldlr\textsuperscript{-/-}} mice on the WTD (8 weeks). Spleens were fixed and sectioned. Oil Red O staining was performed to assess lipid accumulation.
Supplemental Figure VII

Figure VII. Lipoprotein cholesterol distribution in control and MAC-ABC^{DKO} BM transplanted Ldlr^{-/-} mice on the Western-type diet (8 weeks). Plasma was pooled (n=13-17 per pool), lipoproteins were size-fractionated by fast performance liquid chromatography and the individual fractions were assayed for cholesterol.
Figure VIII. Contribution of ABCA1 and ABCG1 deficiency in BM cells to atherogenesis. 1. *Abca1*<sup>−/−</sup>*Abcg1*<sup>−/−</sup> haematopoietic stem and progenitor cells (HSPC) show increased proliferation, stimulating monocyte production. 2. *Abca1*<sup>−/−</sup>*Abcg1*<sup>−/−</sup> monocytes and macrophages in the spleen show increased expression of M-CSF and MCP-1, increasing monocyte production in the BM and monocyte release from the BM, respectively. 3. *Abca1*<sup>−/−</sup>*Abcg1*<sup>−/−</sup> macrophages in the atherosclerotic plaque show increased foam cell formation and levels of MIP-1α and MCP-1, which enhances monocyte infiltration into the plaque.