Deficiency of ABCA1 and ABCG1 in Macrophages Increases Inflammation and Accelerates Atherosclerosis in Mice

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ABSTRACT

Rationale: Plasma HDL levels are inversely correlated with atherosclerosis. Although it is widely assumed that this is due to the ability of HDL 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 MAC-ABC\(^{DKO}\) mice with efficient deletion of the ATP Binding Cassette Transporters A1 and G1 (ABCA1 and ABCG1) in macrophages but not in hematopoietic stem or progenitor populations. MAC-ABC\(^{DKO}\) 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 to controls. On the Western type diet (WTD), MAC-ABC\(^{DKO}\) BM transplanted \(Ldlr^-\) mice had disproportionate atherosclerosis, considering they also had lower VLDL/LDL cholesterol levels than controls. ABCA1/G1 deficient macrophages in lesions showed increased inflammatory gene expression. Unexpectedly, WTD-fed MAC-ABC\(^{DKO}\) BM transplanted \(Ldlr^-\) mice displayed monocytosis and neutrophilia in the absence of HSPC proliferation. Mechanistic studies revealed increased expression of M-CSF and G-CSF in splenic macrophage foam cells, driving BM monocyte and neutrophil production.

Conclusion: These studies 1) show that macrophage deficiency of ABCA1/G1 is pro-atherogenic likely by promoting plaque inflammation and 2) 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.

Keywords: Atherosclerosis, high-density-lipoprotein, inflammation, macrophages, monocytes

Nonstandard Abbreviations:
- ABCA1/G1: ATP Binding Cassette Transporter A1/G1
- APOA1TG: Apolipoprotein A1 Transgenic
- BMT: Bone marrow transplantation
- CCR2: Chemokine receptor 2
- CMP: Common myeloid progenitor
- G-CSF: Granulocyte colony stimulating factor
- GMP: Granulocyte macrophage progenitor
- rHDL: reconstituted High density lipoprotein
- HSPC: Haematopoietic stem and multipotential progenitor cell
- IL-3R\(\beta\): Interleukin 3 receptor common \(\beta\) subunit
- LCM: Laser Capture Microscopy
- LDLr: Low density lipoprotein receptor
- LPS: Lipopolysaccharide
- MAC-ABC\(^{DKO}\): \(LysmCreAbca1^{flo/flo}Abcg1^{flo/flo}\)
- M-CSF: Macrophage colony stimulating factor
- MCP-1: Monocyte chemotactant protein 1
- MIP-1\(\alpha\): Macrophage inflammatory protein 1\(\alpha\)
- RCT: Reverse cholesterol transport
- TLR4: Toll like receptor 4
- VLDL: Very low density lipoprotein
- WTD: Western-type diet
INTRODUCTION

Plasma high-density-lipoprotein (HDL) levels are inversely correlated with the incidence of cardiovascular disease (CVD) in humans. While 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 athero-protection. Infusion or increased production of HDL consistently leads to reduced atherosclerosis in animal models and humans, but the underlying mechanisms of protection remain unclear. A major theory to explain the athero-protective 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. Anti-inflammatory effects mediated by mechanisms independent of cholesterol efflux have also been proposed.

The cholesterol transporters ATP-Binding Cassette A1 and G1 (ABCA1 and ABCG1) mediate active cholesterol efflux to apoA1 and HDL, 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 CVD 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 LysmCreAbca1/Ldlr mice failed to show any difference compared to control Abca1/Ldlr 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 to Ldlr transplanted with Abca1, Abcg1 or wild-type BM. However, further studies revealed an underlying expansion and proliferation of hematopoietic stem and multipotent progenitor cells (HSPCs) in Abca1/Abcg1 BM transplanted Ldlr mice, leading to increased production of monocytes and neutrophils. Thus, the anti-atherogenic 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 Abcg1 mice and crossbred them with LysmCreAbca1 mice to generate LysmCreAbca1/Abcg1 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 only supplement.
Ldlr<sup>−/−</sup> and Ldlr<sup>−/−</sup> APOA1TG mice were transplanted with LysmCreAbca<sup>1+/-</sup>Abcg1<sup>−/−</sup> (MAC-ABCDKO) or Abca<sup>1+/-</sup>Abcg1<sup>−/−</sup> (control) bone marrow (BM). Ldlr<sup>−/−</sup> mice were transplanted with Abca<sup>1+/-</sup>Abcg1<sup>−/−</sup> or wild-type BM or a 1:1 mix of CD45.1 wild-type and CD45.2 CD45.2 MAC-ABC<sup>DKO</sup> BM. All experiments were carried out in the B6 background. Five weeks after BM transplantation (BMT), mice were fed either the chow diet for 15 weeks or the Western-type diet (WTD) for 7-8 weeks (as indicated). Leukocyte subsets were monitored by flow cytometry, cholesterol levels by enzymatic assays, and MCP-1, M-CSF, and G-CSF levels by ELISA. After the indicated period of diet, mice were sacrificed and atherosclerotic lesion area was analyzed in the aortic root. MAC-ABCDKO and control macrophages were characterized for cholesterol efflux and inflammatory gene expression. All protocols were approved by the Institutional Animal Care and Use Committee of Columbia University. The Abca<sup>1+/-</sup>Abcg1<sup>−/−</sup> 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.

RESULTS

ABCA1 and ABCG1 expression in LysmCreAbca<sup>1+/-</sup>Abcg1<sup>−/−</sup> mice.

We assessed the efficiency of Abca1 and Abcg1 deletion in MAC-ABC<sup>DKO</sup> macrophages and in BM progenitor populations. ABCA1 and ABCG1 protein expression were reduced by >95% in thioglycollate-elicited peritoneal macrophages (both P<0.05) (Figure 1A and 1B). MAC-ABC<sup>DKO</sup> BM monocytes showed a >80% reduction in Abca1 and Abcg1 mRNA expression (P<0.05) (Figure 1C and 1D). In MAC-ABC<sup>DKO</sup> neutrophils, there was >50% (P<0.05) reduction of Abca1, while the decrease in Abcg1 was not significant. Importantly, Abca1 and Abcg1 expression were unchanged in the hematopoietic stem and multipotent progenitors (HSPCs), common myeloid progenitors (CMPs), and granulocyte macrophage progenitors (GMPs) (Figure 1C and 1D). In addition, MAC-ABC<sup>DKO</sup> mice showed decreased ABCA1 and ABCG1 expression in macrophage-rich tissues such as spleen and lung and decreased ABCG1 expression in the liver while liver ABCA1 expression was not affected (Supplemental Figure IIA), consistent with observations that hepatic ABCG1 is expressed primarily in Kupffer cells while ABCA1 is expressed primarily in hepatocytes.20 Findings in mice fed the WTD were similar to the chow diet, except that there was also partial reduction of Abca1 and Abcg1 expression in the GMP population and a >90% reduction of Abca1 and Abcg1 in neutrophils (Supplemental Figure IIB and IIC). The more widespread deletion on the WTD may reflect increased liver X receptor-induced expression of Abca1/g1. These findings are consistent with previous reports on the activity of the LysmCre promoter in different cell types.21,22 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 LysmCreAbca<sup>1+/-</sup>Abcg1<sup>−/−</sup> mice as MAC-ABC<sup>DKO</sup> mice, with the caveat that deletion in other cell types may have also contributed to phenotypes.

MAC-ABC<sup>DKO</sup> mice characterization – chow diet.

Cholesterol efflux to apoAI and HDL was decreased by 83% and 68% in MAC-ABC<sup>DKO</sup> thioglycollate-elicited macrophages, respectively (both P<0.0001) (Supplemental Figure IIIA), similar to previous data in Abca<sup>−/−</sup>Abcg1<sup>−/−</sup> macrophages.18 Chow-fed MAC-ABC<sup>DKO</sup> mice showed markedly increased lipid accumulation in thioglycollate-elicited peritoneal macrophages compared to controls (Supplemental Figure IIIB and IIIC). Also chow fed MAC-ABC<sup>DKO</sup> BM and MAC-ABC<sup>DKO</sup> spleens showed prominent lipid accumulation, which was absent in controls.
(Supplemental Figure IIIID and IIIE), and spleens were enlarged (Supplemental Figure IIIF). After LPS treatment, MAC-ABC\textsuperscript{DKO} BM derived macrophages showed increased expression of IL-6, monocyte chemoattractant protein 1 (Mcp-1), macrophage inflammatory protein 1\textalpha (Mip-1\textalpha), granulocyte colony stimulating factor (G-csf), and macrophage colony stimulating factor (M-csf) (all \(P<0.05\)) (Supplemental Figure IIIG), similar to previous studies in Abca1\textsuperscript{-/-}Abcg1\textsuperscript{-/-} macrophages.\textsuperscript{23}

**Atherosclerosis is accelerated after transplantation of MAC-ABC\textsuperscript{DKO} BM into Ldlr\textsuperscript{-/-} on chow diet; comparison with transplantation of Abca1\textsuperscript{-/-}Abcg1\textsuperscript{-/-} BM.**

Previous studies in the B6 background addressing the role of BM ABCA1 and ABCG1 in atherosclerosis have shown that VLDL/LDL cholesterol levels were decreased by \(\sim75\%\) in Ldlr\textsuperscript{-/-} mice transplanted with Abca1\textsuperscript{-/-}Abcg1\textsuperscript{-/-} BM on the WTD.\textsuperscript{24} As a consequence, the increase in atherosclerosis in these mice was not significant.\textsuperscript{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\textsuperscript{-/-} mice were transplanted with MAC-ABC\textsuperscript{DKO}, Abca1\textsuperscript{-/-}Abcg1\textsuperscript{-/-} 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 1). There were relatively minor differences in the VLDL/LDL fraction (\(\sim16\%\) decrease in MAC-ABC\textsuperscript{DKO} BM vs control and \(\sim18\%\) in Abca1\textsuperscript{-/-}Abcg1\textsuperscript{-/-} BM vs wild-type) (Supplemental Figure IVA and IVB). In MAC-ABC\textsuperscript{DKO} BM transplanted Ldlr\textsuperscript{-/-} mice, monocyte levels were similar to controls whereas neutrophil levels were increased by \(\sim50\%\) (\(P<0.05\)) (Figure 2A). Notably, BM HSPCs were not affected as compared to controls (Supplemental Figure IVC). The neutrophilia was likely caused by \(\sim50\%\) (\(P<0.05\)) increased levels of G-CSF in plasma and \(\sim20\%\) (\(P<0.05\)) increased G-csf mRNA in the spleen. We observed no differences in plasma M-CSF levels or splenic M-csf mRNA expression (results not shown). MCP-1 plasma levels were increased by \(\sim57\%\) (\(P<0.001\)) in MAC-ABC\textsuperscript{DKO} BMT Ldlr\textsuperscript{-/-} mice while 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\textsuperscript{-/-} mice, mice transplanted with BM completely deficient in ABCA1/G1 i.e. Abca1\textsuperscript{-/-}Abcg1\textsuperscript{-/-} BM transplanted Ldlr\textsuperscript{-/-} mice, showed increased monocyte levels (\(\sim52\%;\ P<0.01\)) with similar increases in both Ly6-Chi and Ly6-C\textsuperscript{lo} 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 (\(P<0.05\)) (Supplemental Figure IVD), similar to our previous findings.\textsuperscript{19}

Twenty weeks after BMT, mice were sacrificed and atherogenesis was assessed in the aortic root. Deficiency of ABCA1 and ABCG1 in macrophages increased atherosclerotic lesion area by \(\sim73\%\) (\(P<0.05\)) (Figure 2C). Strikingly, BM ABCA1/G1 deficiency increased atherosclerotic lesion area by 2.7-fold as compared to the control group (\(P<0.001\)) (Figure 2C), and by \(\sim54\%\) compared to 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. Whereas controls with detectable atherosclerosis mainly showed macrophage foam cell rich lesions, MAC-ABC\textsuperscript{DKO} BM transplanted Ldlr\textsuperscript{-/-} mice showed an increased number of complex lesions with fibrous caps compared to controls (\(P<0.01\)), and Abca1\textsuperscript{-/-}Abcg1\textsuperscript{-/-} BM transplanted Ldlr\textsuperscript{-/-} mice showed more complex and advanced lesions than controls and MAC-ABC\textsuperscript{DKO} BM transplanted Ldlr\textsuperscript{-/-} mice (both \(P<0.001\)) (Figure 2D). Although both MAC-ABC\textsuperscript{DKO} and Abca1\textsuperscript{-/-}Abcg1\textsuperscript{-/-} BM transplanted Ldlr\textsuperscript{-/-} 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. Since monocytosis is greater and lesions are larger and more advanced in *Abca1*/*Abcg1* BM transplanted mice compared to MAC-ABC<sup>DKO</sup> 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.

Macrophage ABCA1 and ABCG1 deficiency leads to monocytosis and neutrophilia in Ldlr<sup>−/−</sup> mice on the Western-type diet, with reversal by increased HDL.

We next investigated the role of macrophage ABCA1 and ABCG1 in monocytosis and atherosclerosis under more hypercholesterolemic conditions and also assessed the effects of increased HDL levels. *Ldlr<sup>−/−</sup>* and *Ldlr<sup>−/−</sup>APOA1TG* mice were transplanted with MAC-ABC<sup>DKO</sup> or control BM, and fed the WTD. Since *Abca1/g1* expression was not reduced in HSPCs (Supplemental Figure IIB and IIC), we anticipated no major increase in blood leukocytes. However, MAC-ABC<sup>DKO</sup> BM transplanted *Ldlr<sup>−/−</sup>* mice showed increased blood monocyte (2.4-fold) and neutrophil (2.1-fold) levels (both *P*<0.001), with increases in both Ly6-C<sup>lo</sup> and Ly6-C<sup>hi</sup> subsets (Figure 3). Expression of the *APOA1TG* in MAC-ABC<sup>DKO</sup> BM transplanted *Ldlr<sup>−/−</sup>* decreased leukocytosis (monocytes, 43%; neutrophils, 30%) (both *P*<0.001). The *APOA1TG* did not decrease leukocyte levels in control BM transplanted *Ldlr<sup>−/−</sup>* mice (Figure 3). Plasma levels of human apoA1 were similar (251±9 mg/dL; control BM and 201±23 mg/dL; MAC-ABC<sup>DKO</sup> BM) in both *APOA1TG* groups. Thus, feeding the WTD caused MAC-ABC<sup>DKO</sup> BM transplanted *Ldlr<sup>−/−</sup>* mice to develop monocytosis 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<sup>DKO</sup> BM transplanted Ldlr<sup>−/−</sup> mice on the WTD.**

We undertook studies to elucidate the mechanisms underlying the unexpected monocytosis observed in MAC-ABC<sup>DKO</sup> BM transplanted *Ldlr<sup>−/−</sup>* mice on the WTD. 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<sup>DKO</sup> BM transplanted *Ldlr<sup>−/−</sup>* mice (Supplemental Figure VA). HSPC and CMP proliferation were unchanged (Supplemental Figure VA). The increased proliferation was associated with a ~40% increase in GMPs in MAC-ABC<sup>DKO</sup> BM (*P*<0.05), while BM monocyte levels were similar (Supplemental Figure VB and VC). This suggests that monocytosis was due 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).<sup>19</sup> MAC-ABC<sup>DKO</sup> 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<sup>DKO</sup> BM monocytes (37%; *P*<0.01) and GMPs (~10%; *P*<0.05) (results not shown). Blood MAC-ABC<sup>DKO</sup> monocytes showed ~50% increased lipid accumulation (Supplemental Figure VE and VE), and the surface expression of the IL-3Rβ was increased by ~30% (*P*<0.05) (results not shown), but monocyte 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).
Monocytosis and neutrophilia in MAC-ABC^{DKO} BM transplanted Ldlr^{-/-} 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-3Rβ. This would represent a cell autonomous proliferative effect. To assess this, we performed a competitive BMT. Ldlr^{-/-} mice were transplanted with a 1:1 mix of CD45.1 wild-type + CD45.2 MAC-ABC^{DKO} BM or a 1:1 mix of CD45.1 wild-type + 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 + CD45.2 MAC-ABC^{DKO} BM transplanted Ldlr^{-/-} mice, compared to 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^{DKO} monocyte proliferation, this did not lead to an increase in this population in the blood. One possible explanation would be that there was increased turnover of MAC-ABC^{DKO} monocytes due to enhanced apoptosis as shown previously for Abca1^{-/-}Abcg1^{-/-} macrophages.25 However, annexin V staining of blood monocytes showed no difference between MAC-ABC^{DKO} and control monocytes (results not shown). We therefore concluded that a cell-extrinsic mechanism primarily contributed to the monocytosis in WTD-fed MAC-ABC^{DKO} BM transplanted Ldlr^{-/-} mice. Similar data were found in neutrophils (Figure 4C and 4D). Thus, neutrophilia was also regulated by cell-extrinsic factors.

Increased expression 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 (Supplemental Figure VI). G-csf mRNA was ~40% increased in MAC-ABCDKO spleens (P<0.05; Figure 5A). We 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-ABCDKO mice (P<0.05) (Figure 5A). Isolation of splenic cells showed this was due to increased expression 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<0.001; monocytes and P<0.05; macrophages) (Figure 5C). Furthermore, plasma M-CSF levels was 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-ABCDKO BM transplanted Ldlr^{-/-} mice. G-CSF levels were ~2-fold increased in plasma of WTD-fed MAC-ABCDKO 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-ABCDKO 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-ABCDKO BM transplanted Ldlr^{-/-} mice were 534±23 mg/dL, which was ~54% decreased (P<0.001) compared to cholesterol levels in control BM transplanted Ldlr^{-/-} mice (1162±68 mg/dL). The decrease was found in the VLDL/LDL fraction (Supplemental Figure VII). After 7.5 weeks of Western-type diet, we found similar atherosclerotic lesion areas

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in Ldlr−/− mice transplanted with control or MAC-ABC<sup>DKO</sup> BM (Figure 6A), despite the decreased VLDL/LDL-cholesterol levels in the latter group. However, the onset of atherogenesis occurred at 46% of the cholesterol level in MAC-ABC<sup>DKO</sup> BM transplanted Ldlr−/− mice (Figure 6B), and cholesterol levels appeared to correlate with atherosclerotic lesion area only in control BM transplanted Ldlr−/− mice (r=0.6154; P<0.05) (Figure 6B). Both groups of mice showed mainly macrophage rich lesions (data not shown).

We then performed Laser Capture Microscopy (LCM) and assessed the mRNA expression of pro-atherogenic chemokines in the macrophage population. Interestingly, the cells in lesions from MAC-ABC<sup>DKO</sup> BM transplanted Ldlr−/− mice showed increased mRNA expression of Mip-1α (P<0.05; Figure 6C) and a trend towards increased Mcp-1 mRNA. Both of these chemokines are involved in monocyte recruitment and have been reported to accelerate atherogenesis.<sup>26, 27</sup> The expression level of other inflammatory cytokines was also assessed but no significant increases were found (results not shown), possibly due to the decreased plasma cholesterol levels. As expected, the cells in lesions from MAC-ABC<sup>DKO</sup> BM transplanted Ldlr−/− mice completely lacked Abca1 and Abcg1 mRNA expression (P<0.001 and P<0.05, respectively) (Figure 6C). We also performed LCM 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 anti-atherogenic functions of cholesterol efflux pathways mediated by ABCA1 and ABCG1 (Supplemental Figure VIII). Our findings suggest that the role of ABCA1/G1 in controlling HSPC proliferation has anti-atherogenic 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 anti-atherogenic effect of the cholesterol efflux pathways in macrophages, acting in lesional macrophages to suppress expression 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 anti-atherogenic role of HDL is tied to its ability to promote cholesterol efflux from macrophage foam cells. Importantly, measurements of macrophage reverse cholesterol transport (RCT), 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 RCT and atherosclerosis.<sup>28, 29</sup> 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.<sup>30</sup> We now provide direct evidence that macrophage cholesterol efflux pathways mediated by ABCA1 and ABCG1 are anti-atherogenic in mice. This was indicated by significantly accelerated atherosclerosis in MAC-ABC<sup>DKO</sup> BM transplanted Ldlr−/− mice on a chow diet when MAC-ABC<sup>DKO</sup> 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<sup>DKO</sup> 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 pro-atherogenic 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. Since LysmCre also deletes floxed genes in neutrophils and monocytes and to some extent in GMPs (Figure 1C and 1D and Supplemental Figure IIB and IIC), we cannot completely exclude a role of deficiency of cholesterol efflux pathways in these cell types in our results. However, monocyteosis and neutrophilia appeared 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<sup>DKO</sup> group.

In contrast to the chow diet, WTD-fed MAC-ABC<sup>DKO</sup> BM transplanted Ldlr<sup>−/−</sup> 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 (Supplemental 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 expression in macrophages (Supplemental Figure VIII – Step 2). Thus, M-csf and Mcp-1 mRNA expression were increased in MAC-ABC<sup>DKO</sup> peritoneal macrophages and in splenic monocytes/macrophages of WTD-fed MAC-ABC<sup>DKO</sup> BM transplanted Ldlr<sup>−/−</sup> mice (Figure 5B). These mice also showed increased plasma MCP-1 and M-CSF (Figure 5D and 5E). MCP-1 is a ligand for CCR2 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 expression in cholesterol-laden macrophages in the spleen led to monocytosis in MAC-ABC<sup>DKO</sup> BM transplanted Ldlr<sup>−/−</sup> 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; Supplemental 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 to 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<sup>−/−</sup> mice cell autonomous proliferation and expansion of Apoe<sup>−/−</sup> HSPCs led to increased monocyte levels and increased entry of Apoe<sup>−/−</sup> monocytes into atherosclerotic lesions.

Overall, these studies show an athero-protective 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 apoA1 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 (Supplemental Figure IIIA). This suggests that pharmacological interventions that greatly increase HDL production, such as infusions of apoA1 or rHDL, 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.
SOURCES OF FUNDING
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DISCLOSURES
Alan R. Tall is a consultant to Merck, Roche, Amgen, Arisaph and CSL. John S. Parks is a consultant to Merck and GlaxoSmithKline.

REFERENCES


DOI: 10.1161/CIRCRESAHA.113.301086


FIGURE LEGENDS

Figure 1. Characterization of MAC-ABCDKO mice. A and B. Protein levels of ABCA1 (A) and ABCG1 (B) in wild-type, control, and MAC-ABCDKO macrophages. Thioglycollate-elicited peritoneal macrophages were isolated, incubated with the LXR activator TO901317 (3 µM, 24 h), and ABCA1 and ABCG1 expression were assessed by Western blot. Bands were quantified using Image J and corrected for β-actin expression (bar graphs). C and D. Bone marrow (BM) cells were sorted into RLT-buffer by FACS sorting and the mRNA expression of ABCA1 (C) and ABCG1 (D) were assessed and corrected for the housekeeping gene m36B4. *P<0.05, **P<0.01, ***P<0.001.

Figure 2. Leukocyte subsets and atherosclerosis in chow-fed Ldlr−/− mice transplanted with (A and C-E) MAC-ABCDKO (n=10) or control (n=12) BM or (B and C-E) Abca1−/−Abcg1−/− (n=9) or wild-type (n=7) BM. A and B. Blood leukocyte subsets as assessed by flow cytometry. Mono denotes monocytes and neutro denotes neutrophils. Levels of Ly6-C hi and Ly6-C lo monocyte subsets are shown. C. Atherosclerotic lesion area. Hematoxylin-eosin staining was performed on paraffin sections of the aortic root and atherosclerotic lesion area was assessed. In C, each data point represents an individual mouse. D. Atherosclerotic lesion type as percentage of total number of atherosclerotic lesions. Only segments containing atherosclerotic lesions were included in the analysis. *P<0.05, **P<0.01, ***P<0.001.

Figure 3. Leukocyte subsets in Ldlr−/− or Ldlr−/−APOA1TG mice transplanted with MAC-ABCDKO or control BM (n=13-17 per group) on a Western-type diet (6-8 weeks). Blood monocytes, Ly6-C hi and Ly6-C lo monocyte subsets, and neutrophils were measured by flow cytometry. *P<0.05, **P<0.01.

Figure 4. Monocytosis and neutrophilia in MAC-ABCDKO BM transplanted Ldlr−/− mice on the WTD are cell-extrinsic. Ldlr−/− mice were transplanted with a 1:1 mix of CD45.1 wild type + CD45.2 MAC-ABCDKO BM or 1:1 mix of CD45.1 wild-type + CD45.2 control BM. All parameters were assessed by flow cytometry. A. Total blood monocyte levels. B. CD45.2:CD45.1 monocyte ratio. C. Total blood neutrophil levels. D. CD45.2:CD45.1 neutrophil ratio. *P<0.05, **P<0.01.

Figure 5. Characterization of spleen and plasma of control and MAC-ABCDKO BM transplanted Ldlr−/− mice on the WTD (8 weeks). A. Mcp-1, M-csf, and G-csf mRNA expression in the spleen. B. Mcp-1 and M-csf mRNA expression in FACS-sorted splenic monocytes and macrophages. Mono and macro denote monocytes and macrophages, respectively. C. Monocytes and macrophages as % of total spleen cells assessed by flow cytometry. D-F. Plasma levels of MCP-1 (D), M-CSF (E), and G-CSF (F) assessed by ELISA in Ldlr−/− and Ldlr−/−APOA1TG mice transplanted with control or MAC-ABCDKO BM. n=6-12 mice per group. For A and B, RNA was extracted, and expression of Mcp-1, M-csf, and G-csf was assessed and corrected for the housekeeping gene m36B4. *P<0.05, **P<0.01, ***P<0.001.

Figure 6. Atherosclerosis in control and MAC-ABCDKO BM transplanted Ldlr−/− mice after 7.5 weeks of WTD (n=13-17 mice per group). A. Atherosclerotic lesion area. Haematoxylin-eosin staining was performed on paraffin sections of the aortic root and atherosclerotic lesion area was assessed. B. Relation between atherosclerotic lesion area and cholesterol levels. r denotes the correlation coefficient. In A and B, each data point represents an individual mouse. *P<0.05. C. Inflammatory gene expression in macrophages in atherosclerotic lesions. D. Inflammatory gene expression in macrophages in atherosclerotic lesions of Abca1−/−Abcg1−/− BM transplanted Ldlr−/− mice after 10-11 weeks of Paigen diet. For C and D, LCM was performed to collect macrophages, RNA was isolated and amplified and mRNA expression was assessed and corrected for the housekeeping gene m36B4. *P<0.05, **P<0.01.
Table 1. Plasma cholesterol levels in \( Ldlr^{-/-} \) mice transplanted with MAC-ABC\(^{DKO} \) or \( Abca1^{-/-} Abcg1^{-/-} \) bone marrow on a chow diet.

<table>
<thead>
<tr>
<th>Bone marrow 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(^{DKO} )</td>
<td>211 ± 5*</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. *\( P<0.05 \).
Novelty and Significance

What Is Known?

- Bone marrow expression of the ATP Binding Cassette Transporters ABCA1 and ABCG1 (ABCA1 and ABCG1) decreases atherosclerosis in mice.

- The anti-atherogenic 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.

- ABCA1 and ABCG1 also control proliferation of hematopoietic stem and progenitor cells (HSPCs) and monocytosis.

What New Information Does This Article Contribute?

- Deletion of floxed 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 pro-atherogenic monocytes and neutrophils.

- These findings suggest a role of Abca1/g1 in HSPCs in monocytosis and atherogenesis.

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 anti-atherogenic 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 pro-inflammatory chemokines in plasma that instruct the bone marrow to produce pro-atherogenic 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 anti-atherogenic. 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 one way to monitor the effectiveness of these treatments in humans.
Figure 1

A

ABCA1 protein (% of wild-type)

<table>
<thead>
<tr>
<th></th>
<th>wild-type</th>
<th>control</th>
<th>MAC-ABC&lt;sup&gt;DKO&lt;/sup&gt;</th>
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<tr>
<td>ABCA1</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>β-actin</td>
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</table>

B

ABCG1 protein (% of wild-type)

<table>
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<tr>
<th></th>
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<th>control</th>
<th>MAC-ABC&lt;sup&gt;DKO&lt;/sup&gt;</th>
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<td>ABCG1</td>
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<tr>
<td>β-actin</td>
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C

Abca1 mRNA expression (A.U.)

<table>
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<tr>
<th></th>
<th>HSPC</th>
<th>CMP</th>
<th>GMP</th>
<th>monocytes</th>
<th>neutrophils</th>
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<tr>
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D

Abcg1 mRNA expression (A.U.)

<table>
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<tr>
<th></th>
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<th>GMP</th>
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<th>neutrophils</th>
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<tr>
<td>MAC-ABC&lt;sup&gt;DKO&lt;/sup&gt;</td>
<td><img src="" alt="bar graph" /></td>
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</tr>
</tbody>
</table>
Figure 2

A

![Graph showing cell number distribution across different groups](image)

B

![Graph showing cell number across different groups](image)

C

![Lesion area graph](image)

D

![Percentage of lesion types](image)
Figure 3

- Control BM → Ldlr−/
- Control BM → Ldlr−/APOA1TG
- MAC-ABC^DKO^ BM → Ldlr−/
- MAC-ABC^DKO^ BM → Ldlr−/APOA1TG

**Cell number (×10^6/ml)**

- monocytes
- Ly6-Chi
- Ly6-Clo
- neutrophils

**Significance**

- **p < 0.01
- *p < 0.05

- [Graph showing cell numbers for different conditions]
Figure 4

- CD45.1 wild-type + CD45.2 control BM → Ldlr−/−
- CD45.1 wild-type + CD45.2 MAC-ABC^DKO BM → Ldlr−/−
Figure 5

A

B

mRNA expression (relative to control)

mRNA expression (relative to macrophage control)

C

D

% of spleen cells

mCP-1 (ng/ml)

E

F

plasma M-CSF (ng/ml)

plasma M-CSF (ng/ml)

plasma G-CSF (ng/ml)

plasma G-CSF (ng/ml)

Control BM $\rightarrow$ Ldlr$^{-/-}$

MAC-ABC$^{DKO}$ BM $\rightarrow$ Ldlr$^{-/-}$

Control BM $\rightarrow$ Ldlr$^{-/-}$

MAC-ABC$^{DKO}$ BM $\rightarrow$ Ldlr$^{-/-}$

MAC-ABC$^{DKO}$ BM $\rightarrow$ Ldlr$^{-/-}$-APOA1TG

0.08
0.12
0.16

0.04
0.08
0.12

0.8
1.2
1.6

0.8
1.2
1.6
**Figure 6**

**A**

Lesion area (×10^4 μm^2/section)

- control BM → Ldlr−/−
- MAC-ABC<sup>DKO</sup> BM → Ldlr−/−

**B**

Lesion area (×10^4 μm^2/section) vs. cholesterol (mg/dl)

- MAC-ABC<sup>DKO</sup> BM → Ldlr−/−
  - r = 0.1520
- control BM → Ldlr−/−
  - r = 0.6154

*Note:* Lesion area is measured in ×10^4 μm^2 per section, and cholesterol levels are in mg/dl.
Figure 6

C  Control BM $\rightarrow Ldlr^{-/-}$
MAC-ABCDKO BM $\rightarrow Ldlr^{-/-}$

D  Wild-type BM $\rightarrow Ldlr^{+/+}$
Abca1^{-/-}Abcg1^{-/-} BM $\rightarrow Ldlr^{+/+}$

<table>
<thead>
<tr>
<th>mRNA expression (relative to control)</th>
<th>MIP-1α</th>
<th>MCP-1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type BM</td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td>Abca1^{-/-}Abcg1^{-/-} BM</td>
<td>1</td>
<td>3</td>
</tr>
</tbody>
</table>

- ABCA1
- ABCG1
- MIP-1α
- MCP-1

Control (AU)

$*$ * ***
Deficiency of ABCA1 and ABCG1 in Macrophages Increases Inflammation and Accelerates Atherosclerosis in Mice
Marit Westerterp, Andrew J. Murphy, Mi Wang, Tamara A. Pagler, Yuliya Vengrenyuk, Mojdeh S Kappus, Darren J. Gorman, Prabhakara R. Nagareddy, Xuewei Zhu, Sandra Abramowicz, John S. Parks, Carrie L. Welch, Edward A. Fisher, Nan Wang, Laurent Yvan-Charvet and Alan R. Tall

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SUPPLEMENTAL MATERIAL

Animals and Diets

Ldlr<sup>−/−</sup>, APOA1TG, and CD45.1 mice were purchased from Jackson Laboratories (Bar Harbor, ME). Ldlr<sup>−/−</sup> and APOA1TG were intercrossed to obtain Ldlr<sup>−/−</sup> and Ldlr<sup>−/−</sup>APOA1TG littermates. Abca1<sup>−/−</sup>, Abcg1<sup>−/−</sup> were generated as described previously and backcrossed into the C57BL/6J background for at least 10 generations. Abca1<sup>fl/fl</sup> mice<sup>2</sup> had been backcrossed into the C57BL/6J background for at least 10 generations. LysmCre mice<sup>3</sup> were kindly provided by Dr. Tabas. We generated Abcg1<sup>fl/fl</sup> mice directly into the C57BL/6J background (see below). Abca1<sup>fl/fl</sup> and Abcg1<sup>fl/fl</sup> mice were intercrossed to generate Abca1<sup>fl/fl</sup>Abcg1<sup>fl/fl</sup> (control) mice, which were crossbred with LysmCre mice to generate LysmCre:Abca1<sup>fl/fl</sup>Abcg1<sup>fl/fl</sup> (MAC-ABC<sup>DKO</sup>) 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<sup>fl/fl</sup>Abcg1<sup>fl/fl</sup> 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<sup>fl/fl</sup> mice

Three loxP sites and one neomycin resistance gene were placed around exon 3, the exon that is deleted in general Abcg1<sup>−/−</sup> mice obtained from DeltaGen.<sup>4</sup> 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.<sup>5</sup> 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<sup>fl/fl</sup> 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<sup>DKO</sup> 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<sup>DKO</sup> 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<sup>DKO</sup> mice and cultured in DMEM supplemented with 10% FCS, 1% pen strep. Cells were then incubated with TO901317 (3 μM), 2 μCi/ml [3H]-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 δH-activity was counted in the cell lysate and in the medium. Cholesterol efflux is expressed as ([δH-activity-medium]/[δH-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}\textsubscript{a}, \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 FACS\textregistered\textsuperscript{Aria}, 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{-}, Sac1\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{hi}Ly6-C/G\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/G\textsuperscript{hi}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.\textsuperscript{5} 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, 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 APOA1TG 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 $\chi^2$ 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

6. Han S, Liang CP, DeVries-Seimon T, Ranalletta M, Welch CL, Collins-Fletcher K, Accili D, Tabas I, Tall AR. Macrophage insulin receptor deficiency increases er stress-
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Supplemental Figure I

Figure I. *Abcg1* <sup>flox/flox</sup> 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.
Figure II. ABCA1 and ABCG1 expression in MAC-ABC<sup>DKO</sup> mice. A. ABCA1 and ABCG1 expression in tissues of MAC-ABC<sup>DKO</sup> 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<sup>-/-</sup> transplanted with MAC-ABC<sup>DKO</sup> 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

Figure III. Characterization of MAC-ABC<sup>DKO</sup> mice on chow diet. A. Cholesterol efflux in MAC-ABC<sup>DKO</sup> 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-ABC<sup>DKO</sup> 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-ABC<sup>DKO</sup> 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-ABC<sup>DKO</sup> 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-ABC<sup>DKO</sup> 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.
Supplemental Figure V

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 V. Characterization of MAC-ABCDKO 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.
Figure VI. Lipid accumulation in the spleen of MAC-ABC<sup>DKO</sup> BMT Ldlr<sup>/−</sup> 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\textsuperscript{DKO} BM transplanted \textit{Ldlr}\textsuperscript{-/-} 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.