Tissue-Specific Roles of ABCA1 Influence Susceptibility to Atherosclerosis

Liam R. Brunham, Roshni R. Singaraja, MyNgan Duong, Jenelle M. Timmins, Catherine Fivet, Nagat Bissada, Martin H. Kang, Amrit Samra, Jean-Charles Fruchart, Bruce McManus, Bart Staels, John S. Parks, Michael R. Hayden

Objective—The ATP-binding cassette transporter, subfamily A, member 1 (ABCA1) plays a key role in HDL cholesterol metabolism. However, the role of ABCA1 in modulating susceptibility to atherosclerosis is controversial.

Methods and Results—We investigated the role of ABCA1 in atherosclerosis using a combination of overexpression and selective deletion models. First, we examined the effect of transgenic overexpression of a full-length human ABCA1-containing bacterial artificial chromosome (BAC) in the presence or absence of the endogenous mouse Abca1 gene. ABCA1 overexpression in the atherosclerosis-susceptible Ldlr<sup>−/−</sup> background significantly reduced the development of atherosclerosis in both the presence and absence of mouse Abca1. Next, we used mice with tissue-specific inactivation of Abca1 to dissect the discrete roles of Abca1 in different tissues on susceptibility to atherosclerosis. On the Apoe<sup>−/−</sup> background, mice lacking hepatic Abca1 had significantly reduced HDL cholesterol and accelerated atherosclerosis, indicating that the liver is an important site at which Abca1 plays an antiatherogenic role. In contrast, mice with macrophage-specific inactivation of Abca1 on the Ldlr<sup>−/−</sup> background displayed no change in atherosclerotic lesion area.

Conclusions—These data indicate that physiological expression of Abca1 modulates the susceptibility to atherosclerosis and establish hepatic Abca1 expression as an important site of atheroprotection. In contrast, we show that selective deletion of macrophage Abca1 does not significantly modulate atherogenesis. (Arterioscler Thromb Vasc Biol. 2009;29:548-554.)

Key Words: lipid and lipoprotein metabolism ■ genetically altered mice ■ pathophysiology of atherosclerosis

The ATP binding cassette transporter, subfamily A, member 1 (ABCA1) regulates the rate-limiting step in the biogenesis of high-density lipoprotein (HDL) particles by mediating the efflux of cellular cholesterol and phospholipids to apolipoprotein A-I (apoA-I) and therefore plays a critical role in reverse cholesterol transport. Mutations in ABCA1 in humans cause Tangier disease (TD), characterized by nearly absent HDL cholesterol and lipid accumulation in tissue macrophages. Several recent genome-wide association studies have identified common variants in ABCA1 as a significant source of variation in plasma HDL cholesterol levels across multiple ethnic groups, establishing ABCA1 as a major gene influencing HDL levels in humans. Despite the known role of ABCA1 in determining plasma HDL levels, the impact of ABCA1 on atherosclerosis remains controversial and incompletely understood. TD patients have increased risk of coronary artery disease relative to related controls, though perhaps not as great a risk as would be expected based on their extremely low HDL cholesterol levels. In contrast, a recent study suggested that low HDL caused by loss-of-function mutations in ABCA1 does not contribute to risk of cardiovascular disease in the general population. Studies in mouse models have also yielded conflicting results. Mice lacking Abca1 globally exhibit no change in susceptibility to atherosclerosis. However, deletion of Abca1 in bone marrow–derived hematopoietic cells results in increased atherosclerosis, and overexpression of bone marrow Abca1 reduces atherosclerosis. Conversely, transgenic mice significantly overexpressing Abca1 in liver and macrophages have increased plasma HDL cholesterol and apolipoprotein B (apoB) levels, yet have increased atherosclerosis on genetic backgrounds lacking either apolipoprotein E (Apoe<sup>−/−</sup>) or the low density lipoprotein receptor (Ldlr<sup>−/−</sup>). These data led to the hypothesis that hepatic Abca1 may actually play a proatherogenic role in vivo by virtue of its effect on the metabolism of apoB-containing lipoproteins.

Here we addressed the role of ABCA1 in atherosclerosis using two complimentary approaches. We first determined...
the effect of physiological overexpression of a full-length ABCA1-containing bacterial artificial chromosome (BAC) on the Ldlr−/− background, in the presence or absence of the endogenous mouse Abca1 gene. We then investigated in which tissues ABCA1 exerts its effect on atherosclerosis using mice with tissue-specific inactivation of Abca1. We hypothesized that ABCA1 in either the liver, by virtue of its key role in HDL biogenesis,17 or the macrophage, because of its role in removal of vessel wall cholesterol, could impact atherosclerosis. We therefore studied mice that specifically lack either hepatic or macrophage Abca1, on the Apoe−/− and Ldlr−/− backgrounds, respectively. Our results indicate that ABCA1 overexpression protects against atherosclerosis on the Ldlr−/− background, and that hepatic Abca1 is an important site of ABCA1-mediated atheroprotection.

Methods and Materials

Detailed descriptions of animals, reagents, and methods are described in the supplemental methods section (available online at http://atvb.ahajournals.org). All studies were approved by institutional review boards. Protein expression lipoprotein analysis and atherosclerosis measurements were performed as previously described.18–20

Results

Previous studies have examined atherosclerosis in ABCA1 BAC transgenic mice on the Apoe−/− background.20 However, subsequent to this study, it was found that the BAC studied lacked exon 1 of the ABCA1 gene.21 We therefore used recently described transgenic mice containing a full-length human ABCA1 BAC including 25 kilobases of upstream promoter background, and that hepatic Abca1 is an important site of ABCA1-mediated atheroprotection.

Expression of human ABCA1 in Ldlr−/− mice did not result in significant differences in atherosclerotic lesion area (Ldlr−/−: 25.6±4.0 mm² versus BAC+: Ldlr−/−: 39.8±12.8 mm², n=5, P=ns; Figure 2), likely reflecting the small increase in ABCA1 protein observed in these mice, as well as the relatively small lesion area observed. On a WTD, where ABCA1 protein expression was further increased, a significant 2-fold reduction in lesion area was observed in the Ldlr−/− mice expressing the ABCA1 BAC (Ldlr−/−: 491.1±173.30 mm² versus BAC+: Ldlr−/−: 211.5±115.4 mm², n=5, P=0.02; Figure 2).

Because both human and mouse ABCA1 are present in the Ldlr−/− and BAC+: Ldlr−/− mice, and because both genes respond to feeding of WTD (Figure 1), the reduced susceptibility to atherosclerosis we observed in these mice could be accounted for by either the human ABCA1 transgene, the endogenous murine Abca1 gene, or a combination of both. To determine the antiatherogenic properties of human ABCA1 without the potential contribution of the endogenous mouse gene, we next assessed atherosclerosis in mice globally deficient for mouse Abca1 with or without the human ABCA1 BAC. Ldlr−/−: Abca1−/− double-knock-out mice had reduced plasma total cholesterol, HDL cholesterol, and triglyceride levels compared to Ldlr−/− mice (supplemental Figures I and II), consistent with previous reports.5 When the ABCA1 BAC transgene was introduced to the Ldlr−/−: Abca1−/− double-knock-out mice, plasma HDL cholesterol, apoa-I and apoa-II levels were significantly increased on both the chow and WTD (supplemental Figure II), indicating that human ABCA1 expression alone was sufficient to raise plasma HDL cholesterol levels in this genetic background. On the WTD, the expression of ABCA1 also resulted in increased triglycerides and LDL cholesterol, with no change in VLDL cholesterol levels (supplemental Figure II).

Expression of human ABCA1 in the Ldlr−/−: Abca1−/− double-knock-out chow-fed mice resulted in a significant decrease in atherosclerotic lesions (Ldlr−/−: Abca1−/−: 18.2±6.5 mm² versus BAC+: Ldlr−/−: Abca1−/−: 40.0±1.0 mm², n=5, P=0.02). This difference was also evident when the mice were fed the WTD (Ldlr−/−: Abca1−/−: 560.2±143.5 mm² versus BAC+: Ldlr−/−: Abca1−/−: 292.9±31.2 mm², n=5, P=0.04; Figure 3). These data indicate that full-length human ABCA1 expression significantly reduces susceptibility to atherosclerosis in the absence of mouse Abca1.

We next investigated in which specific tissues ABCA1 exerts its major antiatherosclerotic effect by studying mice with tissue-specific deletion of Abca1. We hypothesized that the liver, which is the major site of HDL biogenesis in mice,17 would be an important site for ABCA1-mediated atheroprotection. To test this hypothesis, we generated mice that specifically lack hepatic Abca1 on the Apoe−/− background. Mice deficient for hepatic Abca1 (Abca1−/−)17 on a mixed 129SvEv/C57BL6 genetic background were bred to congenicity on the C57BL6 background by crossing them to wild-type C57BL6 mice through the use of a speed congenic approach.

Hepatic Abca1 protein expression was markedly reduced in Apoe−/−: Abca1−/− mice (Figure 4A). The expression of the scavenger receptor class B, 1 (Srb1) in liver was equivalent between genotypes (Figure 4A). Thioglycollate-elicted perito-
neal macrophages from $\text{Apoe}^{/-}$ and $\text{Apoe}^{/-}\text{Abca1}^{/-/-}$ mice continued to express Abca1, indicating that the deletion of Abca1 was specific to the liver.

The Table shows plasma lipid and apolipoprotein concentrations in 4-hour fasted 12-week-old $\text{Apoe}^{/-}$ and $\text{Apoe}^{/-}\text{Abca1}^{/-/-}$ mice. Plasma total cholesterol and phospholipid levels were reduced by 30% and 20%, respectively, in mice lacking hepatic Abca1 (Table). Plasma HDL cholesterol and apoA-I levels were reduced by $\approx 50\%$ in $\text{Apoe}^{/-}\text{Abca1}^{/-/-}$ mice. Plasma LDL and VLDL cholesterol levels were reduced by $\approx 25\%$ and $\approx 35\%$, respectively (Table), as has been previously observed in models of selective hepatic Abca1 deficiency.\textsuperscript{17,22} Plasma triglyceride levels were not significantly different between genotypes.

Mice lacking hepatic Abca1 displayed a significant $\approx 75\%$ increase in aortic atherosclerotic lesion area compared to $\text{Apoe}^{/-}$ mice ($44.4\pm 30\text{ mm}^2$ versus $73.3\pm 34\text{ mm}^2$, $n=4$ to $5$, $P<0.01$; Figure 4C). The increase in atherosclerotic lesion area was observed in both male and female mice. $\text{Apoe}^{/-}\text{Abca1}^{/-/-}$ mice also displayed a significant increase in both aortic total cholesterol ($P<0.05$) and free cholesterol ($P<0.001$) levels (Figure 4D).
data indicate that hepatic Abca1 plays a key role in protection against atherosclerosis.

To determine whether hepatic lipid content is impacted by absence of hepatic Abca1 and subsequent reduction in hepatic HDL biogenesis, we measured the concentration of lipids in livers of these mice. We observed no difference in the concentration of total or free cholesterol between genotypes (supplemental Figure IVA). Similarly, phospholipids and triglyceride levels were not altered (supplemental Figure IVB). Notably, hepatic Srb1 expression was not altered (Figure 4A) suggesting that Srb1-mediated uptake of HDL was not altered in the absence of hepatic Abca1.

To assess the contribution of macrophage Abca1 to susceptibility to atherosclerosis, we next examined aortic atherosclerotic lesion area in mice lacking Abca1 in macrophages on the Ldlr−/− background. Mice lacking macrophage Abca1 have been recently described23 and have complete loss of macrophage efflux but no significant change in plasma lipid levels. At 16 weeks of high-fat diet feeding, lesion area was not significantly altered in Ldlr−/−; Abca1−/−/− compared to Ldlr−/− mice (Figure 5A; 540.8±24 mm² v. 484.9±48 mm², n=12; P=0.3). Similarly, aortic lesion area was not significantly altered by the absence of macrophage Abca1 at 12 or 20 weeks of age (data not shown), indicating that macrophage Abca1 does not have a major impact on atherosclerosis.

Discussion

Despite universal acceptance of the crucial role of ABCA1 in maintenance of plasma HDL levels, significant controversy still exists as to its role in influencing susceptibility to atherosclerosis. To determine the role of ABCA1 in atherosclerosis we examined the effects of overexpression or deletion of ABCA1 in different animal models. We first crossed mice expressing a full-length human ABCA1 BAC to Ldlr−/− mice and found that the expression of ABCA1 in this

Figure 2. Atherosclerosis in BAC+/−; Ldlr−/− and Ldlr−/− control littermate mice. Aortas isolated from BAC+/−; Ldlr−/− and Ldlr−/− mice were fixed in 4% paraformaldehyde and sections were stained with Oil Red O for neutral lipids. Ldlr−/− (A) and BAC+/−; Ldlr−/− (B) on chow diet. Ldlr−/− (C) and BAC+/−; Ldlr−/− (D) on WESTERN type diet (WTD). E, Lesions were quantified from 4 slices from each of 5 mice per condition.

Figure 3. Atherosclerosis in BAC+/−; Ldlr−/−; Abca1−/− and Ldlr−/−; Abca1−/− control littermate mice. Aortas isolated from BAC+/−; Ldlr−/−; Abca1−/− and Ldlr−/−; Abca1−/− mice were fixed in 4% paraformaldehyde and sections were stained with Oil Red O for neutral lipids. Ldlr−/−; Abca1−/− (A) and BAC+/−; Ldlr−/−; Abca1−/− (B) on chow diet. Ldlr−/−; Abca1−/− (C) and BAC+/−; Ldlr−/−; Abca1−/− (D) on Western type diet (WTD). E, Lesions were quantified from 4 slices from each of 5 mice per condition.
background did not result in major alterations in plasma lipids, but did result in a significant reduction in atherosclerotic lesion area when fed a WTD. Assessing the effect of human ABCA1 expression in the absence of mouse Abca1 in Ldlr−/−; Abca1−/− double knock-out mice again revealed a significant reduction in atherosclerosis in mice expressing ABCA1 accompanied by an increase in plasma HDL cholesterol levels. These data establish that physiological expression of appropriately regulated ABCA1 reduces atherosclerosis in mice, consistent with previous data.20

We next used mice with tissue-specific inactivation of Abca1 to determine the specific tissue sites at which Abca1 impacts atherogenesis. The two major candidate sites at which Abca1 might modulate susceptibility to atherosclerosis are the liver, because of its major effect on HDL levels,17 and the macrophage, implicated in previous studies to impact atherogenesis.8–10 Abca1−/−; Abca1−/−−/− L mice developed significantly more atherosclerotic burden compared to control Abca1−/− mice, establishing the liver as an important site of ABCA1-mediated atheroprotection. In these mice, it seems likely that the significant reduction in HDL is the major mechanism by which Abca1 modulates susceptibility to atherosclerosis. In contrast, Ldlr−/−; Abca1−/−−/− M mice displayed no increased susceptibility to atherosclerosis at any time point studied, indicating that macrophage Abca1 does not exert a major effect on atherosclerosis.

Our data showing no change in atherosclerosis in mice lacking macrophage Abca1 are unexpected based on previous studies using bone marrow transplantation which showed increased atherosclerosis in lethally-irradiated recipients of Abca1-deficient bone marrow,50 and which thus extrapolated that deficiency of Abca1 in macrophages was responsible for this effect. A number of substantial methodological differences are likely to explain these divergent results. First, bone marrow transplantation studies involve the depletion of Abca1 in all hematopoietic cells. In contrast, the myeloid specific M lysozyme promoter used to drive Cre expression in Ldlr−/−; Abca1−/−−/− M mice is restricted to macrophages and neutrophils34 and leads to very low levels of target-gene deletion

### Table. Plasma Lipid and Apolipoprotein Concentrations in Abca1−/− and Abca1−/−−/− L Mice

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Abca1 Genotype</th>
<th>TPC (mg/dl)</th>
<th>LDL Cholesterol (mg/dl)</th>
<th>HDL Cholesterol (mg/dl)</th>
<th>LDL:HDL Ratio</th>
<th>VLDL Cholesterol (mg/dl)</th>
<th>PL (mg/dl)</th>
<th>TG (mg/dl)</th>
<th>ApoA-I (mg/dl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>−/−</td>
<td>+/+</td>
<td>380 ± 19</td>
<td>169.6 ± 6</td>
<td>29.6 ± 12</td>
<td>5.9 ± 4</td>
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<td>258.4 ± 16</td>
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<tr>
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<td>L−/−</td>
<td>263 ± 20</td>
<td>124.1 ± 10</td>
<td>14.1 ± 8</td>
<td>8.9 ± 3</td>
<td>116.5 ± 11</td>
<td>204.2 ± 16</td>
<td>44.9 ± 8</td>
<td>44.9 ± 8</td>
</tr>
<tr>
<td>−/−</td>
<td>L−/−−/−</td>
<td>0.0006</td>
<td>0.0009</td>
<td>0.04</td>
<td>0.08</td>
<td>0.002</td>
<td>0.03</td>
<td>0.3</td>
<td>0.3 ± 0.09</td>
</tr>
</tbody>
</table>

Values are mean ± SEM. The No. of mice is indicated in parentheses. Measurements were performed on plasma from 4-hour fasted mice. TPC indicates total plasma cholesterol. Differences were calculated with a 2-tailed Student t test.

Figure 4. Characterization of mice lacking hepatic Abca1. Western blot of (A) liver lysates and (B) thioglycollate-elicited peritoneal macrophages from Apoe−/− and Apoe−/−; Abca1−/−−/− L mice. C, Aortic lesion area in Apoe−/− and Apoe−/−; Abca1−/−−/− L mice. D, Aortic total and free cholesterol levels, n=4 to 5 per group. *P<0.05, **P<0.001.
in dendritic cells and virtually none in lymphocytes, where Abca1 is highly expressed. This may suggest that the increased atherosclerosis observed in mice receiving Abca1 null bone marrow is attributable to loss of Abca1 in lymphocyte, rather than myeloid cells. Furthermore, bone marrow transplantation may also lead to repopulation of bone marrow–derived endothelial progenitor cells, thought to be important in restoration of the endothelium after ischemic injury, whereas this cell population would not be impacted in Abca1−/− mice. It is possible that lethal irradiation, the bone marrow transplantation procedure, or loss of Abca1 in some population of hematopoietic cells may lead to an immunologic response, as indicated by the significant leukocytosis in recipients of Abca1 null bone marrow, suggesting the creation of an inflammatory milieu that may itself lead to accelerated atherosclerosis.

A further difference is that bone marrow transplantation studies transplanted bone marrow from mice wild-type for the LDL receptor into an Ldlr−/− recipient. In contrast, macrophages from Ldr−/−; Abca1−/− mice lack both the LDL receptor and Abca1. LDL receptor–deficient macrophages are reported to have significantly reduced Abca1 levels compared to wild-type, implying that Abca1 may already be partially inactivated in macrophages from Ldr−/− mice, and thus limiting the difference that could be expected to be observed between Ldr−/−; Abca1−/− and Ldr−/− mice in terms of macrophage Abca1. Further studies on different athero-susceptible genetic backgrounds may help resolve these issues.

Our data agree with a previous study showing no differences in atherosclerotic lesions in Ldr−/−; Abca1−/− mice when compared to Ldlr−/− mice on a high-fat diet. The decrease in endogenous Abca1 in these mice might be expected to increase atherosogenesis because of the loss of Abca1 in hepatocytes and the resultant decrease in HDL levels. However, these mice also show a significant decrease in plasma total and non-HDL cholesterol and triglycerides, thereby providing an antiatherogenic lipid profile which may compensate for the reduction in HDL.

Our data are in contrast to a study by Joyce et al, who found significant increases in both a proatherogenic lipid profile and in atherosclerosis when mice overexpressing Abca1 were crossed to Ldr−/− mice. These authors also found significant increases in atherosclerotic lesions in Apoe−/− mice expressing Abca1. The reasons for these differences likely reflect the different levels of expression and regulation of Abca1 in these different animal models. Joyce et al used Abca1 cDNA mice which were driven by the ApoE promoter with its hepatic and macrophage elements, whereas in our mice Abca1 was driven by the endogenous promoter, which may be necessary to respond to physiological stimuli and for proper expression, subcellular localization and function of Abca1. The Abca1 BAC mice in this study had a transgene copy number of two, resulting in a small but significant increase in ABCA1 protein (Figure 1). In contrast, mice transgenic for the Abca1 cDNA driven by the ApoE promoter had a copy number of 30. High levels of expression of an Abca1 cDNA driven by the CMV promoter may result in nonphysiological subcellular localization of Abca1 and has previously been shown to result in a significant increase in not only HDL cholesterol but in proatherogenic apoB-containing lipoproteins as well, which may explain why atherosclerosis was increased in this model.

The relationship between ABCA1 and the metabolism of apoB-containing lipoproteins is not fully understood. Ldr−/−; Abca1−/− mice have reduced levels of LDL and VLDL cholesterol, consistent with previous reports of mice lacking hepatic Abca1. Mice lacking intestinal Abca1 also have reduced levels of LDL cholesterol. The reduction in apoB levels in conditions of Abca1 deficiency is hypothesized to be secondary to the reduction in HDL, resulting in a smaller pool of plasma cholesterol for transfer to apoB-containing lipoproteins and their subsequent hypercatabolism. Whether ABCA1 also influences apoB-containing particle metabolism more directly is not known.

In summary, we have demonstrated that physiological expression or deletion of ABCA1 modulates susceptibility to atherosclerosis in mice. Our results identify the liver as an important site at which Abca1 inhibits atherosogenesis, suggesting that hepatic ABCA1 may be an appropriate therapeutic target for raising HDL levels and reducing atherosclerosis in humans. Our study does not provide support for the previously held view that macrophage Abca1 influences atherosogenesis. Whether Abca1 in other tissues, such as the intestine, lymphocytes, endothelium, or adipose tissue, also influences atherosclerosis is not known, but can now be investigated with the use of tissue-specific deletion models of Abca1.

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Disclosures

None.

References


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Brunham et al.

Supplemental Information and Data

Material and Methods:

Generation of mice

Human \textit{ABCA1} transgenic mice were generated as previously described \textsuperscript{1} and were crossed to \textit{Ldlr}^{−/−} mice (Jackson laboratories, Bar Harbor, Maine) in order to generate \textit{BAC+;Ldlr}^{−/−} mice that overexpress \textit{ABCA1}, and \textit{Ldlr}^{−/−} littermates. \textit{Abca1}^{−/−} mice (provided by Dr. Omar Francone, Pfizer Global Research and Development) were crossed to the \textit{Ldlr}^{−/−} mice to generate double knock-out \textit{Ldlr}^{−/−};\textit{Abca1}^{−/−} mice. These mice were crossed to \textit{BAC transgenic mice and resulted in BAC+;Ldlr}^{−/−};\textit{Abca1}^{−/−} mouse and \textit{Ldlr}^{−/−};\textit{Abca1}^{−/−} control littermates. Mice were group housed and given access to diet and water \textit{ad libitum}. Age matched male mice were fed a high fat western type diet containing 0.25% cholesterol and 15% cocoa butter (Purified diet W, Hope Farms, The Netherlands) or control chow diet (Autoclavable Lab Diet 5010, Picolab) for 10 weeks.

For the generation of mice lacking hepatic \textit{Abca1} (\textit{Abca1}^{−/−}) and Apoe (\textit{Apoe}^{−/−}), we performed a speed backcrossing assay using ABI PRISM© Mapping Primers (Applied Biosystems (ABI), Foster City, CA), informative between the C57BL6 and 129Sv/Ev strains, and spaced at an average distance of 20 cM across the genome (excluding the X
Supplement Material

and Y chromosomes). PCR fragments were analyzed using Gene Mapper 3.0 (ABI, Foster City, CA).

Mice lacking macrophage Abca1 (Abca1$^{−/−}$) have been previously described and were crossed to Ldlr$^{−/−}$ mice to generate the mice in this study on a mixed 90% C57BL6 background. Ldlr$^{−/−}$;Abca1$^{−/−}$ mice and Ldlr$^{−/−}$ controls were fed a chow diet up until 8 weeks of age, then fed a high-fat diet containing 10% palm oil and 0.2% cholesterol. Mice were sacrificed at 12, 16 or 20 weeks of age and atherosclerosis quantified as below.

All animal protocols were approved by the institutional animal care committee.

**Protein expression**

Mice were sacrificed at the time points indicated and tissues were harvested and flash frozen. Tissue lysates were prepared as previously described. Proteins were separated on polyacrylamide gels, transferred onto PVDF membranes and probed with anti-ABCA1 polyclonal antibody generated as previously described. Anti-GAPDH was used as a protein loading control (Chemicon, Temecula, CA).

**Lipid and lipoprotein analysis**

Blood was collected from mice at sacrifice and plasma isolated. Plasma lipoprotein and lipid (total cholesterol, free cholesterol, triglycerides) concentrations were determined by enzymatic assays adapted to microtiter plates using commercially available reagents.
(BioMerieux, Lyon, France for total cholesterol RTU and triglycerides PAP 1000; Wako Chemicals GmbH, Neuss, Germany for free cholesterol). Fast Protein Liquid Chromatography was performed as previously described. Cholesterol concentrations were determined in the eluted fractions. Plasma levels of apoA-I, apoA-II, apoC-III and apoB were determined by an immunonephelometric assay using specific mouse antibodies developed in rabbits.

Total and free aortic cholesterol content was measured by gas liquid chromatography after lipid extraction of the 10% neutral buffered formalin fixed aorta as described previously. Liver lipid analysis was performed by enzymatic assay of detergent extracted liver.

**Atherosclerotic lesion quantification**

At sacrifice, mice were perfused transcardially with 4% paraformaldehyde and the hearts with attached aortas were isolated and stored in 4% PFA. Tissues were embedded in Optimal Cutting Temperature (Tissuetek) media in a plastic mold, frozen and sixteen 10µm sections were cut. For Oil Red O (ORO) staining, the sections were rinsed in water and isopropanol, and stained in 0.25% ORO for 20 minutes, followed by an isopropanol rinse. Sections were counterstained in Gill’s hematoxylin for 1 minute, and mounted. Transverse sections were obtained from the apex of the heart moving toward the aortic region, with sections beginning at the point where all three aortic valve cusps became clearly visible. Every fourth section was placed on a slide for ORO staining of neutral
lipid, and counter stained with hematoxylin, such that each slide had four sections 40µm apart.

Lesion areas were photographed using an Axioskop2 (Zeiss) microscope, with a SPOT (Diagnostic Instruments, Inc.) camera, and Northern Eclipse software (Empix Imaging) was used for quantification of ORO positive areas within the lesion sites. The total ORO staining area within the sinuses of valsalva of four sections were averaged to provide the lesion area for each mouse.

**Statistical analysis**

Values are presented as mean ± standard error of the mean. Differences between groups were calculated with a two-tailed Student’s t-test with a P value of 0.05 considered statistically significant. Calculations were performed using Graphpad Prism software.
Supplement Material

Reference List


Supplemental Figure Legends:

Supplemental Figure 1. Plasma lipid and apolipoprotein levels in BAC+;Ldlr\(^{-/-}\) and 
Ldlr\(^{-/-}\) control littermate mice. Total, HDL, LDL and VLDL cholesterol levels were 
quantified in on chow and WTD. n=5 for each group of mice.

Supplemental Figure 2. Plasma lipoprotein and apolipoprotein levels in BAC+;Ldlr\(^{-/-}\)
;Abca1\(^{-/-}\) and Ldlr\(^{-/-}\);Abca1\(^{-/-}\) control littermate mice. Total, HDL and non-HDL 
cholesterol levels were quantified on chow and WTD. n=5 for each group of mice.

Supplemental Figure 3. Hepatic lipid content in Apoe\(^{-/-}\);Abca1\(^{+/+}\) and Apoe\(^{-/-}\)
;Abca1\(^{-/-}\) mice. (A) Hepatic total and free cholesterol concentration. (B) Hepatic 
phospholipids and TG content. n=3 mice per group.
Supplement Material

Total Cholesterol

Triglycerides

VLDL-C

LDL-C

HDL-C

ApoA-I

ApoA-II

ApoC-III

ApoB

Supplemental Figure 1
Supplemental Material

$Ldlr^{-/-}; Abca1^{-/-}$

BAC+$Ldlr^{-/-}; Abca1^{-/-}$

**Total cholesterol**

- **Triglycerides**
  - **VLDL-C**
  - **LDL-C**
  - **HDL-C**

**ApoA-I**

- **ApoA-II**

- **ApoC-III**

- **ApoB**

- **Supplemental Figure 2**
Supplemental Figure 3

A

\[ \text{\(\mu g\) lipid/mg protein} \]

- **Total cholesterol**
- **Free cholesterol**

**Legend:**
- Apoe\(^{-}\); Abca1\(^{+/+}\)
- Apoe\(^{-}\); Abca1\(^{-/-}\)

B

\[ \text{\(\mu g\) lipid/mg protein} \]

- **Phospholipid**
- **Triglyceride**

**Legend:**
- Apoe\(^{-}\); Abca1\(^{+/+}\)
- Apoe\(^{-}\); Abca1\(^{-/-}\)