Activation of Liver X Receptor Decreases Atherosclerosis in Ldlr\textsuperscript{−/−} Mice in the Absence of ATP-Binding Cassette Transporters A1 and G1 in Myeloid Cells

Mojdeh S. Kappus, Andrew J. Murphy, Sandra Abramowicz, Vusisizwe Ntonga, Carrie L. Welch, Alan R. Tall, Marit Westerterp

**Objective**—Liver X receptor (LXR) activators decrease atherosclerosis in mice. LXR activators (1) directly upregulate genes involved in reverse cholesterol transport and (2) exert anti-inflammatory effects mediated by transrepression of nuclear factor-κB target genes. We investigated whether myeloid cell deficiency of ATP-binding cassette transporters A1 and G1 (ABCA1/G1), principal targets of LXR that promote macrophage cholesterol efflux and initiate reverse cholesterol transport, would abolish the beneficial effects of LXR activation on atherosclerosis.

**Approach and Results**—LXR activator T0901317 substantially reduced inflammatory gene expression in macrophages lacking ABCA1/G1. Ldlr\textsuperscript{−/−} mice were transplanted with Abca1\textsuperscript{−/−}Abcg1\textsuperscript{−/−} or wild-type bone marrow (BM) and fed a Western-type diet for 6 weeks with or without T0901317 supplementation. Abca1/g1 BM deficiency increased atherosclerotic lesion complexity and inflammatory cell infiltration into the adventitia and myocardium. T0901317 markedly decreased lesion area, complexity, and inflammatory cell infiltration in the Abca1\textsuperscript{−/−}Abcg1\textsuperscript{−/−} BM–transplanted mice. To investigate whether this was because of macrophage Abca1/g1 deficiency, Ldlr\textsuperscript{−/−} mice were transplanted with LysmCreAbca1\textsuperscript{fl/fl}Abcg1\textsuperscript{fl/fl} or Abca1\textsuperscript{−/−}Abcg1\textsuperscript{−/−} BM and fed Western-type diet with or without the more specific LXR agonist GW3965 for 12 weeks. GW3965 decreased lesion size in both groups, and the decrease was more prominent in the LysmCreAbca1\textsuperscript{fl/fl}Abcg1\textsuperscript{fl/fl} group.

**Conclusions**—The results suggest that anti-inflammatory effects of LXR activators are of key importance to their antiatherosclerotic effects in vivo independent of cholesterol efflux pathways mediated by macrophage ABCA1/G1. This has implications for the development of LXR activators that lack adverse effects on lipogenic genes while maintaining the ability to transrepress inflammatory genes. (Arterioscler Thromb Vasc Biol. 2014;34:279-284.)

**Key Words:** atherosclerosis ■ ATP-binding cassette transporters ■ inflammation ■ liver X receptor ■ macrophages

Liver X receptors (LXR) are ligand-activated transcription factors belonging to the nuclear hormone receptor superfamily.\textsuperscript{1} LXR-α is expressed in the liver, adipose, intestine, kidney, and macrophages, whereas expression of LXR-β is nearly ubiquitous.\textsuperscript{2} LXRs are activated by oxysterols formed in response to increased intracellular cholesterol levels.\textsuperscript{3} Synthetic compounds such as T0901317 (T0) and GW3965 (GW) have also been established as active oral LXR agonists.\textsuperscript{5,6} Further investigation into the role of LXR in cholesterol homeostasis led to the discovery that synthetic LXR activators decrease atherosclerosis in multiple mouse models, including Apoe\textsuperscript{−/−} and Ldlr\textsuperscript{−/−} mice.\textsuperscript{7,8} Two principal properties of LXR activators that may contribute to their antiatherogenic effects are (1) direct upregulation of genes involved in macrophage cholesterol efflux and reverse cholesterol transport (RCT) such as the ATP-binding cassette transporters A1 and G1 (ABCA1 and ABCG1)\textsuperscript{9} and (2) anti-inflammatory effects mediated by a distinct molecular mechanism involving SUMOylation of LXR and transrepression of nuclear factor-κB (NF-κB) target genes.\textsuperscript{10,11} Although (1) is known to be antiatherogenic, the role of (2) in mediating antiatherogenic effects of LXR activators has not been explored. Therefore, we investigated whether myeloid or macrophage deficiency of ABCA1 and ABCG1, 2 principal targets of LXR as well as key components of the cholesterol efflux pathway, would abolish the beneficial effects of LXR activation on atherosclerosis.

**See accompanying editorial on page 242**

**Materials and Methods**

GW was kindly provided by Dr Jon Collins, Glaxo Smith Kline. Materials and Methods are available in the online-only Supplement.
Nonstandard Abbreviations and Acronyms

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Acronym</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABCA1/G1</td>
<td>ATP-Binding Cassette Transporter A1/G1</td>
<td></td>
</tr>
<tr>
<td>BM</td>
<td>bone marrow</td>
<td></td>
</tr>
<tr>
<td>GW</td>
<td>GW3965</td>
<td></td>
</tr>
<tr>
<td>HDL</td>
<td>high-density lipoprotein</td>
<td></td>
</tr>
<tr>
<td>LDLr</td>
<td>low-density lipoprotein receptor</td>
<td></td>
</tr>
<tr>
<td>LPS</td>
<td>lipopolysaccharide</td>
<td></td>
</tr>
<tr>
<td>LXR</td>
<td>liver X receptor</td>
<td></td>
</tr>
<tr>
<td>Mcp-1</td>
<td>monocyte chemotactic protein 1</td>
<td></td>
</tr>
<tr>
<td>Mip-1α</td>
<td>macrophage inflammatory protein 1α</td>
<td></td>
</tr>
<tr>
<td>RCT</td>
<td>reverse cholesterol transport</td>
<td></td>
</tr>
<tr>
<td>T0</td>
<td>T0901317</td>
<td></td>
</tr>
<tr>
<td>TG</td>
<td>triglyceride</td>
<td></td>
</tr>
<tr>
<td>VLDL</td>
<td>very-low-density lipoprotein</td>
<td></td>
</tr>
<tr>
<td>WTD</td>
<td>Western-type diet</td>
<td></td>
</tr>
</tbody>
</table>

Results

LXR Activation Decreases Inflammatory Gene Expression in Lipopolysaccharide-Stimulated Macrophages Lacking ABCA1 and ABCG1 In Vitro

We have shown that the ability of LXR activator to increase cholesterol efflux to HDL or apoA1 is abolished in macrophages lacking ABCA1 and ABCG1.1,11 To determine whether LXR activator treatment retained its anti-inflammatory effects, we treated LysmCreAbca1fl/flAbcg1fl/fl control macrophages with T0, and then stimulated them with lipopolysaccharide. We subsequently examined expression of the inflammatory genes Il-1β, Il-6, Mcp-1, and Mip-1α using qPCR. As reported, lipopolysaccharide-induced inflammatory gene expression was increased in LysmCreAbca1fl/flAbcg1fl/fl macrophages.11 T0 treatment led to significantly decreased inflammatory gene expression (Il-1β, 33%; Il-6, 36%; Mcp-1, 42%; and Mip-1α, 35% [all P<0.05]) in LysmCreAbca1fl/flAbcg1fl/fl macrophages indicating that its anti-inflammatory effects occur independent of ABCA1/G1 expression (Figure 1). Interestingly, LXR activators showed similar effects in Abca1fl/flAbcg1fl/fl macrophages (Figure 1); however, the effect was less pronounced, and statistical significance was only reached for Mip-1α (P<0.05). This is consistent with the known anti-inflammatory effects of LXR activators, which have been demonstrated in vitro and in vivo.10,11,14 The more pronounced anti-inflammatory effects in macrophages lacking ABCA1/G1 may reflect their higher baseline inflammatory gene expression.

LXR Activation Decreases Atherosclerosis in Ldlr−/− Mice Transplanted With Abca1−/−Abcg1−/− Bone Marrow

Ldlr−/− mice were transplanted with either wild-type or Abca1−/−Abcg1−/− bone marrow (BM) and fed a Western-type diet (WTD) for 6 weeks supplemented with or without the LXR activator T0 (10 mg/kg). Reconstitution of the BM was >90% (results not shown). Cholesterol and triglyceride (TG) levels were measured after 3 weeks of WTD feeding with or without T0 (Figure I in the online-only Data Supplement). BM Abca1−/−/G1−/− deficiency decreased plasma cholesterol and TG levels by 64% and 31%, respectively (both P<0.001), which was mainly confined to the VLDL/LDL fraction for cholesterol (Figure II in the online-only Data Supplement). The decrease in plasma lipids has been previously observed in WTD-fed Ldlr−/− mice transplanted with Abca1−/−Abcg1−/− BM and may partly reflect increased uptake of LDL by a non-Ldlr-dependent mechanism in the expanded myeloid cell compartment of these mice as has been shown in humans with myeloproliferative diseases.16 We also found an ≈82% decrease in mRNA expression of sterol regulatory element–binding protein-1c in the liver of Ldlr−/− mice transplanted with Abca1−/−Abcg1−/− BM compared with their controls (P<0.001; Figure IIIA in the online-only Data Supplement). This could perhaps reflect effects of decreased adipose tissue and insulin sensitization in myeloid ABCA1/G1 deficiency,17 presumably decreasing plasma insulin levels and hepatic Akt signaling. In wild-type BM-transplanted mice, chronic treatment with T0 caused a decrease in TG and cholesterol levels in VLDL/LDL, as reported; however, in Abca1−/−Abcg1−/− BM-transplanted mice, T0 treatment caused a slight increase in TG and cholesterol levels in VLDL/LDL (Figures I and II in the online-only Data Supplement). In both groups of mice, T0 increased liver TG accumulation by 2.5-fold (P<0.001; Figure IIIB in the online-only Data Supplement).

After 4 weeks of diet and after 2 consecutive IP injections of T0 or vehicle, blood samples were taken to analyze leukocyte subsets. Monocytes and neutrophils were assessed as a percentage of total leukocytes (Figure IV in the online-only Data Supplement). Abca1−/−Abcg1−/− BM-transplanted mice showed a nonsignificant ≈31% increase in monocytes and a ≈147% increase in neutrophils (P<0.001) as compared...
with wild-type BM-transplanted mice. Although the LXR activator T0 had little effect in wild-type BM-transplanted mice, it increased monocyte activity by 8% in Abca1−/−Abcg1−/− BM-transplanted mice (P<0.05), mainly because of an increase in the Ly6−Chit subpopulation (P<0.01; Figure IV in the online-only Data Supplement). The increase in blood lipids and monocytes resulting from T0 treatment would not be expected to have a beneficial effect on atherosclerosis.

After 6 weeks of diet, all mice were euthanized, and the aortic root was collected to analyze atherosclerotic lesions. Similar to a previous report in this model,15 atherosclerotic lesion area was slightly but not significantly increased in Ldlr−/− mice transplanted with Abca1−/−Abcg1−/− BM compared with wild-type mice (Figure 2A and Figure V in the online-only Data Supplement). The nonsignificant increase in lesion area was likely because of the marked lowering of plasma lipids in the Abca1−/−Abcg1−/− group; the lowering of plasma lipids does not occur in the Ldlr−/− background.12,18 In the control group, the LXR activator did not affect lesion area (Figure 2A and Figure V in the online-only Data Supplement). This may be attributed to the short time course of WTD feeding and to the fact that these were early foam cell lesions. The impact of T0 treatment was much greater in the aortic root was collected to analyze atherosclerotic lesions. This may be attributed to the short time course of WTD feeding and to the fact that these were early foam cell lesions. The impact of T0 treatment was much greater in the aortic root than in the BM-transplanted group, which showed a 28% decrease in atherosclerotic lesion area in response to the LXR activator T0 (P<0.05; Figure 2A).

We then analyzed the sections for lesion severity and distinguished 3 lesion types: sections containing no lesions or valve lesions, sections containing foam cell-rich lesions, and sections containing advanced lesions. We quantified lesion severity expressed as number of observations. Differences of distribution of lesions between groups are indicated. n=6 to 9 mice per group. *P<0.05, **P<0.01, ***P<0.001.

**Figure 2.** Liver X receptor activation decreases atherosclerosis in Ldlr−/− mice transplanted with Abca1−/− Abcg1−/− bone marrow (BM). Ldlr−/− mice were transplanted with wild-type or Abca1−/− Abcg1−/− BM and fed WTD with or without T0 supplementation for 6 weeks. A, Atherosclerotic lesion area in the aortic root. Each data point represents a single mouse. B, Lesion severity expressed as number of observations. Differences of distribution of lesions between groups are indicated. n=6 to 9 mice per group. *P<0.05, **P<0.01, ***P<0.001.

**Figure 3.** Liver X receptor activation decreases inflammatory cell infiltration in the myocardium and adventitia of Ldlr−/− mice transplanted with Abca1−/− Abcg1−/− bone marrow. Inflammatory nuclei in the adventitia of hematoxylin and eosin–stained sections were quantified. n=6 to 9 mice per group; **P<0.01.

**LXR Activation Decreases Atherosclerosis**

As previously reported,12 hematoxylin and eosin staining of the aortic sinus revealed that Abca1−/− Abcg1−/− BM-transplanted mice had increased inflammatory cell infiltration in the adventitia and surrounding myocardium, and this was not found in wild-type BM-transplanted mice. Staining of aortic root sections with Mac3 confirmed that these cells were indeed inflammatory cells (macrophages). LXR activator treatment virtually abolished the appearance of inflammatory cells in the adventitia and surrounding myocardium (90% decrease; P<0.01; Figure 3 and Figure VI in the online-only Data Supplement). These results suggest that LXR activation decreases inflammation in vivo in the setting of hematopoietic Abca1/g1 deficiency.
macrophage Abca1/g1 deficiency. We used the GW compound to activate LXR, which has been reported to induce lesser hepatic steatosis than the T0 compound, and does not exhibit the off-target effects of T0 such as activation of the farnesoid X receptor, the pregnane X receptor, and the retinoic acid receptor–related orphan receptor. Eight-week-old Ldlr<sup>−/−</sup> mice were transplanted with LysmCreAbca1<sup>fl/fl</sup>Abcg1<sup>fl/fl</sup> or Abca1<sup>+/+</sup>Abcg1<sup>fl/fl</sup> BM. At 5 weeks after transplantation, BM reconstitution was >90% (results not shown), and the mice were fed WTD with or without GW supplementation (10 mg/kg) for a period of 12 weeks. Similar to our previous observations, macrophage Abca1/g1 deficiency decreased plasma cholesterol levels by ≈48% in WTD-fed Ldlr<sup>−/−</sup> mice (P<0.001), and there was a tendency to decreased TG levels (Figure VII in the online-only Data Supplement). There was ≈47% decreased sterol regulatory element–binding protein-1c mRNA in the liver from LysmCreAbca1<sup>fl/fl</sup>Abcg1<sup>fl/fl</sup> BM–transplanted WTD-fed Ldlr<sup>−/−</sup> mice (P<0.01), concomitant with decreased liver TG levels (57%; P<0.01; Figure VIII in the online-only Data Supplement). GW increased liver TG levels in both groups of mice (P<0.001; Figure VIIIIB in the online-only Data Supplement), albeit to a lower extent than T0 (Figure III in the online-only Data Supplement). As we reported previously, LysmCreAbca1<sup>fl/fl</sup>Abcg1<sup>fl/fl</sup> BM–transplanted WTD-fed Ldlr<sup>−/−</sup> mice showed increased monocyte and neutrophil levels. These increases were not affected by GW treatment (Figure IX in the online-only Data Supplement).

After 12 weeks of WTD, mice were euthanized, and atherosclerosis was assessed in the aortic root. Macrophage Abca1/g1 deficiency did not affect atherosclerotic lesion size (Figure 4A and Figure X in the online-only Data Supplement), likely because of the ≈50% decreased cholesterol levels, as we observed previously in this model. Under these conditions, the control group developed more advanced lesions than the LysmCreAbca1<sup>fl/fl</sup>Abcg1<sup>fl/fl</sup> group (Figure 4B). GW treatment significantly decreased lesion size and lesion severity in both groups of mice (Figure 4B). Again, the reduction in lesion size seemed to be greater in the LysmCreAbca1<sup>fl/fl</sup>Abcg1<sup>fl/fl</sup> BM–transplanted Ldlr<sup>−/−</sup> mice (Figure 4A). Macrophage Abca1/g1 deficiency increased plasma monocyte chemotactic protein 1 (MCP-1) levels (3.7-fold; P<0.001; Figure 4C). GW treatment decreased plasma MCP-1 in mice lacking Abca1/g1 in macrophages (Figure 4C), further substantiating the observation that the antiatherogenic effect of LXR activation may reflect its anti-inflammatory effects.

Discussion

It is well known that LXR activation causes increased expression of genes involved in cholesterol efflux and RCT such as ABCA1 and ABCG1 in macrophages. A therapeutic agent with the ability to increase cholesterol efflux would have obvious positive implications for the treatment of atherosclerosis because it would decrease the number of lipid-laden macrophages or foam cells embedding themselves into the artery wall. Indeed LXR activators have been shown to decrease atherosclerosis in multiple mouse models, and this has been assumed to be mainly because of its effects on cholesterol efflux and reverse cholesterol transport.

LXR also mediates a distinct mechanism of transrepression of the expression of inflammatory genes. When LXR becomes bound by a ligand, it can then be conjugated to a SUMO protein. This process targets the SUMOylated LXR to the promoters of NF-κB target genes where they prevent the signal-dependent removal of corepressor complexes required for transcriptional activation. Thus, inflammatory genes downstream of NF-κB are maintained in a repressed state. Previous studies have shown beneficial effects of LXR activator treatment in skin inflammation models, rheumatoid arthritis models, and hepatic injury caused by endotoxemia. However, the role of inflammatory gene suppression in atherosclerosis has not been addressed.

ABCA1 and ABCG1 are important targets of LXR activation as well as key players in macrophage cholesterol efflux.
LXR has multiple gene targets in addition to ABCA1 and ABCG1, that may affect cholesterol homeostasis, including inducible degrader of the Ldlr (IDOL), a molecule that acts to suppress LDL uptake. In our experiments, BM donor cells induced hepatic ABCG5/8 and seem to stimulate RCT, which supports survival of macrophages and of macrophage RCT in vivo. In addition to its beneficial effects, LXR activators have also been shown to induce expression of lipogenic genes and cause hepatic steatosis. An additional potential concern is that LXR activators may induce hepatic IDOL expression leading to reduced levels of LDLRs and increased plasma LDL levels. Because we have shown the anti-inflammatory effects of LXR are of key importance to its antiatherosclerotic effects in vivo, independent of the ABCA1/G1-mediated cholesterol efflux pathway, it should be possible to develop selective LXR activators that mediate inflammatory transpression without direct targeting of cholesterol efflux, IDOL, and lipogenic genes. These could act as antiatherogenic agents without inducing the adverse effect of fatty liver. Indeed compounds that have selective anti-inflammatory effects with minimal induction of lipogenic gene expression in cultured hepatocytes have been developed, but their role in treating atherosclerosis has not yet been assessed. Our studies suggest that LXR activators selective for their transpressive effects should be further developed and tested for effects on atherosclerosis, especially in models of advanced, complex disease.

**Sources of Funding**

M.S. Kappus was supported by the Sarnoff Cardiovascular Research Foundation. M. Westerterp was supported by Postdoctoral Fellowship 09POST2110109 from the American Heart Association and by NWO – VENI grant 916.11.072 from The Netherlands Organization of Scientific Research. This project was supported by the National Institutes of Health grant HL107653 (to A.R. Tall).

**Disclosures**

A.R. Tall is a consultant to Merck, Roche, Amgen, Arisaph, and CSL. The authors thereby report no conflicts.

**References**


8. Terasaka N, Hiroshima A, Koiyama T, Ubukata N, Morikawa Y, Nakai D, Inaba T. T-0901317, a synthetic liver X receptor ligand, inhibits and initiation of RCT. We showed that the ability of LXR activator to increase cholesterol efflux to apoA1 and HDL was abolished in Abca1−/−Abcg1−/− deficient macrophages, but that anti-inflammatory effects were actually increased in these cells (Figure 1). In the absence of ABCA1 and ABCG1, LXR activation by T0 exerted potent antiatherosclerotic effects, reducing early lesion area, complexity, and inflammation (Figure 2). In a longer term study, LXR activation by GW decreased lesion size and proatherogenic plasma MCP-1 levels in Ldlr−/− mice with macrophase Abca1/g1 deficiency (Figure 4). Our findings thus suggest that the antiatherosclerotic effects of LXR activation in macrophase and BM Abca1/g1 deficiency were because of anti-inflammatory effects of LXR presumably mediated by the well-established transpression mechanism.

This interpretation was supported by the marked decrease in inflammatory nuclei in the adventitia, and myocardium of Abca1−/−Abcg1−/− BM-transplanted mice treated with T0 for 6 weeks (Figure 3), and by the decreased MCP-1 levels in macrophase Abca1/g1−/− deficient mice treated with GW for 12 weeks (Figure 4C). It is notable that effects of LXR activator treatment on atherosclerosis were more pronounced in Abca1−/−Abcg1−/− BM-transplanted and macrophase Abca1/g1−/− deficient mice than in controls. This likely reflects the fact that macrophase inflammatory gene expression, lesional inflammatory features, and lesion complexity were increased in mice transplanted with Abca1−/−Abcg1−/− BM, and suggests that the effects of LXR activators on lesion inflammation and complexity may be at least as important as their effects on macrophase foam cell formation. Whereas we also observed increased inflammation in macrophase Abca1/g1 deficiency compared with controls (Figure 4C), the atherosclerotic lesion complexity was not more advanced than in controls (Figure 4B), likely reflecting the difference in plasma cholesterol levels between these 2 groups.

Several other potential explanations for our findings need to be considered. LXR activation has been shown to increase expression of apoptosis inhibitor expressed by macrophages (AIM; also called Spto or Atp6), which supports survival of macrophages in response to apoptosis-inducing stimuli, such as oxidized lipids. As a consequence AIM−/−Ldlr−/− mice show decreased atherogenesis. Because LXR treatment reduced atherogenesis in BM and macrophase Abca1/g1 deficiency in our study, it is unlikely that this effect was mediated by AIM.

LXR has multiple gene targets in addition to ABCA1 and ABCG1 that may affect cholesterol homeostasis, including inducible degrader of the Ldlr (IDOL), a molecule that acts to suppress LDL uptake. In our experiments, BM donor cells were Ldlr−/−, and thus induction of IDOL could act to suppress Ldlr in BM cells. However, Herijgers et al. have demonstrated that there was no difference in mean atherosclerotic lesion area in Ldlr−/− or Ldlr−/− BM-transplanted Ldlr−/− mice when fed a high-cholesterol diet, likely because of downregulation of Ldlr by the diet, and thus this scenario is also unlikely. LXR activators have been shown to exert modest antiatherogenic effects independent of LXR expression in BM-derived cells. This could reflect beneficial effects on expression of ABCA1 and ABCG1 in vascular endothelial cells. LXR activators also induce hepatic ABCG5/8 and seem to stimulate RCT at the level of the small intestine and such effects cannot be excluded in our study. However, studies in isolated macrophages and of macrophase RCT in vivo indicate a major role of ABCA1 and ABCG1 in cholesterol efflux and initiation of RCT from macrophages. Thus, it seems improbable that stimulating cholesterol flux across the liver or intestine would lead to increased cholesterol removal from macrophage foam cells in the absence of upregulating the initial step of macrophase reverse cholesterol transport.

In addition to its beneficial effects, LXR activators have also been shown to induce expression of lipogenic genes and cause hepatic steatosis. An additional potential concern is that LXR activators may induce hepatic IDOL expression leading to reduced levels of LDLRs and increased plasma LDL levels. Because we have shown the anti-inflammatory effects of LXR are of key importance to its antiatherosclerotic effects in vivo, independent of the ABCA1/G1-mediated cholesterol efflux pathway, it should be possible to develop selective LXR activators that mediate inflammatory transpression without direct targeting of cholesterol efflux, IDOL, and lipogenic genes. These could act as antiatherogenic agents without inducing the adverse effect of fatty liver. Indeed compounds that have selective anti-inflammatory effects with minimal induction of lipogenic gene expression in cultured hepatocytes have been developed, but their role in treating atherosclerosis has not yet been assessed. Our studies suggest that LXR activators selective for their transressive effects should be further developed and tested for effects on atherosclerosis, especially in models of advanced, complex disease.
Activation of Liver X Receptor Decreases Atherosclerosis in Ldlr\(^{-/-}\) Mice in the Absence of ATP-Binding Cassette Transporters A1 and G1 in Myeloid Cells
Mojdeh S. Kappus, Andrew J. Murphy, Sandra Abramowicz, Vusisizwe Ntonga, Carrie L. Welch, Alan R. Tall and Marit Westerterp

*Arterioscler Thromb Vasc Biol*. 2014;34:279-284; originally published online December 5, 2013;
doi: 10.1161/ATVBAHA.113.302781

*Arteriosclerosis, Thrombosis, and Vascular Biology* is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2013 American Heart Association, Inc. All rights reserved.
Print ISSN: 1079-5642. Online ISSN: 1524-4636

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://atvb.ahajournals.org/content/34/2/279

Data Supplement (unedited) at:
http://atvb.ahajournals.org/content/suppl/2013/12/05/ATVBAHA.113.302781.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in *Arteriosclerosis, Thrombosis, and Vascular Biology* can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to *Arteriosclerosis, Thrombosis, and Vascular Biology* is online at:
http://atvb.ahajournals.org//subscriptions/
MATERIALS AND METHODS

Animals and Diets
Abca1−/− mice in the DBA background were from the Jackson Laboratory (Bar Harbor, ME) (stock number 001140). Abcg1−/− mice in the C57BL/6J background were purchased from DeltaGen. We generated Abca1−/− Abcg1−/− mice in the mixed DBA-C57BL/6J background as described previously and backcrossed these mice into the C57BL/6J background for at least 10 generations. Female Ldlr−/− mice were purchased from The Jackson Laboratory (stock number 002207). Abca1flox/flox (Abca1fl/fl) mice were from Dr. Parks and Abcg1flox/flox (Abcg1fl/fl) mice were generated at Columbia University. We generated Abca1fl/fl Abcg1fl/fl and LysmCreAbca1fl/fl Abcg1fl/fl mice as described previously. All mice were housed at Columbia University Medical Center according to animal welfare guidelines. All protocols were approved by the Institutional Animal Care and Use Committee of Columbia University. Animals had ad libitum access to both food and water. Mice were fed chow diet (Purina Mills diet 5053) until 5 weeks after bone marrow transplantation at which point they were fed WTD (0.2% cholesterol, 21% from fat adjusted calorie diet, TD 88137, Harlan Teklad) supplemented with or without T0 (Cayman) (10 mg/kg) or with or without GW3965 (10 mg/kg). GW3965 was kindly provided by Dr. Jon Collins, Glaxo Smith Kline. Additionally, after 4 weeks of treatment, mice on the T0-supplemented diet received 2 consecutive intraperitoneal injections of T0 in DMSO (25 mg/kg) with an interval of 24 h, whereas the control groups received DMSO only.

Bone Marrow Transplantation
At 8 weeks of age, Ldlr−/− mice were transplanted with either wild-type, Abca1−/− Abcg1−/−, Abca1fl/fl Abcg1fl/fl or LysmCreAbca1fl/fl Abcg1fl/fl bone marrow. All bone marrow donors were males and all recipient mice females. Recipient mice received water supplemented with 10 mg/ml neomycin and 10 μg/ml polymyxin B for 1 week before and 2 weeks after bone marrow transplantation. Ldlr−/− recipient mice were irradiated with 2 consecutive doses of 6.5 Gy with a 4 h time interval and were injected intravenously with 5*10^6 bone marrow cells from the aforementioned donors at 24 h after the irradiation. Bone marrow cells were isolated from the femur and tibias of bone marrow donor mice and injected into the tail vein of recipient mice. Recipient mice were allowed to recover for 5 weeks after the bone marrow transplantation before WTD feeding. Before the start of the WTD, peripheral blood was collected and DNA isolated (MO BIO blood DNA isolation kit, Carlsbad, CA) of Abca1−/− Abcg1−/− and wild-type bone marrow transplanted mice to determine the efficiency of bone marrow reconstitution by quantification of wild-type ABCG1 DNA compared with m36B4. For the bone marrow transplantation with LysmCreAbca1fl/fl Abcg1fl/fl and Abca1fl/fl Abcg1fl/fl donors, the efficiency of reconstitution was assessed by quantifying Y chromosome expression compared with m36B4. Samples were run on a Stratagene Mx3000P (Agilent Technologies). Primer sequences are shown in Supplemental Table 1.

Lipid Analyses
Mice were fasted for 4 hours and blood samples were obtained from the tail vein into heparin-coated tubes. Plasma was separated by centrifugation. To assess lipoprotein cholesterol distribution by fast performance liquid chromatography (FPLC), pooled plasma (n=6-9 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, which was also used to measure plasma total cholesterol. Total plasma triglyceride (TG) levels were determined using an enzymatic kit from Wako.
Atherosclerotic Lesion Analysis
Mice were euthanized in accordance to the American Veterinary Association Panel on Euthanasia. Hearts and aortas were isolated and fixed in phosphate-buffered formalin. Hearts were dehydrated and embedded in paraffin, and were cross-sectioned throughout the aortic root area (5 μm sections). Haematoxylin-eosin (H&E) 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). Mac-3 antibody (BD Biosciences) was used to stain macrophages in sections. The typing of lesions was done according to the typing for humans proposed by the American Heart Association,4 which has been adapted to categorize lesions in mice.5 In this study, we discerned sections showing no lesions or valve lesions (type 0), sections showing foam cell rich lesions (type 1-3), and sections showing advanced lesions with foam cells in the media and presence of fibrosis, cholesterol clefts, mineralization and/or necrosis (type 4-5).

Cholesterol Efflux Assay
Bone marrow was isolated from Abca1fl/fl Abcg1fl/fl and LysmCreAbca1fl/fl Abcg1fl/fl mice and differentiated into macrophages by culture in DMEM 10% FBS, 1% pen-strep, supplemented with 20% L-cell medium. After 7 days, bone marrow cells had fully differentiated into macrophages and were ready for use in experiments. For cholesterol loading, cells were cultured for 24 h in DMEM supplemented with 0.2% BSA, 50 μg/ml acetylated LDL, and 2 μCi/ml of [3H]-cholesterol, with or without 3 μM T0. Cholesterol efflux was performed for 6 h in DMEM containing 0.2% BSA in the presence or absence of HDL (50 μg/ml) or apoA1 (25 μg/ml). The cholesterol efflux was calculated as the percentage of the radioactivity released from the cells in the medium relative to the total radioactivity in cells plus medium.6

Inflammatory Gene Expression in Macrophages
Bone marrow derived macrophages from Abca1fl/fl Abcg1fl/fl and LysmCreAbca1fl/fl Abcg1fl/fl mice were incubated with or without the LXR agonist T0 (3 μM) for 18 h and then stimulated with or without lipopolysaccharide (LPS) (50 ng/ml) for 4 h. Total RNA was then extracted using a RNeasy Mini Kit (Qiagen) and cDNA was synthesized using SuperScriptase III (Invitrogen). Levels of Il-1β, Il-6, Monocyte chemoattractant protein-1 (Mcp-1), and Macrophage inflammatory protein-1α (Mip-1α) 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. The primer sequences are shown in Supplemental Table 1.

Liver Triglyceride Content
Liver lipids were extracted according to the method of Folch et al.7 Lipids were then dissolved in ethanol and total TG levels were determined using an enzymatic kit from Wako. Protein levels were determined and liver TG was corrected for liver protein.

Liver Sterol Regulatory Element Binding Protein-1c Expression
Livers were homogenized in Qiazol. Total RNA was extracted using a RNeasy Mini Kit (Qiagen) and cDNA was synthesized using SuperScriptase III (Invitrogen). Expression level of Sterol Regulatory Element Binding Protein-1c (SREBP-1c) mRNA was assessed using qPCR on a Stratagene Mx3000P (Agilent Technologies), and initial differences in RNA quantity were corrected for using the housekeeping gene m36B4. The primer sequences are shown in Supplemental Table 1.
Monocyte Chemoattractant Protein 1 Plasma Levels
After 12 weeks of diet, blood was obtained from the retro-orbital plexus into heparin-coated tubes. Plasma was separated by ultracentrifugation and levels of MCP-1 were measured using enzyme linked immunosorbent assay (ELISA) (R&D Systems).

Flow Cytometry
For peripheral blood leukocytes analysis, 100 μl of blood was collected into EDTA tubes via tail bleeding. Tubes were immediately put on ice and all subsequent steps were performed on ice. Red blood cells were lysed (BD Pharm Lyse, BD Biosciences) and white blood cells were centrifuged, washed, and resuspended in HBSS (0.1% BSA, 5 mM EDTA). Cells were stained with a cocktail of antibodies against CD45-APC-Cy7, Ly6-C/G-PerCP-Cy5.5 (BD Pharmingen), and CD115-APC (eBioscience). Samples were analyzed on a BD LSRII cell analyzer running with DiVa software (BD Biosciences). Data were analyzed using FlowJo software (Tree Star Inc.) Monocytes were identified as CD45hiCD115hi and further identified into Ly6-C^hi and Ly6-C^lo. Neutrophils were identified as CD45hiCD115loLy6-C/G^hi (Gr-1).

Statistical Analysis
All data are presented as mean ± SD. To define differences between datasets, the t-test or one-way analysis of variance (ANOVA) with a Bonferroni multiple comparison post-test was used. The Mann Whitney nonparametric test was used to assess differences in atherosclerotic lesion size or inflammatory cell infiltration in the adventitia/myocardium. The chi square test was used to determine differences in atherosclerotic lesion severity (GraphPad Prism, version 5.01, San Diego, CA).
Supplemental Table 1. Primer sequences. Tm=60°C. m36B4 was used for normalization.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward (F) / (Reverse (R))</th>
<th>Primer sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>m36B4</td>
<td>F</td>
<td>CCT GAA GTG CTC GAC ATC AC</td>
</tr>
<tr>
<td></td>
<td>R</td>
<td>CCA CAG ACA ATG CCA GGA C</td>
</tr>
<tr>
<td>ABCG1</td>
<td>F</td>
<td>GGG ATC TCT GGG AAA TTC AAC AGT G</td>
</tr>
<tr>
<td></td>
<td>R</td>
<td>GTG AGC AGA GCT TCT GGT AGC AAA C</td>
</tr>
<tr>
<td>IL-1β</td>
<td>F</td>
<td>TGT TCT TTG AAG TTG ACG GAC CCC</td>
</tr>
<tr>
<td></td>
<td>R</td>
<td>GAT GCT CTC ATC AGG ACA GCC CA</td>
</tr>
<tr>
<td>IL-6</td>
<td>F</td>
<td>ACA ACC ACG GCC TTC CCT ACT T</td>
</tr>
<tr>
<td></td>
<td>R</td>
<td>CAC GAT TTC CCA GAG AAC ATG TG</td>
</tr>
<tr>
<td>MCP-1</td>
<td>F</td>
<td>AGG TCC CTG TCA TGC TTC TG</td>
</tr>
<tr>
<td></td>
<td>R</td>
<td>AAG GCA TCA CAG TCC GAG TC</td>
</tr>
<tr>
<td>MIP-1α</td>
<td>F</td>
<td>ACT GCC TGC TGC TTC TCC TAC A</td>
</tr>
<tr>
<td></td>
<td>R</td>
<td>AGG AAA ATG ACA CCT GGC TGG</td>
</tr>
<tr>
<td>SREBP-1c</td>
<td>F</td>
<td>GCG CCA TGG ACG AGC TG</td>
</tr>
<tr>
<td></td>
<td>R</td>
<td>TTG GCA CCT GGG CTG CT</td>
</tr>
<tr>
<td>Y-chromosome</td>
<td>F</td>
<td>CTA AGC CAT GTA CCA CCT</td>
</tr>
<tr>
<td></td>
<td>R</td>
<td>CTC TCT CTT TAT CAA TTT TTT CT</td>
</tr>
</tbody>
</table>
REFERENCES


Supplemental Figure I. Plasma lipid levels in Ldlr<sup>−/−</sup> mice transplanted with wild-type or Abca1<sup>−/−</sup>Abcg1<sup>−/−</sup> bone marrow (BM) after 3 weeks of WTD with or without T0 supplementation. Mice were fasted for 4 h and blood was drawn from the tail vein. Plasma was separated by ultracentrifugation. A. Triglycerides. B. Cholesterol. n=6-9 mice per group. ***P<0.001.
Supplemental Figure II. Lipoprotein cholesterol distribution in Ldlr⁻/⁻ mice transplanted with wild-type or Abca1⁻/⁻Abcg1⁻/⁻ bone marrow (BM) after 3 weeks of WTD with or without T0 supplementation. Mice were fasted for 4 h and blood was drawn from the tail vein. Plasma was separated by ultracentrifugation, pooled, and lipoproteins were size-fractionated by fast performance liquid chromatography on a Superose 6 column. n=6-9 mice per pool.
Supplemental Figure III. Liver SREBP-1c mRNA expression and TG content in Ldlr⁻/⁻ mice transplanted with wild-type or Abca1⁻/⁻Abcg1⁻/⁻ bone marrow (BM) after 6 weeks of WTD with or without T0 supplementation and 2 injections of T0. A. Livers were lysed in qiazol, RNA was extracted, and SREBP-1c mRNA expression was assessed and corrected for the housekeeping gene m36B4. B. Lipid was extracted from the liver by the Folch extraction method and the TG concentration was determined and corrected for liver protein. n=6-9 mice per group. ***P<0.001.
Supplemental Figure IV. Monocytes, neutrophils, and monocyte subpopulations in Ldlr−/− mice transplanted with wild-type or Abca1−/−Abcg1−/− bone marrow (BM) after 4 weeks of WTD with or without T0 supplementation and 2 injections of T0. Blood was drawn from the tail vein and leukocyte levels were assessed by flow cytometry. A. Monocytes. B. Neutrophils. C. Ly6C-lo and (D) Ly6C-hi monocytes n=6-9 mice per group. *P<0.05, **P<0.01, ***P<0.001.
Supplemental Figure V. LXR activation decreases atherosclerosis in *Ldlr*⁻/⁻ mice transplanted with *Abca1*⁻/⁻*Abcg1*⁻/⁻ BM. *Ldlr*⁻/⁻ mice were transplanted with wild-type or *Abca1*⁻/⁻*Abcg1*⁻/⁻ BM and fed WTD with or without T0 supplementation for 6 weeks. Sections were made of the aortic roots and stained with H&E. Representative pictures are shown.
Supplemental Figure VI. LXR activation decreases inflammatory cell infiltration in the myocardium and adventitia of Ldlr<sup>-/-</sup> mice transplanted with Abca1<sup>-/-</sup>Abcg1<sup>-/-</sup> BM. Representative pictures of H&E stained sections (A-B) and Mac3 (macrophage) stained sections (C-D) are shown. Arrows indicate the inflammatory cell infiltrate.
Supplemental Figure VII. Plasma lipid levels in \textit{Ldlr}^{-/-} mice transplanted with wild-type or \textit{Abca1}^{-/-}\textit{Abcg1}^{-/-} bone marrow (BM) after 12 weeks of WTD with or without GW supplementation. Mice were fasted for 4 h and blood was drawn from the tail vein. Plasma was separated by ultracentrifugation. \textbf{A}. Triglycerides. \textbf{B}. Cholesterol. \(n=11-13\) mice per group. *\(P<0.05\), ***\(P<0.001\).
**Supplemental Figure VIII**

Liver Srebp-1c mRNA expression and TG content in Ldlr<sup>-/-</sup> mice transplanted with Abca<sup>1fl/fl</sup>Abcg<sup>1fl/fl</sup> or LysmCreAbca<sup>1fl/fl</sup>Abcg<sup>1fl/fl</sup> bone marrow (BM) after 12 weeks of WTD with or without GW supplementation. A. Livers were lysed in qiazol, RNA was extracted, and SREBP-1c mRNA expression was assessed and corrected for the housekeeping gene m36B4.

**B.** Lipid was extracted from the liver by the Folch extraction method and the TG concentration was determined and corrected for liver protein. n=8 mice per group. *P<0.05, **P<0.01, ***P<0.01.
Supplemental Figure IX. Monocytes, neutrophils, and monocyte subpopulations in \( Ldlr^{-/-} \) mice transplanted with \( Abca1^{fl/fl} Abcg1^{fl/fl} \) or \( LysmCreAbca1^{fl/fl} Abcg1^{fl/fl} \) bone marrow (BM) after 10 weeks of WTD with or without GW supplementation. Blood was drawn from the tail vein and leukocyte levels were assessed by flow cytometry. 

A. Monocytes. B. Neutrophils. C. Ly6C-lo and D. Ly6C-hi monocytes. n=11-12 mice per group. *\( P<0.05 \), ***\( P<0.001 \).
Supplemental Figure X. LXR activation decreases atherosclerosis in Ldlr−/− mice transplanted with BM deficient in macrophage Abca1 and Abcg1. Ldlr−/− mice were transplanted with Abca1fl/fl Abcg1fl/fl or LysmCreAbca1fl/fl Abcg1fl/fl BM and fed WTD with or without GW supplementation for 12 weeks. Sections were made of the aortic roots and stained with H&E. Representative pictures are shown.