Macrophage Liver X Receptor Is Required for Antiatherogenic Activity of LXR Agonists


Objective—Complications of atherosclerotic cardiovascular disease due to elevated blood cholesterol levels are the major cause of death in the Western world. The liver X receptors, LXα and LXβ (LXRs), are ligand-dependent transcription factors that act as cholesterol sensors and coordinately control transcription of genes involved in cholesterol and lipid homeostasis as well as macrophage inflammatory gene expression. LXRs regulate cholesterol balance through activation of ATP-binding cassette transporters that promote cholesterol transport and excretion from the liver, intestine, and macrophage. Although LXR agonists are known to delay progression of atherosclerosis in mouse models, their ability to abrogate preexisting cardiovascular disease by inducing regression and stabilization of established atherosclerotic lesions has not been addressed.

Methods and Results—We demonstrate that LXR agonist treatment increases ATP-binding cassette transporter expression within preexisting atherosclerotic lesions, resulting in regression of these lesions as well as remodeling from vulnerable to stable lesions and a reduction in macrophage content. Further, using macrophage-selective LXR-deficient mice created by bone marrow transplantation, we provide the first evidence that macrophage LXR expression is necessary for the atheroprotective actions of an LXR agonist.


Key Words: atherosclerosis ■ LXRs ■ nuclear receptors ■ reverse cholesterol transport

E levated plasma non–high-density lipoprotein cholesterol (non–HDL cholesterol) levels, a major risk factor in the development of cardiovascular disease, increase macrophage cholesterol accumulation and foam cell formation in the underlying epithelium of large arteries. Progressive recruitment of macrophages to this site, combined with their uptake of oxidized low-density lipoprotein (LDL) particles, leads to the development of atherosclerotic lesions. In addition to lipid loading, progression of early lesions to complex lesions (plaques) is a chronic inflammatory process in which monocyte-derived macrophages play a key role.1 The liver X receptors LXα and LXβ (LXRs) are members of the nuclear receptor family that control transcription of genes that coordinately regulate cholesterol transport and lipid metabolism in 3 major sites (macrophage, liver, and intestine) and play a critical role in the maintenance of cholesterol homeostasis.2 Recently, we demonstrated that reconstitution of macrophages from mice deficient in LXα and β (LXαβ−/−) by bone marrow transplantation increased atherosclerosis in apolipoprotein E−/− (apoE−/−) and LDL receptor–null (LDLR−/−) mice fed a high-fat cholesterol-containing (“Western”) diet, signifying that macrophage LXR expression plays a critical role in the inhibition of atherogenesis.3

See page 10

LXRs modify the atherogenic program in the macrophage through regulation of genes involved in both inflammation and cholesterol elimination pathways. LXR agonists negatively regulate macrophage inflammatory gene expression,4 and recent data indicate that pathogens that contribute to the initiation and progression of atherosclerosis interfere with macrophage cholesterol metabolism by inhibition of the LXR signaling pathway.5 Further, LXRs directly regulate the expression of ATP-binding cassette transporters ABCA1 and ABCG1 and apoE, which mediate cellular cholesterol efflux in the presence of acceptors, such as HDL. This process of reverse cholesterol transport is necessary for the maintenance of normal cellular cholesterol balance.6–9 Mutations in the ABCA1 gene produce dysregulation of cellular cholesterol efflux, resulting in Tangier disease, characterized by low HDL levels and cholesterol accumulation in macrophages.10,11 Similarly, sterol/agonist–inducible cholesterol efflux is defective in LXαβ−/− macrophages and results in cellular cholesterol accumulation.3,12 These observations confirm the central importance for macrophage LXRs as cellular cholesterol sensors in the context of the atherogenic response to elevated plasma cholesterol.
In the liver, LXRs regulate genes involved in cholesterol homeostasis, including cholesterol 7α-hydroxylase (Cyp7a), the rate-limiting enzyme that promotes conversion of cholesterol to bile acids in the liver for excretion into bile, and ABCG5 and ABCG8, which regulate cholesterol trafficking.\textsuperscript{1,3-15} LXRs also regulate fatty acid metabolism in the liver through control of sterol regulatory element–binding protein (SREBP)-1c, a key factor involved in regulating the expression of several hepatic lipogenic enzymes, including acetyl coenzyme A carboxylase, fatty acid synthase, and stearoyl coenzyme A desaturase-1.\textsuperscript{16-18} Synthetic LXR agonists increase plasma triglyceride levels\textsuperscript{17,19} and reduce dietary cholesterol absorption.\textsuperscript{8} Thus, hepatic LXR activity can influence atherosclerosis by modifying processes that directly impact plasma lipid levels.

Recent atherosclerosis prevention studies have demonstrated that synthetic LXR agonists inhibit the initiation and development of atherosclerosis,\textsuperscript{19,20} even in the absence of major changes in plasma lipid levels. Although these protective effects of LXR agonist treatment on atherosclerosis are clear, there is no information regarding the potential benefit of LXR agonist therapy in the more clinically relevant setting of preexisting hypercholesterolemia and atherosclerotic lesions. Also, although the preventative effect of LXR agonist treatment was largely attributed to increases in ABCA1 and ABCG1 expression in the intestine and artery wall,\textsuperscript{6} the metabolic effects of LXR agonist treatment on atherosclerosis are stabilizing preexisting atherosclerotic lesions and determined whether macrophage LXR expression is essential for the atheroprotective effects of the LXR agonist.

**Methods**

**Animals and Diets**

Homozygous male LDLR\textsuperscript{−/−} mice (C57BL/6 genetic background) purchased from The Jackson Laboratory (Bar Harbor, Me) were bred at X-Ceptor Therapeutics Inc. Male C57BL/6 mice were purchased from the Harlan Laboratory Inc (San Diego, Calif). Homozygous male LXR\textsuperscript{αβ−/−}double knockout mice (LXR\textsuperscript{αβ−/−}) on the C57BL/6 genetic background were from a colony established and maintained at X-Ceptor Therapeutics Inc.\textsuperscript{13} Mice were fed standard chow ad libitum until study, at which time LDLR\textsuperscript{−/−} mice were fed a Western-type diet (21% fat wt/wt, 0.15% cholesterol wt/wt; Purina Laboratory Chow, St Louis, Mo). LDLR\textsuperscript{−/−} mice (8 weeks of age) housed in microisolator cages were lethally irradiated with 900 rads (9 Gy) from a cobalt \(\gamma\)-source. Bone marrow cells were harvested from 6- to 8-week-old LXR\textsuperscript{αβ−/−} mice, and C57BL/6 donor mice by flushing tibia and femurs with RPMI medium 1640 supplemented with 2% FBS and heparin (5 U/mL). Cells were washed, suspended in fresh medium, and counted. Recipient LXR\textsuperscript{−/−} mice were transplanted with \(3\times10^6\) bone marrow cells through tail vein injection within 6 hours after irradiation.

**Lipid and Lipoprotein Analyses**

Nonfasting mice were anesthetized with isoflurane, and blood samples were obtained from the retro-orbital plexus. Plasma total cholesterol and triglyceride levels were determined by colorimetric enzymatic assays that were adapted to 96-well plate formats (Infinity Total Cholesterol Reagent or Infinity Triglyceride Reagent, Sigma), using a Multiskan plate reader. Plasma HDL cholesterol was determined by precipitating non–HDL cholesterol (Wako Diagnostic 278-67409) and then assaying the remaining HDL cholesterol by means of the Infinity Total Cholesterol Reagent. Terminal plasma samples from mice treated with vehicle or LXR agonist in the lesion regression study were pooled (6 to 12 mice in each group) and fractionated by fast protein liquid chromatography (FPLC) gel filtration (Amersham Pharmacia Biotech) on Superose 6 columns, and cholesterol concentration of each fraction was determined as above.

**RNA Isolation and Analysis of Gene Expression by Quantitative RT-PCR**

Atherosclerotic lesions were fresh dissected from the length of the aorta at the termination of the lesion regression study, starting from aortic arch and finishing at the terminal abdominal aorta. Lesions were visualized using a stereo-microscope (Leica MZ12) and harvested using a sterile scalpel to ensure the material collected contained only atherosclerotic lesion and not underlying aortic tissue. Bone marrow was collected at the termination of the bone marrow transplantation study. Total RNA from tissues was isolated from these tissues using RNeasy kits (Qiagen Inc) according to the supplier’s total RNA isolation procedure. Because of the low recovery of total RNA from the microdissected atherosclerotic lesions, samples were pooled (5 mice per pool) to create 2 independent samples (\(n=10\) mice total) for each treatment condition. Quantitative RT-PCR was performed using a Perkin-Elmer/ABI 7700 Prism. DNase-treated RNA samples were reverse transcribed, followed by quantitative polymerase chain reactions following standard protocols and conditions as recommended by the manufacturer (RNase-free DNase, Roche; Superscript II Reverse Transcriptase and Taq Polymerase, Life Technologies). Levels of cyclophilin were measured in all samples, and results are presented as number of target mRNA transcripts per cyclophilin mRNA transcript.

**Analysis of Atherosclerosis and Lesion Composition**

The extent of atherosclerosis in en face mouse aortic preparations was quantified by computer-assisted image analysis of Sudan IV stained aortas using Image-Pro Plus image analysis software (Media Cybernetics).\textsuperscript{21,22} Immunohistochemical analysis of frozen aortic root sections was performed as described previously.\textsuperscript{23} The total lesion area in oil red O–stained aortic root sections was determined on digitized root images from 5 equally spaced sections per mouse. For detection of macrophages, aortic sections were incubated with macrophage–specific antibody to mouse macrophages (MOMA-2, Accurate Chemicals). Collagen in lesion was detected using trichrome stain (Sigma). The macrophage- and collagen-positive areas in digitized color images of stained aortic root sections (3 equally spaced sections per mouse; \(n=5\) per group) were quantified period when mice received either vehicle or the LXR agonist (\(n=12\) per group) as described above.

**Bone Marrow Transplantation**

Recipient male LDLR\textsuperscript{−/−} mice (8 weeks of age) housed in microisolator cages were lethally irradiated with 900 rads (9 Gy) from a cobalt \(\gamma\)-source. Bone marrow cells were harvested from 6- to 8-week-old LXR\textsuperscript{αβ−/−} mice, and C57BL/6 donor mice by flushing tibia and femurs with RPMI medium 1640 supplemented with 2% FBS and heparin (5 U/mL). Cells were washed, suspended in fresh medium, and counted. Recipient LXR\textsuperscript{−/−} mice were transplanted with \(3\times10^6\) bone marrow cells through tail vein injection within 6 hours after irradiation.
using Image-Pro Plus image analysis software (Media Cybernetics), and the data are expressed as percent of total lesion area.

**Statistical Analyses**
Results were analyzed by 1- or 2-way ANOVA with Newman–Keuls Multiple Comparisons (for 1-way ANOVA) or Bonferroni (for 2-way ANOVA) posttests, or Student unpaired t test, using GraphPad Prism (GraphPad Software Inc).

**Results**

**LXR Agonist Induced Regression of Established Atherosclerotic Lesions**
To examine effects of LXR activation on regression of atherosclerosis in a model with preexisting atherosclerotic lesions, male LDLR<sup>−/−</sup> mice were fed a Western diet and then treated with the LXR agonist or vehicle for 6 weeks, while they continued to consume the Western diet ad libitum (Figure 1A). En face analysis of lesion area at baseline, before the initiation of LXR agonist treatment, revealed that LDLR<sup>−/−</sup> mice developed advanced lesions throughout the aortic arch and distal portions of thoracic and abdominal aortic regions, which continued to progress in the vehicle-treated group during the 6-week treatment period (Figure 1B and 1C). LXR agonist treatment resulted in a 70% reduction of lesion area in comparison to vehicle-treated controls (Figure 1B, middle versus bottom, and 1C), demonstrating a preventive effect of the LXR agonist on lesion development. More importantly, LXR agonist treatment resulted in significantly 62% reduction in lesion areas compared with mice assessed at baseline (Figure 1B, top versus middle and bottom, and 1C), demonstrating that the LXR agonist induced regression of preexisting atherosclerotic lesions. When atherosclerosis was measured by quantitation of aortic root sections, once again a significant delay in disease progression was observed in LXR agonist–treated mice (vehicle=1.9×10<sup>9</sup> μm<sup>2</sup>, n=8; T0901317=1.5×10<sup>8</sup> μm<sup>2</sup>, n=8, *P*<0.01); however, lesion area in both agonist- and vehicle-treated mice was significantly greater than that of the baseline control (7.4×10<sup>7</sup> μm<sup>2</sup>, n=8, *P*<0.01 versus vehicle- and agonist-treated groups). The ability to selectively observe regression in the en face analysis most likely arises from the difference in the type of information provided by the 2 methods. The root section quantitation provides cross-sectional informational from a single area and also includes the necrotic core of the lesion which is not a target for reverse cholesterol transport. In contrast, the en face analysis provides an aggregate measure of the length of the aorta covered by lesions.

**LXR Activation Alters Composition of Atherosclerotic Lesions**
Immunostaining of aortic root lesions with the macrophage-specific antibody MOMA-2 showed a significant 48% reduction of macrophage-positive areas in LXR agonist–treated mice (Figure 2A versus 2B and Table 1). This effect was accompanied by a 34% increase in collagen content of lesions in LXR agonist–treated mice (Figure 2C versus 2D and Table 1). Interestingly, the increases in collagen were observed above necrotic core and in fibrous cap regions of atherosclerotic lesions (Figure 2C versus 2D). The reduction of macrophage content and an increase in collagen content of lesions is consistent with stabilization of vulnerable lesions that may protect advanced necrotic lesions from plaque rupture and thrombosis.

**LXR Regulation of Gene Expression in Atherosclerotic Lesions**
To further evaluate the effects of LXR agonist treatment within atherosclerotic lesions, gene expression was assessed in RNA isolated from micro-dissected aortic lesions from LDLR<sup>−/−</sup> mice on Western diet for 8 weeks, after treatment with vehicle or LXR agonist (10 mg/kg per day for 6 weeks). These aortas were separate from those examined by en face atherosclerosis analysis. Expression of the macrophage marker CD68<sup>25</sup> in these aortic lesions was significantly reduced by 45% in LXR agonist–treated mice compared with vehicle-treated controls (Figure 3A and 3B). In addition, LXR agonist treatment significantly increased ABCA1 mRNA levels in atherosclerotic lesions by 67% (Figure 3A). There was also a slight (≈10%) increase in ABCG1 mRNA levels in the atherosclerotic lesions, which did not attain statistical significance (data not shown). Consistent with the immunohistochemical data presented earlier, these results demonstrate that LXR agonist treatment reduces the inflammatory tone of atherosclerotic lesions while inducing expression of genes that promote reverse cholesterol transport from macrophages.

**LXR Agonist Treatment Ameliorates Hypercholesterolemia in LDLR<sup>−/−</sup> Mice**
Plasma FPLC analysis revealed a substantial effect of LXR agonist treatment across the lipoprotein cholesterol profile (Figure 3B). LXR agonist treatment significantly reduced plasma total cholesterol levels by ≈28% (Figure 3C), along with a nonsignificant reduction of HDL cholesterol after 6 weeks of treatment (Figure 3D). As expected, the LXR agonist increased plasma triglyceride concentrations (Figure 3E).

**Critical Role of Macrophage LXR in Antiatherogenic Activity of LXR Agonist**
In previous studies, we demonstrated that macrophage LXRαβ deficiency leads to increased atherosclerosis in apoE<sup>−/−</sup> and LDLR<sup>−/−</sup> mice. Because LXRs are expressed in multiple tissues and coordinately regulate several target genes involved in cholesterol homeostasis and inflammatory signaling, it is not clear which site(s) of LXR activity is critical to the inhibitory effects of LXR agonists on atherosclerosis. To address whether macrophage LXR activity contributes to the atheroprotective effects of LXR agonists, we created LDLR<sup>−/−</sup> mice with bone marrow macrophage LXRαβ deficiency using bone marrow transplantation (BMT). Irradiated LDLR<sup>−/−</sup> mice were transplanted with C57Bl/6, LXRαβ<sup>−/−</sup>, or LDLR<sup>−/−</sup> bone marrow (C57Bl/6→LDLR<sup>−/−</sup>, LXRαβ<sup>−/−</sup>→LDLR<sup>−/−</sup>, and LDLR<sup>−/−</sup>→LDLR<sup>−/−</sup>, respectively). The C57Bl/6→LDLR<sup>−/−</sup> and LDLR<sup>−/−</sup>→LDLR<sup>−/−</sup> groups represent controls in which macrophages are positive for LXR expression while being either positive (C57Bl/6→LDLR<sup>−/−</sup>→LDLR<sup>−/−</sup>) or negative for LXR expression (C57Bl/6→LDLR<sup>−/−</sup>→LDLR<sup>−/−</sup>).
LDLR<sup>−/−</sup>) or negative (LDLR<sup>−/−</sup>→LDLR<sup>−/−</sup>) for LDLR expression, whereas macrophages in mice in the LXRαβ<sup>−/−</sup>→LDLR<sup>−/−</sup> group are negative for LXR expression but positive for LDLR expression. Mice were allowed to recover from the irradiation and transplant procedure for 4 weeks and were subsequently placed on Western diet and treated with either vehicle or LXR agonist for 8 weeks (Figure 4A).

Plasma cholesterol levels at the termination of the study revealed that all mice were considerably hypercholesterolemic (Table 2). There were no significant alterations in plasma total or HDL cholesterol levels in any of the BMT conditions treated with the LXR agonist, relative to their vehicle-treated controls, with the exception of total cholesterol in the LDLR<sup>−/−</sup>→LDLR<sup>−/−</sup> group. In this BMT condition, mice treated with the LXR agonist had significantly lower plasma cholesterol levels than did their vehicle-treated LDLR<sup>−/−</sup>→LDLR<sup>−/−</sup> controls. However, this difference may be caused by the higher plasma cholesterol levels in the vehicle-treated LDLR<sup>−/−</sup>→LDLR<sup>−/−</sup> mice relative to the other vehicle-treated BMT conditions, because the plasma cholesterol levels in the LXR agonist–treated LDLR<sup>−/−</sup>→LDLR<sup>−/−</sup> mice are not different from LXR agonist–treated mice in the other BMT conditions. Plasma triglyceride levels were elevated by LXR agonist treatment in all 3 BMT groups (Table 2).

The effects of BMT and LXR agonist treatment on atherosclerosis were determined by en face quantification of aortic lesion areas. As previously reported,<sup>3</sup> vehicle-treated LXRαβ<sup>−/−</sup>→LDLR<sup>−/−</sup> mice developed significantly more atherosclerosis than did vehicle-treated C57Bl/6→LDLR<sup>−/−</sup> mice (Figure 4B), reflecting the antiatherogenic activity of macrophage LXR expression. LDLR<sup>−/−</sup>→LDLR<sup>−/−</sup> mice in this study appeared to develop more atherosclerosis than C57Bl/6→LDLR<sup>−/−</sup> mice after 8 weeks of vehicle treatment; however, this difference did not attain statistical significance (Figure 4B), and may reflect the higher plasma total cholesterol levels in vehicle-treated LDLR<sup>−/−</sup>→LDLR<sup>−/−</sup> mice compared with C57Bl/6→LDLR<sup>−/−</sup> mice, as mentioned above (Table 2). LXR agonist treatment for 6 weeks significantly reduced atherosclerosis in mice with macrophages that express LXR (57% reduction in C57Bl/6→LDLR<sup>−/−</sup> and 68% reduction in LDLR<sup>−/−</sup>→LDLR<sup>−/−</sup>, Figure 4B). In contrast, LXR agonist treatment had no inhibitory effect on atherosclerosis in mice with macrophages devoid of LXRs (LXRαβ<sup>−/−</sup>→LDLR<sup>−/−</sup>, Figure 4B). These results demonstrate that LXR activity in the macrophage is obligatory for the LXR agonist–dependent inhibition of atherosclerosis.
TABLE 1. Macrophage and Collagen Content of Atherosclerotic Lesions in LDLR<sup>−/−</sup> Mice After LXR Agonist Treatment

<table>
<thead>
<tr>
<th>Treatment Group</th>
<th>Macrophage Area*</th>
<th>Collagen Content*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>38.5±4.8</td>
<td>17.6±1.5</td>
</tr>
<tr>
<td>T0901317§</td>
<td>20.1±4.7†</td>
<td>23.5±0.8§</td>
</tr>
</tbody>
</table>

*Staining for macrophages (MOMA-2) and collagen (trichrome) within total aortic root lesion areas was quantified by image analysis of 3 equally spaced aortic root sections per mouse (n=5 per group). Values are the percent of the total lesion area stained and are expressed as the mean±SEM, †P<0.05 vs vehicle (Student t test). §LDLR<sup>−/−</sup> mice were fed a Western diet for 8 weeks and subsequently treated with vehicle or the LXR agonist T0901317 (10 mg/kg per day, PO) for 6 weeks.

To determine the impact of macrophage LXR deficiency on LXR agonist–induced gene expression, RNA was isolated from bone marrow cells harvested from each group of mice at the end of the study. In bone marrow cells from C57Bl/6→LDLR<sup>−/−</sup> and LDLR<sup>−/−</sup>→LDLR<sup>−/−</sup> mice, LXRβ mRNA levels were ≈3-fold higher than those of LXRα mRNA, and LXR agonist treatment did not significantly affect mRNA levels of either receptor isotype (data not shown). ABCA1 and ABCG1 mRNA levels were significantly increased by LXR agonist treatment in bone marrow cells from C57Bl/6→LDLR<sup>−/−</sup> and LDLR<sup>−/−</sup>→LDLR<sup>−/−</sup> mice, whereas bone marrow cells from LXRα<sup>−/−</sup>→LDLR<sup>−/−</sup> mice showed no induction of these mRNA transcripts (Figure 4C and 4D). Taken together with the atherosclerosis data, these findings suggest that LXR agonist treatment may delay the progression of atherosclerosis through induction of genes that promote reverse cholesterol transport from the macrophage.

Discussion

These data demonstrate that an LXR agonist induces the regression of preexisting atherosclerotic lesions. Regression was accompanied by remodeling of the lesions in a manner consistent with their stabilization. Our data suggest that the ability of the LXR agonist to induce regression of atherosclerotic lesions may also be linked mechanistically to increased cholesterol efflux from macrophages in the artery wall, specifically through induction of expression of the ABC transporters, including ABCA1 and ABCG1. The recent reports that LXR agonists have antiinflammatory activity and inhibit expression of several proinflammatory genes in macrophages and in the artery wall, combined with their ability to reduce macrophage content of atherosclerotic lesions observed here, suggest a beneficial clinical impact of LXR agonist treatment in preexisting atherosclerosis.

Although a number of pharmacological intervention studies with statins, bile acid-binding resins, fibrates, and ACAT inhibitors have been reported to inhibit atherosclerosis pro-
gression in animal models, regression achieved with pharmacological agents has generally been associated with significantly decreased plasma cholesterol concentrations.\textsuperscript{26,27} Notably, the dramatic effect of LXR agonist treatment on atherosclerotic lesion regression reported here occurred in the presence of only a modest reduction in plasma total cholesterol levels and elevated plasma triglyceride levels. Rapid and substantial lesion regression has been induced in animal models treated with either native\textsuperscript{23} or a naturally occurring mutant of apoA-1\textsuperscript{28,29} in the absence of significant lowering of plasma cholesterol. Recently, a similarly rapid regression of coronary atherosclerosis was demonstrated in humans treated with the apoA-1 Milano variant,\textsuperscript{30} the mechanism of which is presumably related to an increase in reverse cholesterol transport out of the atherosclerotic lesion.\textsuperscript{31} Although possibly affecting the same reverse cholesterol transport process, the LXR agonist and the apoA-I variant represent distinct therapeutic strategies, with the LXR agonist acting directly on the atherosclerotic lesion after oral administration whereas the injected apoA-I molecule acts by drawing cholesterol into the vascular space.

In a previous study with the synthetic LXR agonist GW3965, inhibition of atherosclerosis progression was observed even in the absence of major changes in plasma lipoprotein levels.\textsuperscript{19} The majority of the increased plasma cholesterol in this animal model results from accumulation of LDL, and FPLC fractionation analysis suggests that LXR agonist induced its greatest cholesterol-lowering effect on this fraction. Although somewhat surprising that the total cholesterol lowering was not of greater magnitude, given the plasma FPLC profile, as these 2 measurements are made independently and by different means, the common finding between studies remains that the antiatherogenic effects of an LXR agonist were observed in LDLR\textsuperscript{−/−} mice that were substantially hypercholesterolemic and hypertriglyceridemic. Treatment with the LXR agonist might be expected to shift the non-HDL profile in the plasma, resulting in increases in large apo-B100–containing lipoproteins and decreases in the number of small apo-B100–containing lipoproteins. It is possible that some of the antiatherogenic effects of LXR agonist treatment may have resulted from such a shift, because the small apo-B100–containing particles have been shown to be considerably more atherogenic than the large apo-B100–containing particles.\textsuperscript{32} However, it is unlikely that this effect would completely account for the dramatic regression of lesions observed here over a relatively short (6-week) treatment period, given the significantly longer intervention periods necessary to demonstrate regression of fatty streaks reported across animal models.\textsuperscript{26}

The LDL cholesterol reduction observed in the LDLR\textsuperscript{−/−} mice in the regression study suggests that LDL receptor–independent mechanisms, potentially including upregulation of ABCG5 and ABCG8 and enhanced excretion of cholesterol into bile, are responsible for LXR agonist–mediated plasma cholesterol lowering.\textsuperscript{14,15} However, a similar effect of LXR agonist treatment was not observed in the bone marrow transplant study. The reason for this difference is not known. The design of the 2 studies may contribute to this differential response, because LDLR\textsuperscript{−/−} mice in the regression study were fed the Western diet for a total of 14 weeks, whereas mice in the bone marrow transplant study were fed the Western diet only during the 8-week treatment period. Similar to the plasma cholesterol lowering observed in the lesion regression, there was a significant \textapprox32\% decrease in plasma cholesterol in LDLR\textsuperscript{−/−}→LDLR\textsuperscript{−/−} mice in the bone marrow transplant study, which was not observed in the C57BL/6→LDLR\textsuperscript{−/−} or LXRαβ\textsuperscript{−/−}→LDLR\textsuperscript{−/−} mice treated with LXR agonist. This finding, which appears to stem from the \textapprox50\% higher plasma cholesterol level in vehicle-treated LDLR\textsuperscript{−/−}→LDLR\textsuperscript{−/−} mice compared with the other bone marrow transplant conditions, has been observed previously\textsuperscript{33} and suggests a role for LDLR expression in cells of hematopoietic origin in regulating plasma cholesterol concentrations. This elevated plasma cholesterol level may also contribute to the increased atherosclerotic lesion area observed in the vehicle-treated LDLR\textsuperscript{−/−}→LDLR\textsuperscript{−/−} mice, in comparison to the C57BL/6→LDLR\textsuperscript{−/−} controls. It is clear from the literature that the bone marrow–transplanted LDLR\textsuperscript{−/−} model is not equivalent to its nontransplanted counterpart, at least with respect to atherosclerotic disease progression.\textsuperscript{34} Further studies are required to elucidate precise LXR-dependent mechanism(s) contributing to lowering of plasma cholesterol and the interaction between

### TABLE 2. Terminal Plasma Lipid Levels in Bone Marrow–Transplanted LDLR\textsuperscript{−/−} Mice

<table>
<thead>
<tr>
<th>Bone Marrow Donor</th>
<th>Treatment (n)</th>
<th>Total Cholesterol\textsuperscript{*}</th>
<th>Triglycerides</th>
<th>HDL Cholesterol</th>
</tr>
</thead>
<tbody>
<tr>
<td>C57BL/6</td>
<td>Vehicle (7)</td>
<td>976±158</td>
<td>230±52</td>
<td>72.3±2.5</td>
</tr>
<tr>
<td></td>
<td>T090317† (9)</td>
<td>1054±85</td>
<td>1143±147§</td>
<td>60.8±4.9</td>
</tr>
<tr>
<td>LXRαβ\textsuperscript{−/−}</td>
<td>Vehicle (9)</td>
<td>980±70</td>
<td>262±25</td>
<td>65.0±3.2</td>
</tr>
<tr>
<td></td>
<td>T090317 (11)</td>
<td>1192±40</td>
<td>1177±70§</td>
<td>73.7±2.8</td>
</tr>
<tr>
<td>LDLR\textsuperscript{−/−}</td>
<td>Vehicle (11)</td>
<td>1461±87†</td>
<td>342±22</td>
<td>72.2±6.0</td>
</tr>
<tr>
<td></td>
<td>T090317 (10)</td>
<td>994±82§</td>
<td>978±110§</td>
<td>67.3±3.0</td>
</tr>
</tbody>
</table>

*All data are mean±SEM, in mg/dL.
†LXR agonist T0901317 was administered orally (10 mg/kg per day) for 8 weeks, beginning 4 weeks after irradiation and bone marrow transplantation.
‡P<0.001 vs vehicle-treated LDLR\textsuperscript{−/−} mice transplanted with C57BL/6 or LXRαβ\textsuperscript{−/−} bone marrow (ANOVA, Newman–Keuls).
§P<0.001 vs vehicle-treated group within bone marrow donor condition.
experimental manipulations and LXR agonist induced reductions in circulating cholesterol concentrations.

In addition to reductions in lesion area, our studies demonstrate a decrease in CD68 mRNA expression and macrophage-positive immunostaining in atherosclerotic lesions in mice treated with an LXR agonist. The ability of LXR agonist treatment to reduce macrophage content and increase collagen content of advanced lesions, coupled with the reported antiinflammatory role of LXRs in the artery wall, strongly suggests that LXR agonists can be effective agents to reverse preexisting atherosclerosis. It should be noted that changes in CD68 expression in aortas of mice were not detected by Joseph et al; however, these investigators used a different strain of atherosclerotic mice and LXR agonist than those used here. We have shown that the regression lesions from LXR agonist–treated mice contain fewer macrophages and are more fibrous than lesions from vehicle-treated controls, suggesting that LXR agonist intervention can reduce the inflammatory nature and increase the stability of atherosclerotic lesions.

This study provides important evidence that macrophage expression of LXR is essential for the antiatherogenic effects of LXRs agonists in the artery wall, because the agonist failed to inhibit atherosclerosis in the absence of macrophage LXR expression. Further, the data argue that this effect does not result from activation of LXR-dependent effects in tissues such as liver and intestine (where LXRs regulate target genes that control lipid homeostasis) and that the effect can be appreciated in the absence of significant plasma lipid lowering. Because LXR activation in the liver contributes to plasma triglyceride elevation, LXR agonists that selectively target macrophages can be effective agents to induce ABC transporter expression and to promote reverse cholesterol transport. It should be noted that changes in macrophage LXR expression in aortas of mice were not detected by Joseph et al; however, these investigators used a different strain of atherosclerotic mice and LXR agonist than those used here. We have shown that the regression lesions from LXR agonist–treated mice contain fewer macrophages and are more fibrous than lesions from vehicle-treated controls, suggesting that LXR agonist intervention can reduce the inflammatory nature and increase the stability of atherosclerotic lesions.

This study provides important evidence that macrophage expression of LXR is essential for the antiatherogenic effects of LXRs agonists in the artery wall, because the agonist failed to inhibit atherosclerosis in the absence of macrophage LXR expression. Further, the data argue that this effect does not result from activation of LXR-dependent effects in tissues such as liver and intestine (where LXRs regulate target genes that control lipid homeostasis) and that the effect can be appreciated in the absence of significant plasma lipid lowering. Because LXR activation in the liver contributes to plasma triglyceride elevation, LXR agonists that selectively target macrophages can be effective agents to induce ABC transporter expression and to promote reverse cholesterol transport in the artery wall without undesirable liver effects. In conclusion, the data presented here demonstrate the obligatory role of macrophage LXR expression for the antiatherogenic activity of LXR agonists and suggest that selectively targeting macrophage LXR activity may provide novel promising therapeutic agents for the treatment of atherosclerosis.

Acknowledgments
We thank Dr B. Wagner, M. Petrowski, and J. Morisugu for quantitative polymerase chain reaction analyses; Dr A. Johnson for providing T0901317; Dr J. Witztum for FPLC plasma fractionation; Dr D.J. Mangelsdorf for the provision of the LXRα−/− and LXRβ−/− founder mice; and Drs P. Willy and B. Wagner for critical review of the manuscript.

References
Macrophage Liver X Receptor Is Required for Antiatherogenic Activity of LXR Agonists

Arterioscler Thromb Vasc Biol. 2005;25:135-142; originally published online November 11, 2004;
doi: 10.1161/01.ATV.0000150044.84012.68
Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2004 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/25/1/135

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/