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Effect of Macrophage Overexpression of Murine *Liver X Receptor-α* (*LXR-α*) on Atherosclerosis in LDL-Receptor Deficient Mice

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Background—The nuclear liver X receptor-α (LXR-α) has been implicated in the regulation of intracellular cholesterol homeostasis, inflammatory response, and atherosclerosis susceptibility. The aim of the present study was to test whether transgenic expression of LXR-α might affect these mechanisms and result in a reduction of atherosclerosis.

Methods and Results—We generated mice with macrophage overexpression of mouse *LXR-α*, evidenced by significantly elevated expression levels of LXR-target genes (*ABCA1*, *ABCG1*) in these cells. For atherosclerosis studies, mice were crossed onto the LDL-receptor deficient background. Plasma lipids and lipoproteins as well as liver triglycerides were not significantly different between transgenic animals and nontransgenic controls. However, lesion area at the brachiocephalic artery (BCA) was significantly reduced (−83%, $P=0.02$) in male *LXR-α* transgenic mice. This was associated with a significantly increased cholesterol efflux to acceptor-free media (+24%, $P=0.002$) and ApoA1 containing media (+20%, $P<0.0001$) as well as reduced lipopolysaccharide (LPS)-induced NO-release from macrophages of transgenic animals, providing a potential mechanism for the reduction of atherosclerosis.

Conclusion—Our data show for the first time that transgenic overexpression of *LXR-α* in macrophages has significant antiatherogenic properties. We conclude that overexpression of *LXR-α* in macrophages might be useful as a therapeutic principle for the prevention of atherosclerosis. **(*Arterioscler Thromb Vasc Biol*. 2008;28:2009-2015)**

Key Words: macrophages ■ LXR-α ■ atherosclerosis ■ transgenic mouse

Liver X receptor-α (LXR-α) is a ligand-dependent nuclear receptor playing an important role in cholesterol homeostasis and inflammatory signaling (for review see[1,2]). LXR-α was first identified in liver but is also expressed in the adrenals, intestine, adipose tissue, kidney, lung, and macrophages.[3] In contrast, its homologue LXR-β is expressed more ubiquitously.[3] Both, LXR-α and LXR-β form permissive heterodimers with the retinoid X receptor (RXR), and these dimers are able to activate transcription after binding of ligands for either receptor. Currently identified endogenous ligands of LXRs are oxidized derivatives of cholesterol (oxysterols) such as 22-(R)-hydroxycholesterol, 24(S)-25-epoxycholesterol, 27-hydroxycholesterol, and 6-α-hydroxy bile acids.[4] In addition, a number of highly potent synthetic agonists to LXR have been identified.[5] Activated LXR/RXR heterodimers bind to LXR-responsive elements (LXRE) in DNA consisting of two direct repeats of the core sequence AGGTCA separated by four nucleotides (DR-4).[6] LXREs have been identified in genes such as *ABCA1*,[7] *ABCG1*,[8] *cholesteryl ester transfer protein (CETP)*, *SREBP-1c*, *lipoprotein lipase*, and murine *CYP7A1* (for review see[9]). LXR also upregulates the expression of the entire *ApoE/CI/CIV/CII* cluster via two LXREs identified in the *ApoE* promoter.[9] These data provide functional evidence for the role of LXRs in lipid metabolism and cholesterol homeostasis. Specifically, LXR-α coordinates the physiological response to cellular cholesterol loading by activating ATP-binding cassette transporters ABCA1 and ABCG1, and ApoE in the macrophage, thus implicating an important role in the development of atherosclerosis.[1,4]

The significance of LXR-α in atherogenesis has been addressed in two ways, (1) using LXR-deficient mouse models and (2) by experimental treatment of mice with LXR agonists. The effect of deficiency of LXRs on atherosclerosis has been investigated by transplantation of bone marrow from LXR-α/LXR-β-double–deficient mice onto atherosclerosis-prone apolipoprotein–E deficient ($ApoE^{-/-}$) or LDL-receptor deficient ($LDLR^{-/-}$) mice.[10] This resulted in increased lipid

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Parts of the data have been published in abstract form (AHA Scientific Sessions, Orlando, 2007).

D.T. and D.K. contributed equally to this study.

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accumulation in macrophages and significantly increased atherosclerosis in transplanted animals.[10]

In contrast, treatment of ApoE$^{-/-}$ and LDLR$^{-/-}$ mice with the synthetic LXR-agonist GW3965 led to a ≈50% decrease of lesion area.[5] This finding could be replicated using the LXR-agonist T0901317 in LDLR$^{-/-}$ mice.[11] Moreover, Levine et al showed that treatment of LDLR$^{-/-}$ mice with T0901317 was potent enough to induce regression of preexisting atherosclerotic lesions.[12] Recently, it was shown that GW3965 was capable of reducing atherosclerosis in ApoE$^{-/-}$/LXR-$\alpha^{-/-}$ mice, suggesting an important role of LXR-β in atherogenesis.[13] LXR-agonists have a number of potential antiatherogenic properties, such as enhancement of reverse cholesterol transport.[14] On the other hand, it should be noted that LXR-agonists also induce genes involved in hepatic lipogenesis, leading to elevated plasma triglyceride levels in agonist-treated mice. The latter poses an obstacle to the use of these nonselective LXR-activating compounds as human therapeutics.[2]

Regardless, these studies provide experimental evidence for a key role of the macrophage LXR pathway in atherosclerosis. However, it was not clear whether overexpression of LXR-α in macrophages would exert antiatherogenic effects. The latter might be of particular interest, because this strategy would help to avoid the known side-effects of general LXR-α activation. Thus, the aim of the present study was to investigate the effect of selective transgene expression of LXR-α in macrophages on atherosclerosis development in the LDLR$^{-/-}$ mouse model.

Methods

An expanded methods section can be found in the online supplement (available online at http://atvb.ahajournals.org).

Preparation of Murine LXR-α Construct and Generation of Transgenic Mice

A 1452-bp fragment containing the full-length cDNA of mouse *LXR-α* was amplified from liver (C57BL/6), subcloned into pCRII-TOPO (Invitrogen), and subsequently ligated into the pIIIiLys chicken lysozyme promotor construct[15] using the *Sal*I and *Mlu*I restriction sites (Trenzyme, Konstanz, Germany). The resulting 20-kb construct was linearized with *Xho*I and prepared for microinjection. The full-length cDNA was verified by DNA sequencing. Microinjection of the vector construct was done in oocytes from mice on the C57BL/6 (B6) background. Transgenic founder animals were identified by quantitative fluorogenic polymerase chain reaction (PCR) (see below). The LXR-α transgenic line was designated B6$^{tg\ mLXR\alpha}$.

Crossing and Experimental Procedures Involving Mice

For atherosclerosis studies, B6$^{tg\ mLXR\alpha}$ mice were crossed onto a homozygous B6.LDLR$^{-/-}$ background (The Jackson Laboratory, Bar Harbor, Maine, stock no. 002207) to generate B6.LDLR$^{-/-tg\ mLXR\alpha}$ animals. Female (n=37) and male (n=38) mice were weaned at 28 days of age and fed a semisynthetic diet containing 0.02% cholesterol[16] until they were euthanized at 20 weeks of age. At sacrifice, blood was collected into syringes containing EDTA and the circulatory system was flushed with PBS (20 mL). The heart and brachiocephalic artery (BCA) were removed and snap-frozen in Tissue-tek OCT compound (Sakura Finetek). Liver, kidney, lung, spleen, heart, intestine, aorta, brain, and bone marrow were collected on liquid nitrogen and stored at −80°C.

Bone marrow–derived macrophages were isolated by flushing both femurs of a mouse, washed and cultivated in DMEM containing 30% L-cell conditioned medium, 10% FCS, 1% penicillin/streptomycin and 1% partricin. Cells were expanded for a total of 2 weeks, until they were seeded onto smaller dishes for functional studies.

Experimental studies with animals were approved by the responsible authorities of the state of Saxony (Regierungspräsidium Sachsen TVV 07/02, N1/05).

Histological Analysis

Atherosclerosis quantification at the aortic root and brachiocephalic artery (BCA) was performed as previously described.[16] Complexity of atherosclerotic lesions was assessed according to the Stary-classification, adapted to lesions in mice as suggested by Reddick et al.[17] Sections without atherosclerotic lesions were categorized as stage 0. Early lesions consisting predominantly of foam cells (Stary equivalent type I-II) were classified as stage 1. Moderate lesions with extracellular lipid deposition or thin fibrous cap development (Stary equivalent type III-V) were classified as stage 2, and advanced lesions with complex fibro-proliferative plaques (Stary equivalent type V–VIII) were classified as stage 3.

Expression Studies in Bone Marrow–Derived Macrophages

Bone marrow–derived macrophages were grown in L-cell conditioned medium for 2 weeks and seeded in 35-mm dishes at a density of 1×10^6 cells per dish. Cells were grown for 72 hours, before LXR-was activated by incubating with 10 μmol/L T0901317. Treatment with acLDL was performed as described for cholesterol efflux studies. After 24 hours, RNA from macrophages was isolated and *LXR-α*, *LXR-β*, *ABCA1*, and *ABCG1* expression was determined (see below).

RNA Isolation and Reverse Transcription

Tissue (50 to 100 mg) was homogenized in a Precellys24 homogenizer using CK14 ceramic beads (Brevet Bertin Technologies). Total RNA was isolated using the monophasic phenol-guanidine isothiocyanate TRIzol reagent (Invitrogen), and 2 μg were reverse transcribed into cDNA (SuperScript II RNase H- reverse transcriptase, Invitrogen) using random hexamer primers.

Quantitative Fluorogenic PCR (TaqMan)

Quantitative fluorogenic TaqMan-PCR was performed for detection of the *mLXR-α*-cDNA transgene in the animals' DNA and quantification of mRNA expression of *LXR-α*, *LXR-β*, *ABCA1*, and *ABCG1*. PCR-conditions and sequences of primers and probes are detailed in the online supplement. *GAPDH* was used as housekeeping gene. Cloned cDNA fragments of the template were used as standards in serial dilutions ranging from 100 to 10^7 copies for quantification.

Blood Analyses

Plasma parameters were analyzed in an automated analyzer (Modular PPE, Roche). Lipoproteins were isolated by sequential ultracentrifugation from 60 μL of plasma at densities (d) <1.006 g/mL (very low–density lipoprotein), $1.006\leq d\leq1.063$ g/mL (intermediate-density lipoprotein and low-density lipoprotein), and $d>1.063$ g/mL (high-density lipoprotein) in a LE-80K ultracentrifuge (Beckman) as described.[18]

Cholesterol Efflux

Cholesterol efflux was determined in cultivated bone marrow–derived macrophages from B6.LDLR$^{-/-}$ and B6.LDLR$^{-/-tg\ mLXR\alpha}$ animals, essentially as previously described.[19] Briefly, macrophages (1×10^6) were grown in replicates of six and loaded with medium containing $3.7\text{x}10^4$ Bq/mL ^{3}H-cholesterol and 50 μg/mL acLDL. Cells were washed and incubated with acceptor medium A (control), B (10 μmol/L T0901317), C (100 μg/mL HDL), D (100 μg/mL HDL, 10 μmol/L T0901317), E (10 μg/mL ApoAI), F (10 μg/mL ApoAI, 10 μmol/L T0901317). After 24 hours, the medium was collected and used to determine radioactivity. Cells were washed, lysed, and radioactivity was determined. Efflux was calculated as the ratio of disintegrations per minute (DPM) of medium over total DPM.

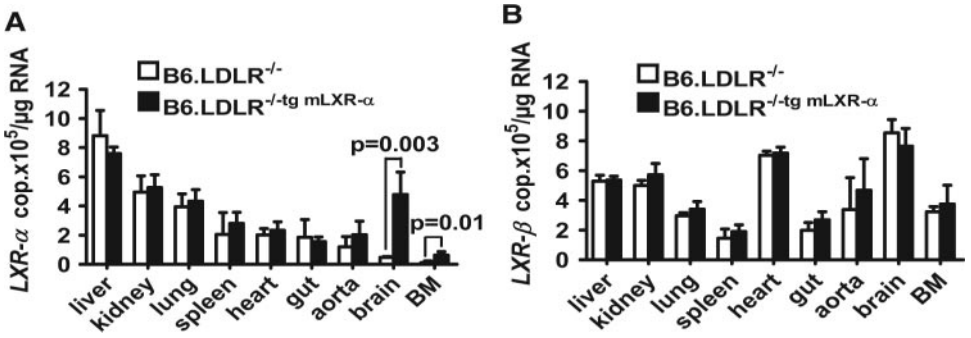

Figure 1. Expression of (A) LXR-α mRNA and (B) LXR-β mRNA in tissue of 5 B6.LDLR$^{-/-\text{tg mLXR-}\alpha}$ mice and 3 B6.LDLR$^{-/-}$ controls. Values are given as mean±SD. No significant differences between strains were noted unless described. BM, bone marrow.

Griess Assay

Bone marrow–derived macrophages were seeded in 96-well plates (80 000 cells per well) and grown for 24 hours. Cells were pretreated with 10 μmol/L T0901317 for 3 hours and subsequently incubated with 100 ng/mL LPS for 24 hours in presence and absence of T0901317. Replicates of 6 were used. 50 μL of the supernatant were used for determination of nitrate using the Griess reagent (G4410, Sigma-Aldrich). NO-production was normalized to cellular protein, determined with the Coomassie Plus Kit (Pierce).

Statistical Analysis

Values are given as mean±SEM unless noted otherwise. Distributions were tested for normality and statistical analysis was done by *t* test and analysis of variance (ANOVA) for normally distributed data and Mann–Whitney and Kruskal-Wallis tests for nonnormally distributed data using the Prism software, version 4.0. The χ^2 test was used to detect group differences of frequency (count) data.

Results

Generation of mLXR-α Transgenic Mice and Expression of mLXR-α mRNA

Mice transgenic for *mLXR-α* under the control of the chicken gene domain for lysozyme were apparently healthy and did not show any gross abnormalities. Transgenic animals were generated on the B6 background and backcrossed to the LDLR$^{-/-}$ background. The lysozyme vector, containing the transgene flanked by chicken lysozyme cis-acting elements, has previously been shown to direct gene expression predominantly to macrophages.[15] To test for expression of the transgene, we examined mRNA expression of the *mLXR-α* transgene by quantitative RT-PCR in 9 different tissues of B6.LDLR$^{-/-\text{tg mLXR}\alpha}$ animals (Figure 1). It should be noted that the transgene expressed the mouse cDNA of *LXR-α* in addition to endogenous *mLXR-α*. Endogenous expression of *mLXR-α* was highest in liver, kidney, and lung and transgenic expression of *mLXR-α* did not lead to increased expression levels in these organs. In contrast, transgenic animals showed significantly elevated expression levels of *mLXR-α* in bone marrow (Figure 1A), bone marrow–derived macrophages (Figure 2A), and interestingly also in brain (Figure 1A). Expression of a transgene, driven by the chicken gene domain of lysozyme in the brain of transgenic mice, has previously been observed.[15] As already mentioned, *LXR-α* expression was not significantly elevated in liver of transgenic animals compared to nontransgenic controls. Elevated LXR-activity

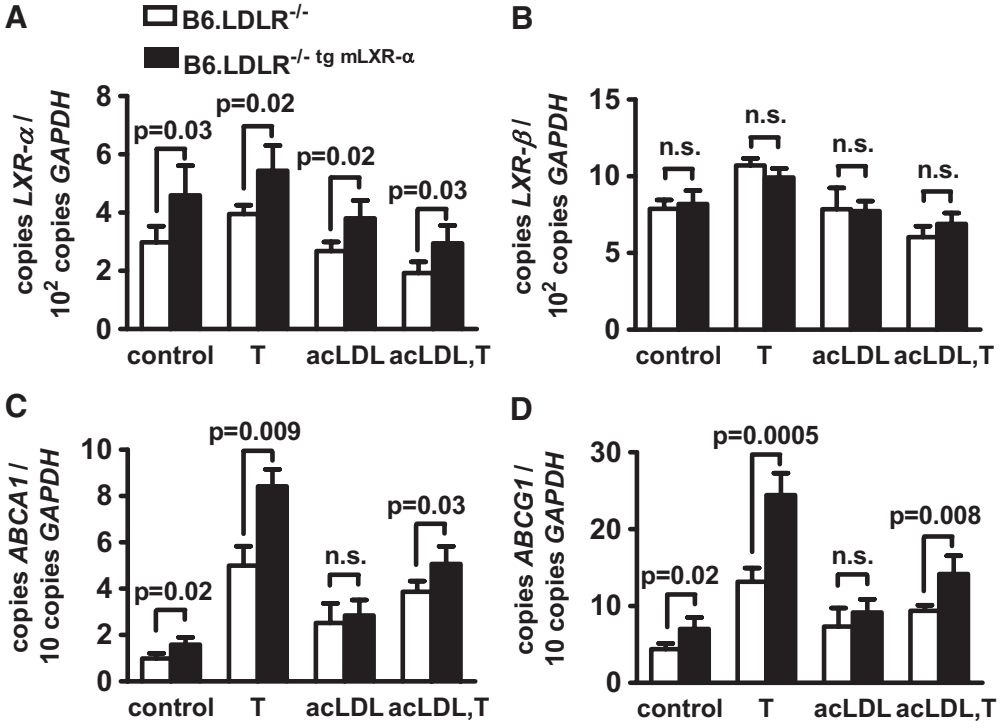

Figure 2. mRNA expression of *LXR-α*, *LXR-β*, and LXR target genes *ABCA1* and *ABCG1* in bone marrow–derived macrophages of B6.LDLR$^{-/-\text{tg mLXR-}\alpha}$ mice and B6.LDLR$^{-/-}$ controls incubated with/without the synthetic LXR-agonist T0901317 (10 μmol/L) and/or acLDL (50 μg/mL). A, *mLXR-α*; B, *mLXR-β*; C, *ABCA1*; D, *ABCG1*. Values are given as mean±SD of quadruplicate dishes. T, T0901317.

Table. Clinical Chemistry and Lipid Parameters in Male mLXR-α Transgenic Mice

Parameters	B6.LDLR$^{-/-}$	B6.LDLR$^{-/-tg\ mLXR-\alpha}$
n	20	21
Body weight, g	29.3±0.79	29.1±0.41
ALAT, μkat/L	0.54±0.09	0.65±0.09
ASAT, μkat/L	2.17±0.24	2.65±0.25
Protein, g/L	46.05±1.16	44.36±0.83
GLDH, μkat/L	0.25±0.07	0.29±0.05
LDH, mmol/L	6.24±0.59	6.80±0.52
ChE, μkat/L	62.58±2.54	57.06±2.10
BUN, mmol/L	11.65±0.44	10.49±0.43
Cholesterol, mmol/L	16.21±1.18	14.46±0.92
VLDL-C, mmol/L	3.81±0.47	3.28±0.59
LDL-C, mmol/L	10.02±1.13	8.62±1.01
HDL-C, mmol/L	2.26±0.10	2.25±0.09
Triglycerides, mmol/L	2.99±0.32	2.65±0.18

n indicates number of animals; ALAT, alanine aminotransferase; ASAT, aspartat aminotransferase; GLDH, glutamate dehydrogenase; LDH, lactat dehydrogenase; ChE, cholinesterase; VLDL-C, very low–density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; HDL-C, high-density lipoprotein cholesterol. Values are mean±SEM. No significant differences were observed between B6.LDLR$^{-/-}$ and B6.LDLR$^{-/-tg\ mLXR-\alpha}$ mice.

and fat-accumulation in liver has been described as a major side-effect in mouse models of LXR-activation by LXR-agonists.[2] We therefore performed oil-red-O staining of frozen sections of liver tissue of transgenic and nontransgenic mice and also measured triglyceride content in liver but found no apparent differences in fat-deposition between strains (data not shown). Because functional redundancy between LXR-α and LXR-β was reported in LXR-deficient mice,[20] expression of *LXR-β* was also tested, but no significant differences were observed (Figure 1B). Thus, *mLXR-α* expression was selectively elevated in macrophages and brain of transgenic mice compared to nontransgenic controls with no significant side-effects such as fat accumulation in the liver.

Expression of *mLXR-α*, *LXR-β*, *ABCA1*, and *ABCG1* in Transgenic Mice

To prove that *mLXR-α* overexpression in macrophages has functional consequences, we next determined mRNA expression levels of *ABCA1* and *ABCG1* as target genes of LXR-α. The *ABCA1* and *ABCG1* promoters have LXR-responsive elements and are upregulated after LXR-activation.[7,8] As shown in Figure 2A, *mLXR-α* expression was elevated in macrophages from B6.LDLR$^{-/-tg\ mLXR\alpha}$ compared to B6.LDLR$^{-/-}$ mice. This was also true after activation of cells with LXR agonist T0901317 and loading with acLDL (Figure 2A). Elevated expression of *mLXR-α* in transgenic macrophages was associated with increased expression levels of its target genes *ABCA1* (Figure 2C) and *ABCG1* (Figure 2D), which were highly expressed in particular in presence of the LXR agonist T0901317 and acLDL loading. In contrast, no significant differences in *LXR-β* expression levels were noted between *LXR-α* transgenic- and nontrangenic macrophages (Figure 2B). These data showed that *mLXR-α* overexpression in macrophages from our animals was indeed functional.

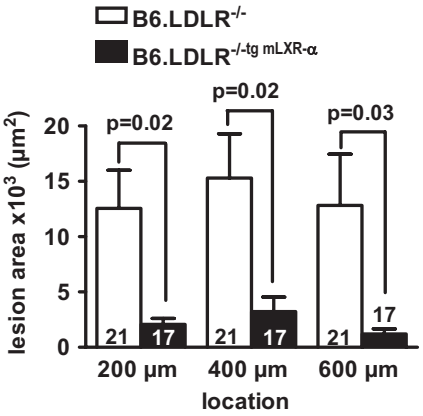

Figure 3. Atherosclerotic lesion area at the BCA in male B6.LDLR$^{-/-tg\ mLXR-\alpha}$ mice and B6.LDLR$^{-/-}$ controls at 20 weeks of age. Sections were quantified 200, 400, and 600 μm proximal to the branching point of the BCA into the subclavian and carotid arteries. Bars represent mean±SEM, and the numbers of animals in the individual groups are given on the bars.

Atherosclerosis Susceptibility in mLXR-α Transgenic Mice

To test whether macrophage *mLXR-α* overexpression had an effect on atherosclerosis susceptibility, transgenic animals and littermate controls were kept on a standardized semisynthetic diet for 16 weeks and euthanized at 20 weeks of age. At sacrifice, blood was drawn and clinical chemistry parameters were determined. No significant differences were observed for parameters of liver and kidney function (Table). In addition, a detailed analysis of plasma lipid and lipoprotein parameters was performed. No significant differences were noted between B6.LDLR$^{-/-tg\ mLXR-\alpha}$ animals and nontransgenic littermate controls (Table).

To study the effect of the transgene on atherosclerosis development, we determined lesion size at the BCA and aortic root. Lesions at the BCA were quantified at 200, 400, and 600 μm proximal from its branching site into the carotid and subclavian arteries. As shown in Figure 3, lesion area in male B6.LDLR$^{-/-tg\ mLXR-\alpha}$ mice was dramatically reduced at sections of the BCA, located 200 μm (2193±548 μm^2 versus 12563±3440 μm^2; P=0.02), 400 μm (3406±1394 μm^2 versus 15289±3995 μm^2; P=0.02), and 600 μm (1326±5474 μm^2 versus 12822±4637 μm^2; P=0.03) proximal to the bifurcation into the carotid and subclavian arteries. The mean lesion area across the 3 anatomic sites was reduced by 83% in B6.LDLR$^{-/-tg\ mLXR-\alpha}$ compared to B6.LDLR$^{-/-}$ littermate controls (2309±587 μm^2 versus 13558±3640 μm^2; P=0.02). In female mice, overall lesion size was significantly larger than in male mice, and no significant differences between transgenic and nontransgenic mice were observed. We also determined lesion size at the aortic root. Lesion size at this anatomic location showed a trend toward smaller lesions in both sexes of the LXR-α transgenic animals but differences did not reach statistical significance neither in male (transgenic: 79080±11489 μm^2; nontransgenic: 93351±9398 μm^2) nor in female mice (transgenic: 212322±15072 μm^2; nontransgenic: 222125±17124 μm^2).

In addition, the stage of atherosclerotic lesions at the BCA was assessed using a modified Stary classification adapted to

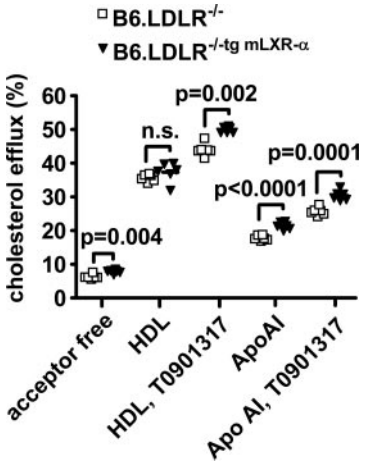

Figure 4. Cholesterol efflux in bone marrow–derived macrophages of B6.LDLR$^{-/-tg\ mLXR-\alpha}$ mice and B6.LDLR$^{-/-}$ controls. Acceptor-free indicates acceptor-free medium; HDL, HDL acceptor medium; HDL-T, HDL acceptor medium with LXR-agonist (T0901317); AI, ApoAI acceptor medium; AI-T, ApoAI acceptor medium with LXR-agonist (T0901317). Data represent means and individual values of 6 replicate cell culture dishes. n.s. indicates not significant.

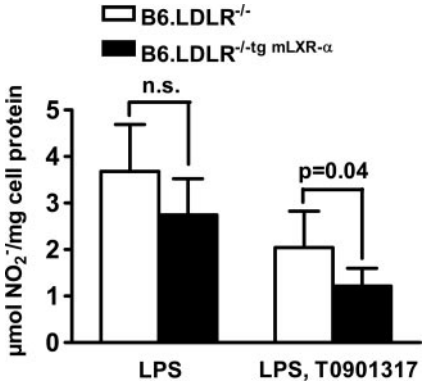

Figure 5. Nitrite production of macrophages from B6.LDLR$^{-/-tg\ mLXR-\alpha}$ and B6.LDLR$^{-/-}$ mice incubated with/without the synthetic LXR-agonist T0901317 (10 μmol/L). Cell were incubated with 100 ng/mL LPS for induction of iNOS. Bars represent mean±SD of replicates of 6 wells.

mouse lesions. Lesion composition was investigated by oil red O staining for lipids. Representative sections are shown in supplemental Figure I. As shown in supplemental Table I, lesions in male B6.LDLR$^{-/-tg\ mLXR-\alpha}$ animals compared to B6.LDLR$^{-/-}$ animals were significantly less advanced. At all 3 locations tested (at 200 μm, 400 μm, and 600 μm), transgenic animals had significantly greater numbers of early lesions (stage 0 and 1), whereas nontransgenic animals had significantly greater numbers of advanced lesions (stage 2 and 3; supplemental Table I).

Cholesterol Efflux in *mLXR-α* Transgenic Mice

One potential mechanism by which LXR-α might prevent atherosclerosis is by promoting cholesterol efflux from macrophages. We thus investigated cholesterol efflux in bone marrow–derived macrophages from B6.LDLR$^{-/-tg\ mLXR-\alpha}$ and B6.LDLR$^{-/-}$ animals. Efflux was studied into media without a cholesterol acceptor and after addition of HDL and ApoAI as cholesterol acceptors. As expected, addition of HDL and ApoAI dramatically increased cholesterol efflux (5.7-fold and 2.8-fold, respectively) compared to acceptor-free media. In some experiments, cells had previously been activated with the LXR agonist T0901317, which led to a further increase of cholesterol efflux. The overall picture showed that cholesterol efflux was enhanced in macrophages from B6.LDLR$^{-/-tg\ mLXR-\alpha}$ compared to B6.LDLR$^{-/-}$ mice (Figure 4).

Inflammatory Response in Macrophages From *mLXR-α* Transgenic Mice

One other potential mechanism by which LXR-α might reduce atherosclerosis is by modulating inflammatory response.[21] To this end, we investigated LPS-induced NO release in LXR-α transgenic macrophages compared to controls. As shown in Figure 5, there was a trend toward lower NO production in transgenic macrophages. NO release was further reduced when cells were incubated with the LXR agonist T0901317. Under these conditions, NO production was significantly lower in macrophages from B6.LDLR$^{-/-tg\ mLXR-\alpha}$ compared to B6.LDLR$^{-/-}$ mice (P=0.04; Figure 5), suggesting an antiinflammatory effect of the transgene.

Discussion

In the present study, we investigated the effect of macrophage overexpression of *LXR-α* on atherosclerosis susceptibility in LDLR$^{-/-}$ mice. As a main finding, we could demonstrate that lesion area at the BCA was significantly reduced (−83%, P=0.02) in male *LXR-α* transgenic mice compared to nontransgenic controls (Figure 3). This was associated with a significant increase of cholesterol efflux to acceptor-free media (+24%, P=0.002) and ApoA1 containing media (+20%, P<0.0001; Figure 4) as well as decreased inflammatory response in macrophages of transgenic animals (Figure 5).

Macrophage expression of *LXR-α* was achieved by transgenic expression of the mouse *LXR-α* cDNA under the control of a chicken lysozyme genomic DNA construct. The chicken lysozyme gene domain has previously been used in a number of studies to direct transgene-expression into macrophages of rabbits[22] and mice.[15,23,24] Consistent with these results, transgenic expression of *LXR-α* in our study was highest in macrophages, bone marrow and interestingly also in brain (Figure 1). Neuronal expression of a transgene driven by the chicken gene domain of lysozyme has previously been observed.[15,23] In these studies, the transgene was expressed in specific neuronal cells in the dentate gyrus of the hippocampus and in the granular layer of the outer cortex.[23] Transgene expression in brain most likely represents a feature of *trans*-species expression of a chicken transgene in mouse.[23] However, transgenic animals did not show any apparent neurological abnormalities. In this context it should be mentioned that *LXR-α* expression was not significantly affected in livers of *LXR-α* transgenic mice. This constitutes a major advantage of our transgenic model of *LXR-α* overexpression compared to the use of synthetic LXR-agonists. These substances have been shown to cause hypertriglyceridemia, and liver-steatosis as side-effects, posing a significant obstacle to the development of these compounds as human therapeutics.[2] Previous studies have shown that treatment of mice with the LXR agonist T0901317 led to increased

expression of *Srebp-1c,* and increased expression of major fatty acid synthesis genes, an effect that was accompanied by a significant increase in plasma triglyceride levels. ApoE$^{-/-}$ and LDLR$^{-/-}$ mice are particularly susceptible to hypertriglyceridemia, because these animals are unable to clear triglyceride-rich VLDL as efficiently as wild-type mice.[25] In our study, the transgene was not significantly expressed in liver (Figure 1), and mice did not show elevated plasma cholesterol, trigycerides, VLDL-, LDL-, and HDL-cholesterol or lipid accumulation in liver, even when crossed onto the LDL-receptor deficient background (Table). However, we could demonstrate that LXR-α overexpression in macrophages resulted in the activation of *LXR-α* target genes such as *ABCA1* and *ABCG1*, whereas expression levels of *LXR-β* remained unchanged (Figure 2). Thus, *LXR-α* expression was elevated in macrophages of transgenic animals and functionally active, whereas no adverse effects on lipid metabolism were observed. It is of interest that overexpression of LXR-α had an effect even in absence of additional ligand, implying that LXR ligands were not limiting in macrophages.

The major finding of the present study was a dramatic reduction of atherosclerosis in the BCA of male B6.LDLR$^{-/-\text{tg mLXR-}\alpha}$ mice compared to B6.LDLR$^{-/-}$ controls. The reduction of atherosclerosis was seen at 3 different anatomic locations in the BCA. These results are consistent with previous work, showing a reduction of atherosclerosis in mice treated with LXR-agonists.[5,11,12] However, it should be noted that pharmacological intervention in these studies led to a generalized ligand-dependent activation of LXR. In contrast, the approach of our study involved specific overexpression and subsequent activation of LXR by physiological LXR-activators (eg, oxysterols) present in the cells.

It is of interest that the reduction of atherosclerosis in our study was only statistically significant in male mice but not in female mice. There is only one previous study where gender-specific effects of pharmacological LXR-modulation on atherosclerosis have been demonstrated.[5] Comparable to our results, these investigators reported a stronger reduction of atherosclerosis in male mice treated with the LXR-agonist GW3945 than in female mice. Three other studies reported only data from male mice on the reduction of atherosclerosis by the LXR agonist T0901317.[11–13] The study by Tangilara et al is difficult to interpret with respect to gender specificity because these investigators transplanted bone marrow from male LXR-α/β deficient mice onto female recipients and also bone marrow from female LXR-α/β deficient mice onto male recipients.[10] Thus, the available data indicate that LXR-activation has a more potent effect on the reduction of atherosclerosis in male mice even though the reasons for the potential gender-dependent effect of LXR on atherosclerosis susceptibility are presently not clear and deserve further study. Another aspect requiring further investigation are differences in the amount of reduction of atherosclerosis in B6.LDLR$^{-/-\text{tg mLXR-}\alpha}$ mice at different anatomic sites of the vasculature. These effects were much stronger at the BCA, and only showed a trend toward smaller lesions at the aortic root. In this regard, a recent study is of particular interest, showing a marked reduction of atherosclerosis at the BCA in mice treated with the LXR agonist T0901317, which was not seen at other sites of the vascular bed including the aortic root.[26] It should be noted that similar site-specific differences of induced mutant mouse models for other candidate genes of atherosclerosis have been previously observed by a number of groups,[27] including ours.[18]

Two major potentially atheroprotective mechanisms of LXR function have been suggested. These include regulation of cholesterol homeostasis and inhibition of inflammatory signaling.[2] The mechanisms underlying the inhibition of inflammatory signaling are poorly understood, and current data suggest that inhibition of the NF-κB pathway is involved.[21] To test this in our transgenic model, we determined LPS-induced NO release from macrophages, which is mediated by NF-κB–dependent activation of iNOS. We could demonstrate that incubation with an LXR agonist significantly reduced NO release from macrophages, which was already known from previous work.[21] In addition, we could show that this reduction was significantly stronger in macrophages from *LXR* transgenic mice, compared to nontransgenic controls (Figure 5). This is of interest, because it is known from pervious work in ApoE$^{-/-}$ deficient mice that deficiency in iNOS results in reduced atherosclerosis.[28] These data suggest that reduced inflammatory response might represent an additional atheroprotective mechanism in mLXR-α transgenic animals.

The other potentially important atheroprotective function of LXR involves regulation of cholesterol homeostasis.[2] Because our data indicated a reduction of lipid-laden macrophages in the vessel wall of B6.LDLR$^{-/-\text{tg mLXR-}\alpha}$ mice (supplemental Figure I), one major focus was to investigate the effect of *LXR-α* transgene expression on cholesterol efflux in macrophages. Here, we demonstrate that cholesterol efflux was in fact enhanced in *LXR-α* transgenic macrophages under several conditions (Figure 4). Cholesterol efflux was further activated after incubation of cells with the synthetic LXR agonist T0901317, as previously shown.[29] However, when incubated with T0901317, efflux from LXR-α transgenic macrophages to HDL and ApoAI containing media was consistently higher compared to nontransgenic controls (Figure 4), thus providing a potential explanation of reduced foam cell formation and atherosclerosis in B6.LDLR$^{-/-\text{tg mLXR-}\alpha}$ mice. Activation of the ABC transporters ABCA1 and ABCG1 by LXR might be critical in enhancing cholesterol efflux. This hypothesis is corroborated by our finding of increased cholesterol efflux to HDL, indicating a role of ABCG1, as well as increased efflux to ApoAI, indicating a role of ABCA1.[30] Both these genes contain LXRE and are upregulated in response to LXR-activation.[7,8] This was supported by increased expression of *ABCG1* and *ABCG1* in *LXR-α* transgenic macrophages (Figure 2). LXR activation also induces upregulation of ApoE,[9] and we and others have shown that ApoE enhances cholesterol efflux from macrophages.[31,32] As a word of caution, the identified atheroprotective mechanisms from in vitro studies in *LXR*-transgenic macrophages do not necessarily extrapolate to the in vivo situation, even though this might be possible. In addition, there are current limitations to the potential use of LXR-overexpression as a therapeutic principle, because to the best of our knowledge, compounds that specifically activate LXR-α expression in macrophages have not been developed and efficient delivery of transgenes in humans is not yet solved. Nevertheless, we believe that LXR-α overexpression in macrophages constitutes an interesting target.

In summary, our data show for the first time that transgenic overexpression of *LXR-α* using a macrophage gene expression construct led to an increase of cholesterol efflux from macrophages, a downregulation of proinflammatory response, and a reduction of atherosclerosis. Transgenic expression of *LXR-α* was primarily directed into macrophages, and animals did not show the attendant side-effects usually seen with generalized LXR-activation by synthetic LXR-agonists. We conclude that macrophage overexpression of *LXR-α* might be useful as a therapeutic principle for the prevention of atherosclerosis.

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Disclosures

None.

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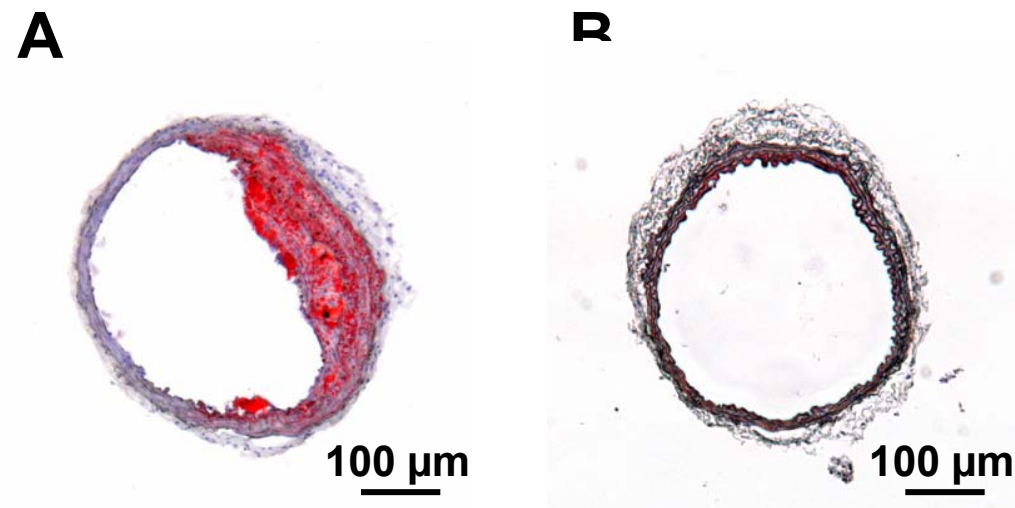
Supplemental Figure I



Representative sections through the BCA of $LDLR^{-/-}$ mice at 20 weeks of age. A, Oil red O staining in B6.$LDLR^{-/-}$ controls and B, B6.$LDLR^{-/-tg\ mLXR-\alpha}$ mice.

Supplementary Table I: Modified Stary-classification of lesion-complexity in male B6.LDLR$^{-/-}$ and B6. LDLR$^{-/-tg\ mLXR-a}$ at the brachiocephalic artery

Location		Stage 0	Stage 1	Stage 2	Stage 3	Mice (n)	p
200 µm	LDLR$^{-/-}$	5%	38%	33%	24%	21	0.004
	LDLR$^{-/-tg\ mLXR-\alpha}$	28%	67%	5%	0%	17	
400 µm	LDLR$^{-/-}$	5%	29%	28%	38%	21	0.005
	LDLR$^{-/-tg\ mLXR-\alpha}$	33%	50%	17%	0%	17	
600 µm	LDLR$^{-/-}$	19%	29%	24%	28%	21	0.004
	LDLR$^{-/-tg\ mLXR-\alpha}$	53%	47%	0%	0%	17	

Percentages of total sections per group at distinct stages of atherosclerosis; n, number of mice.