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Abstract

Background: The nuclear liver X receptor-α (LXR-α) has been implicated in the regulation of intracellular cholesterol homeostasis and atherosclerosis susceptibility by up-regulating proteins involved in reverse cholesterol transport. Previous atherosclerosis studies were based on knockout mouse models of LXR and treatment of mice with LXR agonists. The aim of the present study was to test whether increased expression of LXR-α might result in an improvement of cholesterol homeostasis and reduction of atherosclerosis.

Methods and Results: To address this question, we generated mice with macrophage specific over-expression of mouse LXR-α under the control of a chicken lysozyme genomic DNA construct. Macrophages from transgenic mice indeed showed significantly elevated expression levels of the LXR-target genes (ABCA1) compared to non-transgenic controls. To assess the effect of the transgene on atherosclerosis susceptibility, mice were crossed onto the LDL-receptor deficient background. Plasma total cholesterol, triglycerides, LDL- and HDL-cholesterol concentrations were not significantly different between transgenic animals and non-transgenic 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%, $p<0.0001$) from macrophages of transgenic animals, thus providing a potential mechanism for the reduction of atherosclerosis.

Conclusions: Our data show for the first time that transgenic over-expression of LXR-α using a macrophage-specific gene expression construct has significant anti-atherogenic

properties. We conclude that over-expression of LXR-α in macrophages might be useful as a therapeutic principle for the prevention of atherosclerosis.

Condensed Abstract:

We demonstrate for the first time that macrophage-specific over-expression of murine LXR-α in $LDR^{-/-}$ mice reduces the development of peripheral atherosclerosis and enhances cholesterol efflux in bone marrow derived macrophages.

Key words:

macrophages, LXR-α, atherosclerosis, transgenic mouse

Introduction

Liver X receptor-α (LXR-α) is a ligand-dependent nuclear receptor playing an important role in cholesterol homeostasis and inflammatory signalling (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-alpha-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] LXRE 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 up-regulates 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 important role of LXRs in lipid metabolism and cholesterol homeostasis. Specifically, LXR-α coordinates the physiologic response to cellular cholesterol loading by activating ATP-binding cassette transporters ABCA-1 and ABCG-1, 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-α agonists. The effect of deficiency of LXRs on atherosclerosis has been addressed by transplantation of bone marrow from LXR-α/LXRβ-double deficient mice onto either ApoE$^{-/-}$ or LDLR$^{-/-}$ mice.[10] This resulted in increased lipid accumulation in macrophages and significantly increased atherosclerosis in transplanted animals.[10]

In contrast, treatment of atherosclerosis-prone apolipoprotein E deficient (ApoE$^{-/-}$) and LDL-receptor deficient (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 T-0901317 in LDLR$^{-/-}$ mice.[11] More recently, Levine et al showed that treatment of LDLR$^{/}$ mice with T-0901317 was potent enough to induce regression of preexisting atherosclerotic lesions.[12] LXR-agonists have a number of potential anti-atherogenic properties such as enhancement of reverse cholesterol transport.[13] 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 over-expression of LXR-α in macrophages would exert anti-atherogenic effects. The latter might be of particular interest, since 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

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

A 1452 bp fragment containing the full length cDNA of mouse LXR-α was amplified from liver (C57BL/6). A *Sal*I restriction site (underlined) was inserted into the forward primer 5'-G GGT GGG GTC GAC TGA GAA GCA GTC-3' and a *Mlu*I (underlined) restriction site was inserted into the reverse primer 5'-C TCC ACG CGT GGG AAG CAG-3' for cloning of the resulting PCR-fragment. To allow subsequent subcloning, an additional *Sal*I restriction site (underlined) within the LXR-α cDNA was removed using Quick Change Multi Site-Directed Mutagenesis Kit (Stratagene). The mutation primer (5'-GAA ATG CCA GGA GTG TCG TCT TCG CAA ATG CCG CC-3') was designed to insert a tolerable thymine instead of adenine (box). The cDNA fragment was then subcloned into pCR-TOPO (Invitrogen) and subsequently ligated into the pIIIiLys chicken lysozyme promotor construct[14] 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. To identify transgenic founder animals, DNA was isolated from tail tips of the resulting offspring using the DNeasy Blood & Tissue Kit (Qiagen) and detected by quantitative fluorogenic 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 LDL

receptor deficient (B6.LDLR$^{-/-}$) background (The Jackson Laboratory, stock no. 002207) and B6.LDLR$^{-/-tg\ mLXR\alpha}$ animals were generated. Female (n=37) and male (n=38) mice were weaned at 28 days of age and fed a semisynthetic diet containing 0.02 % cholesterol until they were sacrificed at 20 weeks of age.[15] One day before animals were killed, food was removed from the cage at 8 p. m. and access to water was allowed ad libitum. The animals were sacrificed 12 hours later. Mice were exsanguinated by left-ventricular puncture and blood was collected into syringes containing EDTA. 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, Japan). Bone marrow, brain, liver, kidney and skeletal muscle were collected on liquid nitrogen and stored at -80°C. Peritoneal macrophages were harvested three days after peritoneal injection of 3% thioglycolate broth (1.5 mL) in selected animals (n=5). The latter animals were not used for additional studies since thioglycolate might have induced a generalized state of inflammation.

Bone marrow derived macrophages were isolated by flushing both femurs of a mouse with 10 mL of ice cold PBS containing 1 IU heparin/mL. After filtering through a 40 µm nylon cell strainer (Becton Dickinson, Heidelberg, Germany) the cell suspension was centrifuged 5 min at 440 g. The cell pellet was resuspended in 1.5 mL DMEM containing 10% FCS, 1% penicillin/streptomycin and 1% partricin. Cells were seeded onto T75 culture flasks and cultivated in DMEM containing 30% L-cell conditioned medium, 10% FCS, 1% penicillin/streptomycin and 1% partricin for one week with a change of medium every 3 days. After one week, cells from each T75 culture flask were scraped off and transferred into two T175 culture flasks. Cells were grown for an additional week until they were scraped off and seeded in smaller dishes for functional studies.

Animals were housed in the Medical Experimental Center at the University of Leipzig in a controlled climatic and specific pathogen free (SPF) environment in rooms with 7 a. m. to 7 p. m. light/dark cycle. 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.[15] Complexity of atherosclerotic lesions was assessed according to the Stary-classification, adapted to lesions in mice as suggested by Reddick et al.[16] 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 x 10^6 cells/dish. After 72 h, the medium was replaced with DMEM containing, 10% FCS, 1% penicillin/streptomycin, 1% partricin. LXR-was activated by incubating with 10 µM T0901317. After 24 hours, RNA from macrophages was isolated and LXR-α and ABCA-1 expression was determined (see below).

RNA Isolation and Reverse Transcription

Total RNA from macrophages and tissue was isolated using the monophasic phenol-guanidine isothiocyanate TRIzol reagent (Invitrogen). RNA was dissolved in DEPC-treated water, RNA-concentration and -quality were determined at 260 nm and 280 nm. RNA (2 µg) was reverse transcribed into cDNA (SuperScript II RNase H- reverse transcriptase, Invitrogen) using random hexamer primers.

Quantitative Fluorogenic PCR (TaqMan)

Quantitative fluorogenic PCR was used for detection of the mLXR-α-cDNA transgene in DNA isolated from tail-tissue in order to determine whether animals carried the transgene. Quantitative fluorogenic PCR was also performed to determine mRNA expression levels of genes of interest in cDNA after reverse transcription.

The following primers and probes were used: mLXR-α forward primer, 5'-TTT-CTT-CAA-GCG-GAT-CTG-TTC-TT-3'; mLXR-α reverse primer, 5'-GAG-TGT-CGA-CTT-CGC-AAA-TGC-3'; mLXR-α probe 5'-FAM-TGA-CAG-CAC-ACA-CTC-CTC-CCT-CAT-GC-TAMRA-3'. ABCA1 forward primer, 5'-GCT-CAA-CTT-TTA-CGA-AGG-CAA-3'; ABCA1 reverse primer, 5'-AGC-GAA-TGT-CCT-TCC-CCA-G-3'; ABCA1 probe 5'-FAM-CCC-CAA-CTT-CTG-GCA-CGG-CCT-AC-TAMRA-3'. β-actin was taken as a standard housekeeping gene: β-actin forward primer 5'-GAG-AAG-CTG-TGC-TAT-GTT-GCT-C-3', β-actin reverse primer 5'-AGG-AAG-ACG-ATG-CGG-CA-3', β-actin probe 5'-FAM-AGA-CTT-CGA-GCA-GGA-GAT-GGC-CA-TAMRA-3'. Cloned cDNA fragments of the template were used as standards in serial dilutions ranging from 100 to 10^7 copies. PCR reactions were prepared in a final volume of 25 µL containing 5 µL of DNA or diluted cDNA (1:15), 5 mmol/L $MgCl_2$, 2.5 µL 10 x AmpliTaq buffer A, 200 µmol/L dNTP, 0.75 U AmpliTaq

Gold, 200 nM fluorescently labeled oligonucleotide probe and 900 nmol/L, each of the specific primers. Reactions were run on an ABI PRISM 7700 Sequence Detector (Applied Biosystems) with online detection of fluorescence. The cycling conditions were as follows: 50°C, 2 min; 95°C, 10 min and 40 two step cycles of 95°C, 15 sec; 60°C, 1 min. Analysis of the data was performed with the ABI PRISM sequence detection software Version 2.0.

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) of <1.006 g/mL (very-low-density lipoprotein), 1.006≤d≤1.063 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.[17] Cholesterol in the lipoprotein fractions was determined enzymatically using a colorimetric method (Roche).

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.[18] Briefly, macrophages (1 x 10^6) were plated in replicates of six onto 12 well plates in 1 mL of DMEM containing 30% L-cell conditioned medium, 10% FCS, 1% penicillin/streptomycin, and 1% partricin. After 2 days of adhesion, cells were washed with serum free medium and incubated with DMEM containing 3.7x10^4 Bq/mL ^{3}H-cholesterol, 30% L-cell conditioned medium, 10% FCS, 1% penicillin/streptomycin, and

1% partricin. After 24 hours, medium was replaced with fresh medium containing $3.7x10^4$ Bq/mL ^{3}H-cholesterol and 50 µg/mL acLDL. After another 24 hours, cells were washed extensively before acceptor media were added. Acceptor medium A) consisted of DMEM containing 0.2% BSA, 1% penicillin/streptomycin, and 1% partricin. In addition, acceptor medium B) contained 10 µmol/L T0901317, C) contained 100 µg/mL HDL, D) contained 100 µg/mL HDL and 10 µmol/L T0901317, E) contained 10 µg/mL ApoAI, and F) contained 10 µg/mL ApoAI and 10 µM T0901317. Acceptor medium (500 µL) was added to each well. After 24 hours, the medium was collected into 1.5 mL tubes, centrifuged at 3000 rpm for 10 minutes and 100 µl of the supernatant were used to determine radioactivity. Cells were washed with serum free medium and lysed overnight with 1 mL lysis buffer (0.1% SDS, 0.1 N NaOH). Radioactivity was determined by scintillation counting of 100 µL of lysed cells or medium in tubes containing 4.5 mL Ultima Gold XR liquid scintillation cocktail (Packard BioScience). Efflux was calculated as follows: Disintegrations per minute (DPM) in cells + DPM in medium were set as total DPM; DPM in medium/ total DPM was set as total efflux.

Statistical Analysis

Values are given as mean±standard error of the 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 non-normally distributed data using the Prism software, version 4.0. The chi-square test was used to detect group differences of frequency (count) data.

Results

Generation of mLXR-α Transgenic Mice and Expression of mLXRα 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.[14] To test for expression of the transgene, we examined mRNA expression of the mLXR-α transgene by quantitative RT-PCR in six different tissues of $B6.LDLR^{-/-tg\ mLXR\alpha}$ 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 muscle 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 peritoneal macrophages, bone-marrow derived macrophages and interestingly also in brain (Figure 1). Expression of a transgene driven by the chicken gene domain of lysozyme in the brain of transgenic mice has previously been observed.[14] As already mentioned, LXR-α expression was not significantly elevated in liver of transgenic animals compared to non-transgenic controls. Elevated LXR-activity 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 non-transgenic mice and found no apparent

differences in fat-deposition between strains (data not shown). Thus, mLXR-α expression was selectively elevated in macrophages and brain of transgenic mice compared to non-transgenic controls with no significant side-effects such as fat accumulation in the liver.

Expression of mLXR-α and ABCA1 in Transgenic Mice

To proof that mLXR-α over-expression in macrophages has functional consequences, we next determined mRNA expression levels of ABCA1, a target gene of LXR-α. The ABCA1-promoter has a LXR-responsive element and is up-regulated following LXR-activation.[7] We thus determined ABCA1 mRNA expression in bone marrow derived macrophages of mLXR-α and non-transgenic B6.LDLR$^{-/-}$ mice after activation with the LXR agonist T0901317 and confirmed that mRNA expression of LXR-α was indeed elevated (Figure 2A). We also showed that elevated LXR-α expression in our transgenic mice was associated with a significant 1.5-fold increase of ABCA1 expression ($p=0.0004$, Figure 2B). These data showed that mLXR-α over-expression in macrophages from our animals was indeed functional.

Atherosclerosis Susceptibility in mLXR-α Transgenic Mice

To test whether macrophage mLXR-α over-expression had an effect on atherosclerosis susceptibility, transgenic animals (n=36) and littermate controls (n=39) were kept on a standardized semisynthetic diet for 16 weeks and sacrificed 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 1). In addition, a detailed analysis of plasma lipid- and lipoprotein parameters was

performed. No significant differences of plasma lipid- and lipoprotein levels were noted between B6.LDLR$^{-/-tg\ mLXR-\alpha}$ animals and nontransgenic littermate controls (Table 1).

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 µm² vs. 12563±3440 µm²; p=0.02), 400 µm (3406±1394 µm² vs. 15289±3995 µm²; p=0.02) and 600 µm (1326±5474 µm² vs. 12822±4637 µm²; p=0.03) proximal to the bifurcation into the carotid and subclavian arteries. The mean lesion area across the three anatomical sites was reduced by 83% in B6.LDLR$^{-/-tg\ mLXR-\alpha}$ compared to B6.LDLR$^{-/-}$ littermate controls (2309±587 µm² vs. 13558±3640 µm²; p=0.02). In female mice, overall lesion size was significantly larger than in male mice, and no significant differences between transgenic and non-transgenic mice were observed. We also determined lesion size at the aortic root. Lesion size at this anatomical location showed a trend towards smaller lesions in both sexes of the LXR-α transgenic animals, but differences did not reach statistical significance neither in male (transgenic: 79080±11489 µm²; non-transgenic: 93351±9398 µm²) nor in female mice (transgenic: 212322±15072 µm²; non-transgenic: 222125±17124 µm²).

In addition, the stage of atherosclerotic lesions at the BCA was assessed using a modified Stary-classification adapted to mouse lesions. Lesion composition was investigated by oil-red O staining for lipids. Representative sections are shown in Figure 4. As shown in Table 2, lesions in male B6.LDLR$^{-/-tg\ mLXR-\alpha}$ animals compared to B6.LDLR$^{-/-}$ animals were significantly less advanced. At all three locations tested (at 200

μm, 400 μm and 600 μm), transgenic animals had significantly greater numbers of early lesions (stage 0 and 1) whereas non-transgenic animals had significantly greater numbers of advanced lesions (stage 2 and 3) (Table 2).

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$^{-/-}$ and B6.LDLR$^{-/-tg\ mLXR-\alpha}$ 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 5). This result was statistically significant for media without acceptor, media containing ApoAI (with and without stimulation of cells with T090137) and media containing HDL (with stimulation of cells with T090137). Under these conditions, efflux from B6.LDLR$^{-/-tg\ mLXR-\alpha}$ macrophages was increased between 13 and 20% compared to B6.LDLR$^{-/-}$ macrophages. These data indicate that the LXR-α transgene indeed enhanced cholesterol efflux from macrophages.

Discussion

In the present study we investigated the effect of macrophage specific over-expression of LXR-alpha 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 non-transgenic 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) from macrophages of transgenic animals (Figure 5).

Macrophage-specific 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 [19] and mice.[14,20,21] Consistent with these results, transgenic expression of LXR-α in our study was highest in peritoneal macrophages, bone-marrow derived macrophages and interestingly also in brain (Figure 1). Expression of a transgene driven by the chicken gene domain of lysozyme in the brain has previously been observed.[14,20] 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.[20] Transgene-expression in brain most-likely represents a feature of *trans*-species expression of a chicken transgene in mouse.[20] Transgenic animals did not show any apparent neurological abnormalities. While increased LXR-expression in brain is an interesting feature, this finding is not likely of direct relevance with respect to the development of atherosclerosis. In this context it should be mentioned that LXR-α expression was not significantly affected in livers of LXR-transgenic mice. This constitutes a major advantage of our transgenic model of

LXR-α over-expression 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 lead 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.[22] 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 1). However we could demonstrate that LXR-α over-expression in macrophages resulted in the activation of LXR-α target genes such as ABCA-1 (Figure 2). Thus, LXR-α expression was elevated in macrophages of transgenic animals and functionally active, while no adverse effect on lipid metabolism were observed.

The major finding of the present study was a dramatic reduction of atherosclerosis in the BCA of male B6.LDLR$^{-/-tg\ mLXR-\alpha}$ mice compared to B6.LDLR$^{-/-}$ controls. The reduction of atherosclerosis was seen at three different anatomical locations in the BCA. These results are consistent with previous work, showing a reduction of atherosclerosis in mice treated with LXR-agonists. However, it should be noted that pharmacological intervention in these studies lead to a generalized ligand-dependent activation of LXR. In contrast, the approach of our study involved specific over-expression and subsequent activation of LXR by physiological LXR-activators (e.g. 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. Two other studies, reported only data from male mice on the reduction of atherosclerosis by the LXR agonist T0901317.[11,12] 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$^{-/-tg\ mLXR-\alpha}$ mice at different anatomical sites of the vasculature. These were much stronger at the BCA, and only showed a trend towards smaller lesions at the aortic root. However, 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,[23] including ours.[17]

Two major potentially athero-protective 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.[24] The other potentially important athero-protective mechanism of LXR involves regulation of cholesterol homeostasis.[2] Since our data indicated a reduction of lipid-laden macrophages in the vessel wall of B6.LDLR$^{-/-tg\ mLXR-\alpha}$ mice (Supplementary 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 5). Cholesterol efflux was further activated after incubation of cells with the synthetic LXR agonist T0901317, as previously shown.[25] However, when incubated with T0901317, efflux from LXR-α transgenic macrophages to HDL and ApoAI containing media was consistently higher compared to non-transgenic controls (Figure 5), thus providing a potential explanation of reduced foam cell formation and atherosclerosis in B6.LDLR$^{-/-tg\ mLXR-\alpha}$ 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, pointing to a role of ABCG1 as well as increased efflux to ApoAI, pointing to a role of ABCA1.[26] Both these genes contain LXRE and are up-regulated in response to LXR-activation.[7,8] LXR-activation also induces up-regulation of ApoE[9] and we and others have shown that ApoE enhances cholesterol efflux from macrophages.[27,28]

In summary, our data show for the first time that transgenic over-expression of LXR-α using a macrophage-specific gene expression construct lead to an increase of cholesterol efflux from macrophages and a reduction of atherosclerosis. Transgenic expression of LXR 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-specific over-expression of LXR-α might be useful as a therapeutic principle for the prevention of atherosclerosis.

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Tables

Table 1: 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
Urea (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±0.1	2.25±0.09
Triglycerides (mmol/L)	2.99±0.32	2.65±0.18

n, 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.

Table 2: Modified Stary-classification of lesion-complexity in male B6.LDLR$^{-/-}$ and B6. LDLR$^{-/-tg\ mLXR-\alpha}$ 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.

Figure Legends:

Figure 1. Expression of mLXR-α mRNA in tissue of B6.LDLR$^{-/-tg\ mLXR-\alpha}$ mice and B6.LDLR$^{-/-}$ controls. Values are given as mean ± SEM of up to 7 animals. PM, peritoneal macrophages; BM, bone marrow; B, brain; L, liver; M, muscle; K, kidney; n.s., not significant.

Figure 2. A, Expression of mLXR-α mRNA in bone marrow derived macrophages of B6.LDLR$^{-/-tg\ mLXR-\alpha}$ mice and B6.LDLR$^{-/-}$ controls activated with the synthetic LXR-agonist T0901317. B, Expression of ABCA-1 mRNA in bone marrow derived macrophages of B6.LDLR$^{-/-tg\ mLXR-\alpha}$ mice and B6.LDLR$^{-/-}$ controls activated with the synthetic LXR-agonist T0901317. Values are given as mean ± SEM of 3 animals.

Figure 3. Atherosclerotic lesion area at the BCA in male B6.LDLR$^{-/-tg\ mLXR-\alpha}$ mice and B6.LDLR$^{-/-}$ controls at 16 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.

Figure 4. Representative sections through the BCA of LDLR$^{-/-}$ mice at 16 weeks of age. A, Oil red O staining in B6.LDLR$^{-/-}$ controls and B, B6.LDLR$^{-/-tg\ mLXR-\alpha}$ mice.

Figure 5. Cholesterol efflux in bone marrow derived macrophages of B6.LDLR$^{-/-tg\ mLXR-\alpha}$ mice and B6.LDLR$^{-/-}$ controls. acceptor-free, 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., not significant.

Figure 1

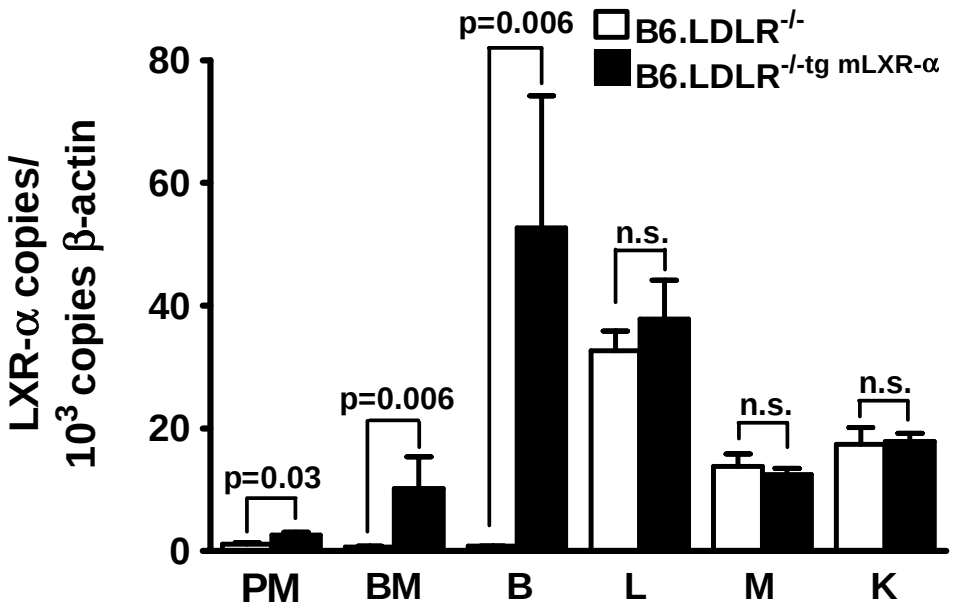

p=0.006
B6.LDLR-/-
B6.LDLR-/-tg mLXR-α
LXR-α copies/
10³ copies β-actin
80
60
40
20
0
p=0.03
p=0.006
n.s.
n.s.
n.s.
PM
BM
B
L
M
K

Figure 2

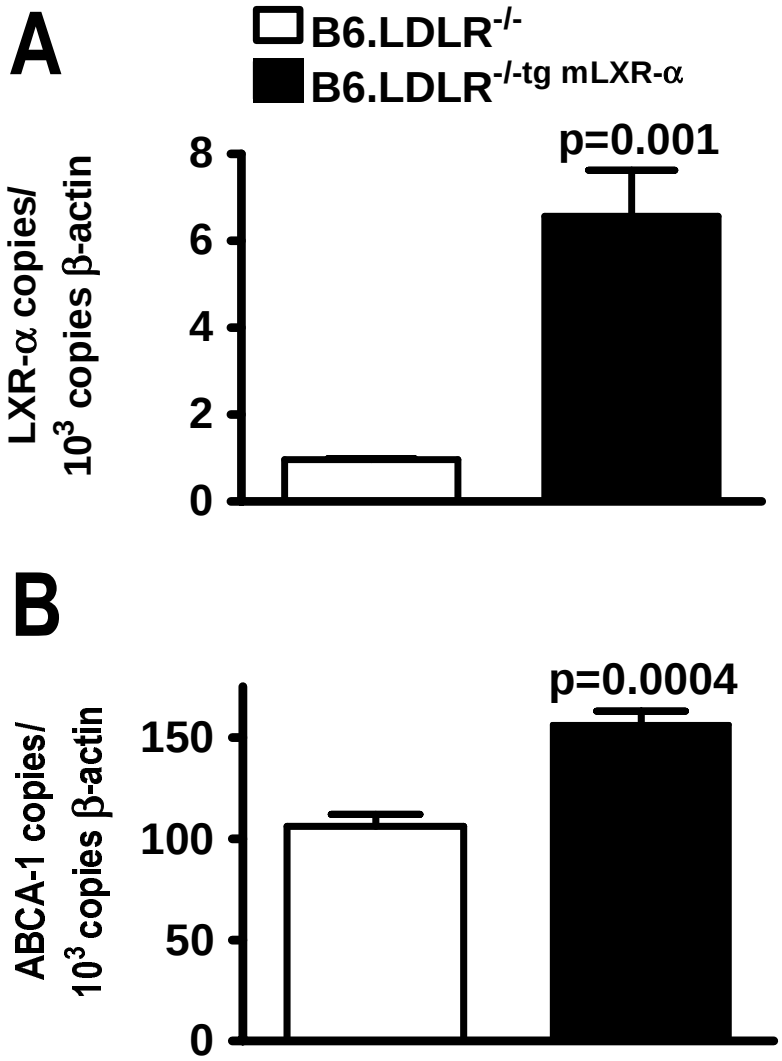

A
B6.LDLR-/-
B6.LDLR-/-tg mLXR-α
p=0.001
LXR-α copies/ 10^3 copies β-actin
8
6
4
2
0
B
p=0.0004
ABCA-1 copies/ 10^3 copies β-actin
150
100
50
0

Figure 3

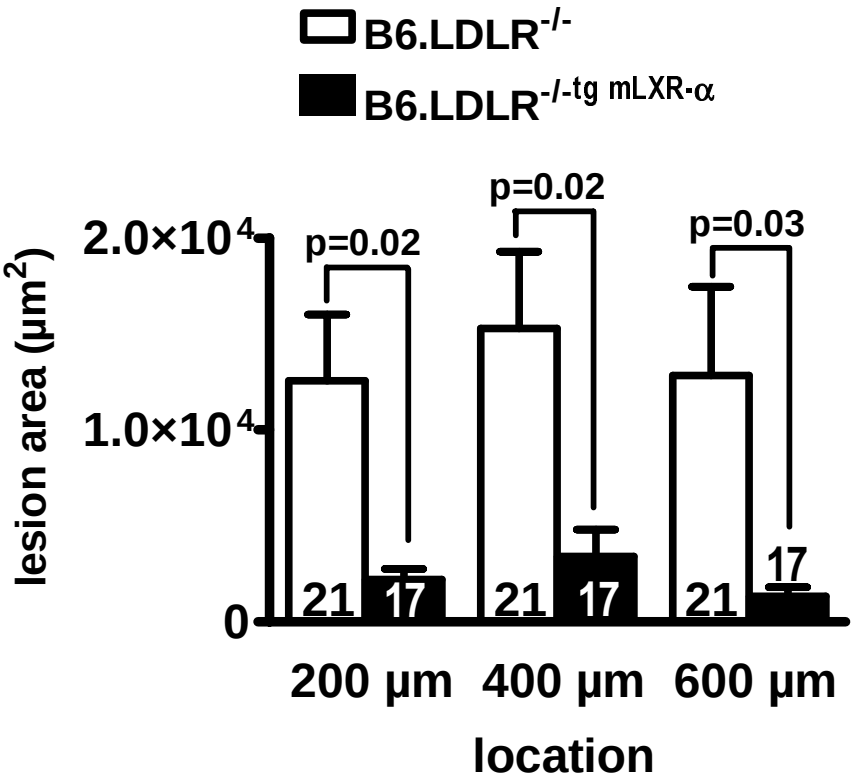

B6.LDLR-/-
B6.LDLR-/-tg mLXR-α
lesion area (μm2)
2.0×10 4
1.0×10 4
0
p=0.02
p=0.02
p=0.03
21
17
21
17
21
17
200 μm
400 μm
600 μm
location

Figure 4

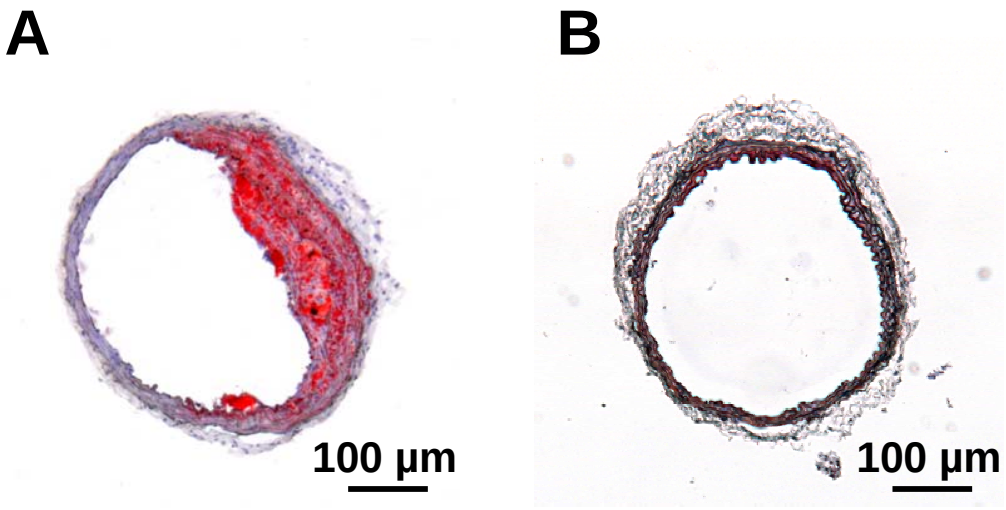
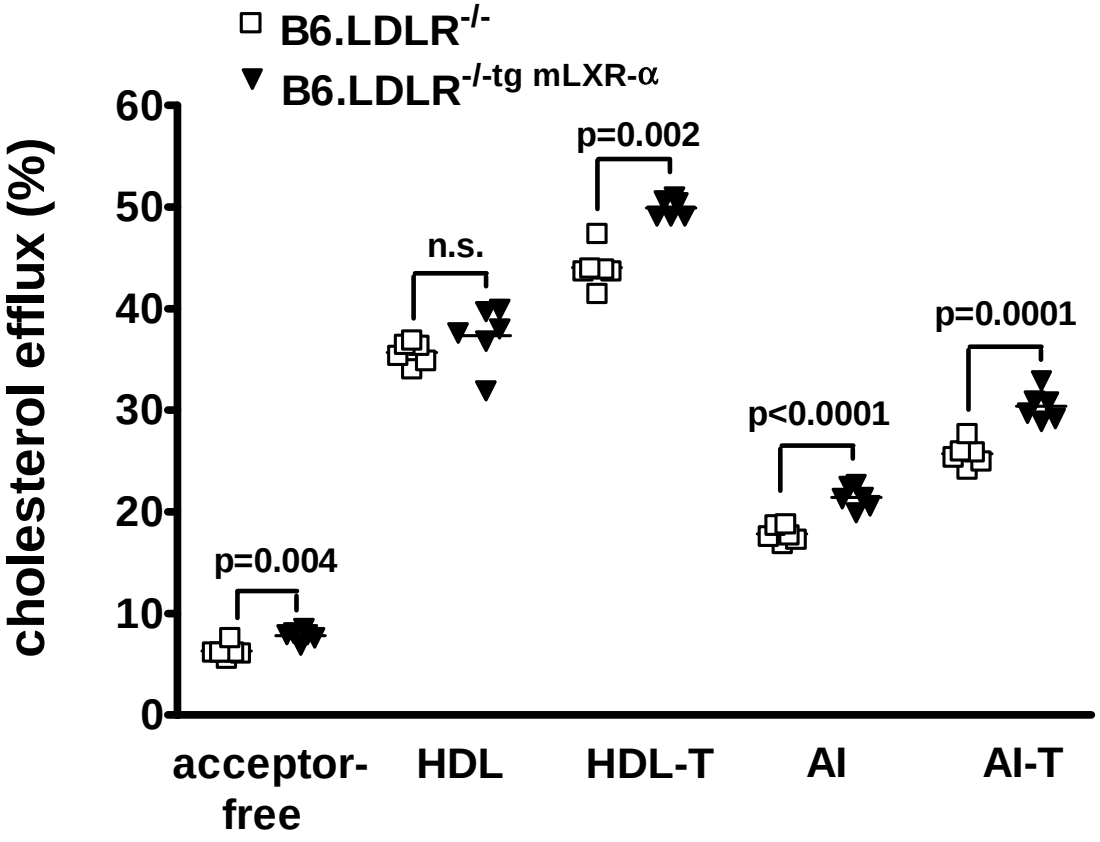


Figure 5


B6.LDLR-/-
B6.LDLR-/-tg mLXR-α
cholesterol efflux (%)
60
50
40
30
20
10
0
p=0.004
n.s.
p=0.002
p<0.0001
p=0.0001
acceptor-free
HDL
HDL-T
AI
AI-T