LXRα Regulates Macrophage Arginase 1 Through PU.1 and Interferon Regulatory Factor 8


Rationale: Activation of liver X receptors (LXRs) inhibits the progression of atherosclerosis and promotes regression of existing lesions. In addition, LXRα levels are high in regressive plaques. Macrophage arginase 1 (Arg1) expression is inversely correlated with atherosclerosis progression and is markedly decreased in foam cells within the lesion.

Objective: To investigate LXRα regulation of Arg1 expression in cultured macrophages and atherosclerotic regressive lesions.

Methods and Results: We found that Arg1 expression is enhanced in CD68+ cells from regressive versus progressive lesions in a murine aortic arch transplant model. In cultured macrophages, ligand-activated LXRα markedly enhances basal and interleukin-4–induced Arg1 mRNA and protein expression as well as promoter activity. This LXRα-enhanced Arg1 expression correlates with a reduction in nitric oxide levels. Moreover, Arg1 expression within regressive atherosclerotic plaques is LXRα-dependent, as enhanced expression of Arg1 in regressive lesions is impaired in LXRα-deficient CD68+ cells. LXRα does not bind to the Arg1 promoter but instead promotes the interaction between PU.1 and interferon regulatory factor (IRF)8 transcription factors and induces their binding of a novel composite element. Accordingly, knockdown of either IRF8 or PU.1 strongly impairs LXRα regulation of Arg1 expression in macrophage cells. Finally, we demonstrate that LXRα binds the IRF8 locus and its activation increases IRF8 mRNA and protein levels in these cells.

Conclusions: This work implicates Arg1 in atherosclerosis regression and identifies LXRα as a novel regulator of Arg1 and IRF8 in macrophages. Furthermore, it provides a unique molecular mechanism by which LXRα regulates macrophage target gene expression through PU.1 and IRF8. (Circ Res. 2011;109:492-501.)

Key Words: atherosclerosis ■ liver X receptor ■ interferon regulatory factor 8 ■ arginase 1 ■ macrophages

Atherosclerosis results from disrupted lipid homeostasis as well as chronic inflammation involving a number of immune pathways.1 As cells involved in both lipid and immune processes, macrophages play a key role throughout atherogenesis from initiation and fatty streak formation to plaque rupture.2 In addition to dissecting the progression of atherosclerosis, there is a growing interest in the mechanisms leading to the regression (or reduced volume) of established atherosclerotic lesions. In humans, plaque regression occurs when LDL-cholesterol levels are aggressively reduced by high doses of statins and/or when HDL-cholesterol is increased.3 To identify pathways leading to lesion regression, a number of experimental models have now been developed.3 In one such model, a segment of plaque-containing aorta from a hyperlipidemic apolipoprotein E-deficient (apoE−/−) mouse is transplanted into a wild-type (WT) recipient, thereby rapidly improving abnormal lipid levels and inflammatory environment.4 In this setting, plaques in the WT recipients undergo a substantial reduction in their foam cell content while increasing the number of smooth muscle cells present in the fibrous cap, consistent with the stabilization and regression of the plaque. Even though the underlying mechanisms and factors involved in atherosclerosis regression are not fully understood, it appears that CD68+ cells of monocytic origin play an important role. In these cells, the chemokine (C-C motif) receptor 7 (CCR7) is induced and functions as a key regulator of plaque regression.5–7

The liver X receptors (LXRs) of the nuclear receptor family are important regulators of macrophage biology.8 Both LXRα and LXRβ (NR1H3 and NR1H2, respectively) are

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activated by oxysterols and by synthetic agonists such as T0901317 (T1317) or GW3965. LXR agonists induce gene expression by heterodimerizing with the 9-cis retinoid X receptor (RXR) and binding to a DNA motif termed LXR response element (LXRE), in regulatory regions of LXR target genes.9 LXR agonists also inhibit target gene expression either by antagonizing the activities of other transcription factors10 or by inhibiting the release of corepressor complexes from target gene promoters.11

LXR agonists mediate the prominent antiatherosclerotic effects of LXR on efflux of cholesterol by activating genes that promote cholesterol efflux, reducing macrophage and lymphocyte activation, inhibiting smooth muscle cell proliferation, and downregulating the expression of endothelial adhesion molecules.8,12,13 Treatment of apoE−/− or LDL receptor (LDLR)−/− atherosclerosis mice models with LXR agonists substantially decreases the development of lesions, and ablation of LXR in hematopoietic cells in these models increases aortic lesion area.8 Moreover, LXR agonists play an important role as modulators of atherosclerosis regression. LXR agonists promote lesion regression in the ApoE3-Leiden mouse model fed a cholesterol-free diet, which is associated with an increase in CCR7 expression and reduced endothelial monocyte adherence.7 Likewise, LXR agonists are required for the CCR7 increase in CD68+ cells and for the maximal CCR7-mediated regression of atherosclerotic lesions in the plaque transplantation regression model.5

In addition to CCR7, LXR agonists may regulate the expression of other important modulators of macrophage biology in lesions undergoing regression. Arginase (Arg1) catalyzes the hydrolysis of L-arginine to urea and L-ornithine, a precursor for the synthesis of collagen and polyamines, which may contribute to plaque stabilization and tissue repair.14,15 Several lines of evidence point to an antiatherosclerotic role for macrophage Arg1. Enhanced levels of this enzyme in macrophages are associated with reduced atherosclerosis in rabbits.16 Moreover, in human atherosclerotic plaques, Arg1 is mainly present in macrophages and non–lipid-laden cells adjacent to the arterial lumen but not in cells surrounding the atherosclerotic lipid core.17

Macrophage Arg1 expression is regulated in response to anti-inflammatory cytokines, such as interleukin (IL)-4 and IL-10,18 STAT6, CAAT/enhancer binding protein-β, and the Ets family transcription factor PU.1 cooperate to regulate the IL-4-dependent induction of Arg1 gene by binding to a series of sites located 3 kbp upstream of its transcription start site.18,19 Macrophage Arg1 mRNA levels are also induced by the nuclear receptors peroxisome proliferator-activated receptor (PPAR)γ and PPARβ.20–22

In the present study, we demonstrate that Arg1 is highly expressed in CD68+ cells from regressive lesions in a transplant model and dissect the role of LXRα on the regulation of Arg1 expression in cultured macrophages as well as in the regressive atherosclerotic plaque. This work not only implicates LXRα in the regulation of Arg1 during atherosclerosis regression but also uncovers a novel mechanism by which LXRα modulates gene expression.

### Methods

**Aortic Plaque Transplantation**

Aortic transplant experiments were carried out as previously described.6,23 ApoE−/− mice fed a Western diet for 16 weeks were divided into a pretransplant group for baseline analysis or a group of donors of aortic arches. Recipients were maintained on a standard chow diet and euthanized 3 days after transplantation. Laser capture microdissection was performed as described.6 All procedures were approved by the Animal Care and Use Committee at New York University School of Medicine.

**Transient Transfections**

RAW-VO cells transfected with pXP2-ARG1prom, pCMV-β-Galactosidase, pcDNA3.1, or pcDNA3.1-FLAG-hLXRα using Turbofect (Fermentas) for 24 hours were treated with 10% fetal bovine serum containing DMEM and vehicle (dimethyl sulfoxide) or T1317 (1 μmol/L) for 18 hours, followed by incubation with or without IL-4 (10 ng/μL) for another 6 hours, and luciferase and β-galactosidase activities were measured.

**Chromatin Immunoprecipitation Assays**

Chromatin immunoprecipitation experiments were performed as described,24 except that cells were cross-linked with 1.5 mmol/L of ethylene glycol-bis(succinimidylsuccinate) for 20 minutes, followed by 10 minutes with 1% formaldehyde solution. The antibodies used were LXRα (ab41902), PU.1 (sc-352), heat shock protein 90 kDa (sc-59577), and IRF8 (sc-6058). The Arg1, ABCA1, or IRF8 gene promoters were amplified with primers shown in Online Table I.

**RNA Analysis**

RNA reverse transcription was performed with the use of a cDNA synthesis kit (Roche) and quantified by PCR, using the Brilliant
Arg1 Expression Is Enhanced in Regressive Atherosclerotic Lesions

Arg1 is expressed in several cell types in the arterial wall such as smooth muscle cells, endothelial cells, or macrophages.14 To address the regulation of Arg1 expression in progressive versus regressive lesions, we used a previously validated surgical transplant atherosclerosis model.4 In this model, the size of apoE−/− mice plaques decreases by 40% and lipid-loaded cell content by 75% when transplanted to WT mice but continues to progress when transplanted into apoE−/− mice.6 Thus, lesions from the thoracic aorta in the apoE−/− mice were transplanted into abdominal aortic segments of either apoE−/− or WT mice (Figure 1A). As control, RNA from CD68+ cells from donor plaque lesions was isolated by laser capture microdissection (LCM), analyzed, and labeled as baseline (Figure 1). No difference in Arg1 expression was observed between CD68+ cells from baseline or recipient mice (progressive plaques), whereas Arg1 levels were dramatically induced in CD68+ cells from lesions transplanted to WT mice (regressive plaques) (Figure 1B and Online Figure I). These results demonstrate that Arg1 gene expression is dynamically regulated within atherosclerotic lesions and is markedly enhanced in plaques subject to a regressive environment. LXRα Induces Macrophage Arg1 Expression

Because ligand-activated LXRα inhibits atherogenesis25,26 and is highly induced in CD68+ cells from plaques undergoing regression,6 we next explored the possibility that LXRα regulates Arg1 expression in macrophages. We first determined Arg1 mRNA levels in the macrophage-derived LXRα-deficient RAW264.7 cell line transduced with either FLAG-LXRα (LXRα) or an empty retroviral vector (VO)24 treated with the LXR ligand T1317. Because Arg1 expression is strongly modulated by IL-4 and the molecular mechanism underlying this regulation has been characterized in detail,18,19 we also examined whether IL-4–induced levels of Arg1 could be regulated by the LXR ligand (Figure 2A). Arg1 mRNA levels were induced by T1317 compared with vehicle treatment in RAW-LXRα cells but not in RAW-VO cells (Figure 2A, inset). In agreement with previous reports,19 Arg1 expression was induced by IL-4 in both cell lines; however, this IL-4 induction was synergistically enhanced in the presence of T1317 in an LXRα-dependent manner. In addition, Arg1 protein was also induced by T1317 (Figure 2B, upper panel) and more potently by a combination of IL-4 and T1317 or GW3965 (Figure 2B, lower panel). Similar results were obtained in bone marrow–derived macrophages, which express LXRα (Online Figure II). Altogether, these data show that both mRNA and protein expression of Arg1 are enhanced by LXRα activation in cultured macrophages.

Arg1 Expression in Regressive Plaques Requires LXRα

To examine the role of myeloid LXRα expression on Arg1 levels in regressive lesions, we compared Arg1 levels in CD68+ cells from WT recipient mice that had been transplanted with plaques from apoE−/− donors repopulated with either apoE−/− or apoE−/−/LXRα−/− bone marrow (Figure 2C). Arg1 expression in regressive plaques was severely impaired in lesions from donor mice lacking LXRα expression in their myeloid cells (Figure 2C), and this reduced Arg1 expression was associated with less plaque regression. These data indicate that LXRα is important in the regulation of Arg1 expression in vivo, within atherosclerotic plaques undergoing regression. Arg1 competition with nitric oxide synthase (NOS) for their common substrate decreases nitric oxide (NO) production.14,15 Macrophage expression of the inducible form of NOS (iNOS) is proatherogenic.27 Thus, an increase in Arg1 expression leading to lower NO levels in...
these cells may result in plaque reduction. As a first attempt to elucidate the functional relevance of LXR-induced Arg1 levels, we evaluated the levels of NO in LXR ligand–treated macrophages on stimulation with lipopolysaccharide (Figure 2D and Online Figure III). In lipopolysaccharide-treated cells, LXR/H9251–induced Arg1 expression was associated with a reduction in NO production, and the combination of IL-4 and LXR ligand further diminished NO levels significantly. Interestingly, this occurred in the absence of changes in the expression of iNOS (Online Figure III), which can also be subject to regulation by LXR,12 indicating that the observed changes in NO are unlikely to be due to LXR modulation of iNOS expression. Thus, LXRα-mediated induction of Arg1 correlates with reduced NO levels, which may in turn affect the progression of the atherosclerotic lesion.

Arg1 Regulation by LXRα Is Mediated by PU.1
We next examined the regulation of a luciferase reporter gene driven by a 4.8-kb fragment of the Arg1 region upstream of the transcription start site.19 As shown in Figure 3A, Arg1 reporter activity was enhanced when cells were activated with T1317 only in the presence of transiently overexpressed LXRα. As expected, Arg1 reporter activity was induced by IL-4 regardless of LXRα expression. Furthermore, the activity of this Arg1 reporter was further increased in an LXRα-dependent manner when cells were cotreated with T1317 and IL-4 compared with IL-4 or T1317 alone. The absence of LXR ligand responsiveness in RAW264.7 cells, which endogenously express LXRα in the absence of LXRα, indicates that the levels of LXRα present in these cells may not be sufficient to drive Arg1 promoter activity because both isotypes are capable of being activated by the T1317 ligand. However, overexpression of LXRβ above the endogenous levels induced Arg1 reporter activity, albeit to a lower extent compared with LXRα (Online Figure IV), indicating that both isotypes when present at high levels are able to stimulate Arg1 transcription.

Positive regulation of target genes by LXRα often involves the binding of the LXR/RXR heterodimer to an LXRE.9 To

Figure 2. LXRα enhances Arg1 expression in macrophages. A, RAW-VO or RAW-LXRα cells were treated as described in the Methods section. Transcripts were analyzed by qRT-PCR. Values indicate expression of target genes normalized to cyclophilin mRNA levels and are presented relative to the expression in dimethyl sulfoxide–treated cells, which was set as 1. Data are mean±SD (n=3). B, RAW-LXRα cells were treated with vehicle dimethyl sulfoxide or LXR agonists T1317 (T), or GW3965 (G), and/or IL-4 for 48 hours. Arg1 protein levels were determined by immunoblotting. Low and high exposures (LE and HE) of the bottom Arg1 immunoblot are shown to visualize full range of Arg1 protein levels. C, Left, overview of the aortic arch transplantations. BM indicates bone marrow. Right, Aortic arch donors were apoE−/− mice subject to a bone marrow transfer from apoE−/− or apoE−/− LXRα−/− mice. After 16 weeks, arches were transplanted into WT recipients. RNA from CD68+ cells was isolated and analyzed for Arg1 mRNA levels as described in Figure 1. Fold changes are presented relative to baseline apoE−/− donors, which was set as 1 (not shown, t test; apoE−/− versus apoE−/− LXRα−/−: *P<0.001). D, RAW-LXRα cells were treated with vehicle (dimethyl sulfoxide) or LXR agonists T1317 (T) and/or IL-4 for 48 hours. Cells were then stimulated with 100 ng/mL lipopolysaccharide. After 24 hours, NO production was measured. Data are mean±SD (n=3). *P<0.05.
identify an LXRE, a series of truncated Arg1 reporter constructs were assayed for their response to ligand-activated LXRα. Ligand-dependent increase of Arg1 reporter activity was abolished only when the −0.336 kb Arg1 reporter was used (Figure 3A), suggesting that the LXR-responsive region is located between 0.8 kb and 0.336 kb upstream of the Arg1 transcription start site. However, no LXRE was predicted in that region by MatInspector. Recruitment of LXRα was further investigated by chromatin immunoprecipitation in RAW-LXRα macrophages using RAW-VO cells as negative control (Online Figure V). As expected, LXRα bound to the promoter of ABCA1, a well-characterized LXR target gene (Online Figure VA) but not to the −0.8/−0.3 kb region in the Arg1 promoter (Online Figure VB). These data suggest that LXRα may not regulate Arg1 expression by a classic LXRE-binding mechanism that and instead, additional LXRE-dependent pathways may be involved.

Analysis of the −0.8/−0.3 kb Arg1 sequence revealed a PU.1 site situated at −0.7 kb (Figure 3B). PU.1 is already known to enhance Arg1 expression by binding to sites located around −3 kb in an Arg1 enhancer.13 Thus, we speculated that Arg1 regulation by LXRα might be mediated through PU.1 and assessed PU.1 binding to the −0.7 kb sequence. In RAW-VO cells, PU.1 occupied the −3 kb region in the Arg1 promoter as expected, as well as the −0.7 kb site in an LXR ligand–dependent manner (Figure 3B). Only in RAW-LXRα cells, the presence of LXRα ligand dramatically increased PU.1 binding to the −0.7 kb site but not to the −3 kb site. Hence, PU.1 binds to an additional site in the Arg1 promoter in an LXRα- and T1317-regulated manner.

To determine whether the LXRα-dependent increase in Arg1 expression is mediated by PU.1, RAW-LXRα cells were transfected with control siRNA oligonucleotides or siRNA targeting PU.1, which efficiently decreased PU.1 protein levels (Figure 3C). PU.1 silencing led to a dramatic decrease (~70%) in the Arg1 induction by T1317 and T1317/IL-4 (Figure 3C and Online Figure VIA). Conversely, PU.1 overexpression in the presence of LXRα potentiated the basal and T1317-induced Arg1 reporter activity (Figure 3D). Altogether, these results strongly suggest that PU.1, in concert with LXRα, mediates the LXR ligand–dependent activation of Arg1 expression through a novel element localized 0.7 kb upstream the Arg1 transcription start site.

**LXRα Enhances IRF8 Binding to the −0.7 kb Site in the Arg1 Promoter**

PU.1 exerts its transcriptional activity with other partners, including the interferon regulatory factor 8 (IRF8).28 IRF8 is a member of the IRF transcription factor family and is implicated in B-cell29 and macrophage30 differentiation during hematopoiesis. Thus, we speculated that LXRα might enhance PU.1 binding to the −0.7 kb site (Figure 3A) by facilitating the recruitment of IRF8. As shown in Figure 4A, IRF8 weakly binds to the −0.7 kb site (Figure 3A) by facilitating the recruitment of IRF8. As shown in Figure 4A, IRF8 weakly binds to the −0.7 kb site (Figure 3A) by facilitating the recruitment of IRF8. As shown in Figure 4A, IRF8 weakly binds to the −0.7 kb site (Figure 3A) by facilitating the recruitment of IRF8. As shown in Figure 4A, IRF8 weakly binds to the −0.7 kb site (Figure 3A) by facilitating the recruitment of IRF8. As shown in Figure 4A, IRF8 weakly binds to the −0.7 kb site (Figure 3A) by facilitating the recruitment of IRF8. As shown in Figure 4A, IRF8 weakly binds to the −0.7 kb site (Figure 3A) by facilitating the recruitment of IRF8. As shown in Figure 4A, IRF8 weakly binds to the −0.7 kb site (Figure 3A) by facilitating the recruitment of IRF8.

### Figure 3. Arg1 regulation by LXRα is mediated through PU.1. A, RAW264.7 cells were transfected with hLXRα or pcDNA3.1 (−) along with the indicated Arg1 reporters. Cells were treated as described in the Methods section. For each reporter, luciferase and β-galactosidase activities were measured and the ratio was compared with the vehicle-treated condition and the absence of LXRα (−), which was set as 1. Data are mean±SD (n=3). B, Upper panel: Location of the previously reported (−3 kb) and newly identified in (−0.7 kb) PU.1 sites in the Arg1 locus. Arrows indicate the position of primers used for chromatin immunoprecipitation assays. Lower panel: RAW-VO or RAW-LXRα cells were incubated with or without T1317 (T) at 1 μmol/L for 2 hours. PU.1 occupancy was assessed by chromatin immunoprecipitation assays. The indicated primers were used to amplify an unrelated site at −5 kb (T) that served as a negative control, the −3 kb (2), and −0.7 kb (3) sites. Shown is a representative experiment of n=4. C, RAW-LXRα cells were transfected with control or PU.1 siRNAs and then treated with vehicle or T ligand for 24 hours. PU.1 and heat shock protein 90 kDa were analyzed by Western blotting. Arg1 mRNA expression was analyzed by qRT-PCR. Values are mean±SD (n=3) and indicate expression normalized to cyclophilin and are presented relative to levels in siRNA-control dimethyl sulfoxide–treated cells, which is set as 1. D, RAW264.7 cells were transfected with a PU.1 expression vector or pcDNA3.1 (−) along with a −4.8 kb Arg1 reporter and LXRα expression vector, and cells were treated as in B. Ratios of luciferase and β-galactosidase activities were compared with those in dimethyl sulfoxide–treated cells in the absence of PU.1 (−), which was set as 1. Each bar is mean±SD of n=3.
ligand–induced expression of Arg1 (Figure 4B), whereas the expression of another well-characterized LXR target gene such as ABCA1 remained unaffected (Online Figure VI.B). Altogether, these results demonstrate that IRF8 occupancy to the −0.7 kb site in the Arg1 promoter is specifically enhanced by ligand-activated LXRα and identifies IRF8 as a novel regulator of Arg1 expression. Further analysis of the sequence at −0.7 kb revealed the presence of an Ets/IRF composite element (Figure 6). Such an element consists of a PU.1 and an IRF element separated by 2 nucleotides and can be bound by either PU.1/IRF8 or PU.1/IRF4 heterodimers. Similar composite sites are present in the promoters of genes involved in immune signaling such as IL-1β<sup>32</sup> and TLR4,<sup>33</sup> among others. To confirm that this sequence within the Arg1 promoter is indeed responsible for its regulation by LXR, luciferase assays were performed using a reporter in which both PU.1 and IRF8 half-sites were mutated. As shown in Figure 4C, LXR ligand–induced activity was abrogated when the composite element was disturbed, indicating that this is the bona fide LXR-responsive sequence.

### LXRα Enhances IRF8 Expression

To dissect the molecular mechanism by which LXRα facilitates IRF8 and PU.1 binding to the Arg1 promoter, immunoprecipitation assays were performed and demonstrated that IRF8 interaction with PU.1 occurs in an LXRα-dependent manner (Figure 4D). The formation of the PU.1/IRF8 complex correlated with an increase in IRF8 levels in LXRα-expressing cells. Thus, we next investigated in more detail whether LXRα regulates the expression of PU.1, IRF8, or both. We examined PU.1 and IRF8 mRNA levels in T1317-treated and/or IL-4–treated cells. Although the expression of PU.1 was unchanged (Online Figure VII), IRF8 mRNA and protein levels were increased in LXRα-expressing macrophages treated with T1317 (Figure 5A and 5B and Online Figure VII). Furthermore, in silico analysis of the IRF8 locus revealed the presence of an LXRE located 20 kb upstream the IRF8 gene transcription start site. Using RAW-VO cells as negative control, we confirmed that LXRα specifically binds to the −20 kb site (Figure 5C). Thus, our findings suggest that the regulation of IRF8 expression by LXRα probably occurs through an LXRE in a distal sequence of the IRF8 gene.

### Discussion

To design strategies aimed at promoting the regression of established atherosclerotic lesions, the mechanisms and factors regulating this process must be defined. Recently, the emigration of monocytic-derived cells from plaques was described as an important feature of plaque regression, and LXRα was shown to play a key role in this process by inducing the expression of the chemokine receptor CCR7. Our study demonstrates that Arg1 expression is also markedly induced in CD68<sup>+</sup> cells from regressive lesions in a regression transplant model, which is consistent with a recent study showing Arg1 expression in regressive atherosclerotic lesions in “Reversa” mice (Ldlr<sup>−/−</sup>ApoB<sup>100/100</sup>Mtp<sup>Mx1Cre</sup><sup>++/+</sup>)<sup>4</sup> and

**Figure 4.** Arg1 regulation by LXRα is mediated through IRF8. **A.** Upper panel: Location of a novel IRF8 response element in the Arg1 promoter. Arrows indicate the position of primers used for chromatin immunoprecipitation assays. **Lower panel:** RAW-VO or RAW-LXRα cell lines were incubated as in Figure 3B. IRF8 occupancy was determined by chromatin immunoprecipitation assays. Primers amplifying the −5 kb (1), −3 kb (2), or −0.7 kb (3) sites were used. Shown is a representative experiment of n=4. **B,** RAW-LXRα cells were transfected with control or IRF8 siRNAs and treated as in Figure 3. IRF8 and heat shock protein 90 kDa and Arg1 expression were analyzed as in Figure 3C. Values indicate expression normalized to cyclophilin and are presented relative to the expression in siRNA-control vehicle-treated cells, which is set as 1. Data are mean±SD (n=3). **C,** Bone marrow–derived macrophages from WT or IRF8<sup>−/−</sup> mice were cultured with T1317 (T) (1 μmol/L) for 72 hours. Expression of the indicated proteins was analyzed by immunoblotting. **D,** RAW264.7 cells were transfected as in Figure 3. For each reporter, luciferase and β-galactosidase activities were measured and the ratio was compared with the vehicle-treated condition in the absence of LXRα (−), which was set as 1. Data are mean±SD (n=3). **D,** RAW-VO or LXRα cells were incubated with dimethyl sulfoxide or LXR agonist T for 24 hours. IRF8 was immunoprecipitated and IRF8-containing complexes were resolved by SDS-PAGE.
with earlier reciprocal findings that Arg1 levels are substantially decreased in foam lipid–loaded cells within progressive lesions.\textsuperscript{17} Our work also identifies LXR\textsubscript{\alpha}/H9251 as an important modulator of Arg1 expression both in vitro and in vivo. Moreover, Arg1 levels are induced in lipid-laden macrophages, as expected from previous reports,\textsuperscript{20–22} and, intriguingly, LXR\textsubscript{\alpha}/H9251 is able to induce Arg1 expression in these cells (Online Figure IIB). This regulation of Arg1 expression in cultured loaded macrophages is somehow contradictory to a study showing weak Arg1 levels in foam cells within the plaque.\textsuperscript{17} In the present report, we focus on the regulation of Arg1 by LXR\textsubscript{\alpha}/H9251 in regressive lesions, and additional experiments will be required to establish whether LXR\textsubscript{\alpha}/H9251 induces Arg1 levels in plaques undergoing atherosclerosis progression.

Arg1 may affect plaque regression. As mentioned, Arg1 decreases the production of NO,\textsuperscript{14,15} which, when synthesized in macrophages, is proatherogenic.\textsuperscript{27} Therefore, a shift in L-arginine availability for macrophage iNOS caused by enhanced Arg1 levels may result in reduced atherosclerosis. Indeed, our studies show that in cultured macrophages, LXR\textsubscript{\alpha}-induced Arg1 expression correlates with a decrease in NO production, whereas iNOS levels remain unaffected (Figure 2D and Online Figure III). In addition, pilot studies suggest that altered NO levels in these cells are mainly due to Arg1, because even in the absence of the second arginase isoform, Arg2, activation of LXR\textsubscript{\alpha}/H9251 leads to decreased NO levels (B. Pourcet, A. Hobbs, and I. Pineda-Torra, unpublished observations). Whether these changes in NO production may lead to plaque reduction awaits further investigation.

An increase in macrophage Arg1 levels has also been shown to be associated with enhanced vascular smooth muscle cell proliferation,\textsuperscript{35} which may contribute to the remodeling of the fibrous cap surrounding the atherosclerotic lesion and thus to plaque stabilization.\textsuperscript{36} Interestingly, in the transplant regression model used in our study, regressing plaques also present an increased number of smooth muscle cells, particularly in the fibrous cap.\textsuperscript{37} Therefore, an induction in macrophage arginase expression may modulate atherosclerosis in part by increasing the stability of the plaque. The impact of Arg1 function on atherosclerosis, however, may be cell type–dependent. In endothelial cells, NO limits atherosclerosis progression\textsuperscript{38} and thus inhibition of arginase activation, and specific knockdown of Arg2 in the endothelium reduces atherosclerosis progression.\textsuperscript{39} Antiatherogenic therapies based on arginase inhibition have been proposed.\textsuperscript{40} However, because LXR\textsubscript{\alpha} activates Arg1 expression through PU.1 and IRF8 (Figure 3, 4), factors exclusively expressed in myeloid- and lymphoid-derived cells,\textsuperscript{31} it is likely that LXR\textsubscript{\alpha} induction of Arg1 expression is restricted to these cells. Consistent with this, Arg1 is mainly present in CD68\textsuperscript{+} cells within the regressive atherosclerotic plaque, where LXR\textsubscript{\alpha} is also abundantly expressed,\textsuperscript{5} whereas only a few endothelial cells lining the lesion appear to express the

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\caption{Figure 5. IRF8 is an LXR\textsubscript{\alpha} target gene. A, RAW-VO and RAW-LXR\textsubscript{\alpha} cells were incubated with vehicle or LXR agonist T (1 \textmu mol/L) for 24 hours. Transcripts were analyzed as in Figure 2A. B, Both cell lines and bone marrow–derived macrophages were treated with vehicle or T1317 (T) (1 \textmu mol/L) for 48 hours. IRF8 and heat shock protein 90 kDa were measured by Western blotting. C, Upper panel: Location of a novel LXRE in the IRF8 locus. Primer pairs used to amplify an upstream \textminus50 kb region that serves as an additional negative control (1) and the \textminus20 kb site (2) are shown. Lower panel: RAW-VO and RAW-LXR\textsubscript{\alpha} cell lines incubated with T (1 \textmu mol/L) for 2 hours. LXR\textsubscript{\alpha} occupancy was determined by chromatin immunoprecipitation assays with primers amplifying the indicated sites. Values were normalized to input chromatin and to signal from RAW-VO cells, which was considered as background and set as 1. Shown is a representative experiment of n=3.}
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enzyme (Online Figure I). By contrast, the expression of Arg2, which is an LXR target gene regulated by a classic LXR-binding mechanism, is altogether absent from regressive lesions, suggesting that regulation of Arg2 within the plaque is more complex and probably requires additional factors.

Our results establish that LXRα does not bind to the Arg1 promoter but instead regulates Arg1 levels by promoting the binding of the hematopoietic transcription factors IRF8 and PU.1 to a novel site located 0.7 kb upstream of the transcription start site in the Arg1 gene. Previous work established that the PU.1/IRF complex is formed before it binds to the promoter and is stabilized by PU.1 phosphorylation. Our own immunoprecipitation studies show that the PU.1/IRF8 interaction is enhanced in the presence of LXRα (Figure 4D), probably in part by inducing the levels of IRF8 expression. Whether this receptor also promotes the binding of PU.1 to other members of the IRF family remains to be investigated.

A different role for PU.1 in LXR target gene expression was shown in a previous study. A large proportion of LXRβ sites in macrophages are within 100 bp of PU.1 elements, and their occupancy requires PU.1. Thus, regulation of a subset of LXRβ target genes (which are also targets for LXRα) is PU.1-dependent. In that report, however, LXR target gene transcription is mediated through binding of each transcription factor (LXR and PU.1) to their respective sites. In this case, PU.1 primes the binding of LXR to its response elements by facilitating the deposition of monomethylated histone marks through collaboration with other yet unknown factors. Interestingly, our data demonstrate that reciprocally, LXRα can modify the binding of PU.1 to certain sequences, such as the composite −0.7 kb site in the Arg1 locus, which are bound by PU.1 in an LXRα-dependent manner (Figure 3B), apparently without LXRα binding (Online Figure V).

Our findings further reveal IRF8 as an important component of the regulation of macrophage Arg1 expression and its regulation by LXR ligands. Interestingly, Arg1 expression appears to be particularly sensitive to IRF8 because an approximately 40% reduction in IRF8 levels results in markedly reduced Arg1 mRNA (Figure 4 and Online Figure VI, D). Consistent with this, pilot studies show that Arg1 levels are dramatically reduced in primary macrophages from IRF8−/− mice even if PU.1 expression remains unaffected (B. Pourcet, X. Xu, and I. Pineda-Torra, unpublished observations). Intriguingly, IRF8 and PU.1 are regulated by each other: The expression of each partner in the heterodimer is critically reading the manuscript and to Xiaoyu Hu for providing the pcDNA3.1-mPU.1 plasmid. We are grateful to I. Rogatsky for

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Disclosures

None.

References


Novelty and Significance

What Is Known?

- The liver X receptors (LXRs) are nuclear receptors that both reduce the progression of atherosclerosis and promote the regression of established atherosclerotic lesions.
- The expression of LXR alpha (LXRα) is high in atherosclerotic lesions undergoing regression.
- Arginase 1 (Arg1) is considered an anti-inflammatory and tissue-remodeling enzyme.
- Arg1 expression is regulated in atherosclerotic plaques.

What New Information Does This Article Contribute?

- We show that Arg1 levels are increased by LXRα in regressive atherosclerotic lesions and in cultured macrophages.
- LXRα regulates Arg1 expression through the binding of the transcription factors PU.1 and IRF8 to a novel site in the Arg1 promoter.
- LXRα also regulates the expression of IRF8 through a newly identified LXR response element in the IRF8 gene.
- LXRα promotes the interaction between PU.1 and IRF8.

Identifying factors that regulate the regression of established atherosclerotic lesions is critical for designing strategies aimed at promoting this process. LXRα is a well-known antiatherogenic nuclear receptor that modulates atherosclerosis regression. This work demonstrates that the expression of Arg1, which is negatively correlated with atherosclerotic lesion progression, is activated by LXRα in regressive lesions as well as in cultured macrophages. At the molecular level, LXRα does not appear to bind to the Arg1 gene, instead, the regulation of Arg1 is mediated through the binding of the hematopoietic transcription factors PU.1 and IRF8 to a novel site in the Arg1 promoter in an LXR ligand-dependent manner. IRF8 was found to be a novel activator of Arg1 expression, and LXRα promotes the interaction between IRF8 and PU.1 in part through the induction of IRF8 gene expression, thus identifying IRF8 as a novel LXRα target gene. Collectively, our findings uncover an original LXRα-modulated mechanistic axis involving LXRα/IRF8/PU.1 in macrophages that could be used to modulate the expression of additional macrophage transcripts and may have important implications for atherosclerosis and other macrophage-centered diseases.
LXRα Regulates Macrophage Arginase 1 Through PU.1 and Interferon Regulatory Factor 8

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SUPPLEMENT MATERIAL

LXRα regulates macrophage arginase 1 via PU.1 and IRF8

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Supplementary Experimental Procedures

Plasmids
Plasmids were prepared in an endotoxin-free manner with a Nucleobond Xtra Midi Plus EF kit (Macherey-Nagel). pcDNA3.1-FLAG-hLXRα was described,\textsuperscript{1} pcDNA3.1-mPU.1 was kindly gifted by A.L. Corbí López and Arg1 luciferase reporters were described elsewhere.\textsuperscript{2}

Cell culture and bone marrow-derived macrophage (BMDM) isolation
RAW-Vector Only (VO) or RAW-LXRα cells were generated and cultured as described.\textsuperscript{1} BMDM were prepared from C57BL/6 mice as described\textsuperscript{1} except that 20% L929-conditioned medium was used as a source of M-CSF. After 7 days of differentiation, BMDM were treated with DMSO or 1 µmol/L T1317 with or IL-4 10 ng/µL without in phenol red free DMEM medium containing 10 % of low-endotoxin FBS and 5% gentamycin. For certain experiments, cells were differentiated for 7 days in the presence of T1317 (1 µmol/L) with or without IL-4 and the medium was supplemented with fresh ligand and/or cytokine every 2-3 days.

Bone marrow transplants
To generate apoE\textsuperscript{-/-} mice with monocyte-derived cells deficient in LXRα, bone marrow was transferred from apoE\textsuperscript{-/-} or LXRα\textsuperscript{-/-}/apoE\textsuperscript{-/-} mice into apoE\textsuperscript{-/-} mice in Dr. P. Tontonoz’s laboratory as described.\textsuperscript{3} Mice were fed a Western diet for 16 weeks before performing the aortic arch transplants.

Transient transfections
RAW-VO cells (2x10\textsuperscript{5} cells) transfected with pXP2-ARG1prom (300 ng), pCMV-β-Galactosidase (100 ng), pcDNA3.1 or pcDNA3.1-FLAG-hLXRα (50 ng) using Turbofect (Fermentas) for 24 h were treated with 10% FBS-containing DMEM and vehicle (DMSO) or T1317 (1 µmol/L) for 18 h, followed by incubation with or without IL-4 (10 ng/µL) for another 6 h. Luciferase and β-galactosidase activities were measured in a Tropix TR717 Microplate luminometer (PE Applied Biosystem) using the Luciferase Reporter Gene Assay (Roche) and Galacto-Light Plus System (Applied Biosystem) kits, respectively.

Protein extraction and Western blot analysis
RAW-VO or RAW-LXRα cells (1.2x10^6 cells) were treated with vehicle or T1317 (1 µmol/L) with or without IL-4 (10 ng/µL) for 48 h and then lysed with RIPA buffer. Antibodies against Arg1, LXRα (Abcam, ab41902), PU.1 (sc-352), Hsp90 (sc-59577) and IRF8 (sc-6058) were used.

**Chromatin immunoprecipitation (ChIP) assays**

ChIP experiments were performed as described except that cells were first cross-linked with 1.5 mmol/L of ethylene glycol-bis(succinimidylsuccinate) for 20 min, followed by 10 min with 1% formaldehyde solution. The following antibodies were employed: LXRα (ab41902), PU.1 (sc-352), Hsp90 (sc-59577) and IRF8 (sc-6058). The Arg1, ABCA1 or IRF8 gene promoters were amplified with primers shown in Online Table I. Crossing threshold (Ct) values were determined for each promoter and normalized to the Ct of Input using the following equation: relative values = 2^{(Ct\text{IP} - Ct\text{input})}.

**RNA analysis**

RNA was extracted using Trizol reagent (Invitrogen) for RAW cells or Absolute RNA Microprep Kit (Stratagene) for BMDM. Reverse transcription was performed using a cDNA synthesis kit (Roche) and quantified by PCR using the Brilliant SYBR Green QPCR Master Mix (Stratagene) on the MX 3000p detection system (Stratagene) using specific primers (Online Table I). Cyclophilin Ct values were determined for each gene and normalized to the Ct of cyclophilin using the following equation: relative values = 2^{(Ct\text{target gene} - Ct\text{Cyclophilin A})}.

**Determination of NO production**

The concentrations of NO₂⁻ and NO₃⁻ were determined by chemiluminescence as described.

**Immunohistochemistry**

Grafted arches were removed after PBS perfusion, frozen, cut in serial sections (6 µm thick) and stained as described. Primary antibodies used were Arginase I (H-52) and ArgII (H-64) from Santa Cruz Biotechnology.

**Coimmunoprecipitation**
RAW-VO or RAW-LXRα cells treated with vehicle or T1317 for 24 h were cross linked for 20 min in PBS with 1.5 mmol/L Ethylene glycol bis[succinimidylsuccinate (Pierce) prior to lysis. IRF8 was immunoprecipitated using an IRF8 antibody and (sc-6058) Protein A agarose slurry (Millipore). Protein extracts were analyzed by immunoblotting with the antibodies described above.

**Site-directed mutagenesis**

The pXP2 -2.8 kb ARG1 PU.1/IRF8 mut reporter vector was generated by using the Quickchange XL-II Site-directed Mutagenesis kit (Strategene, Amsterdam, Netherlands) and primers shown in Online Table I. The mutated DNA was entirely sequenced.

**Statistical methods**

For the aortic plaque transplantation experiments, data are expressed as mean ± SEM and were analysed by 2-tailed Students’s t test. A P value less that 0.05 was considered significant. In vitro data are represented as mean ± SD.
References


Online Figure I: Arginase 1 and 2 expression in progressive vs regressive atherosclerosis plaques. Aortic sections from mice treated as shown in Figure 1 were immunostained for arginase 1 and arginase 2 using a fluorescently labelled secondary antibody. Representative laser-confocal microscopic images are displayed (40x). As a positive control for arginase 2 staining its expression is shown in a kidney section. The wavy lines represent the autofluorescent internal elastic lamina.
Online Figure II: LXRα regulates Arg1 expression in primary murine macrophages.

A. Arg1 protein expression in bone marrow-derived macrophages treated with vehicle (DMSO) with or without mIL-4 (10 ng/µL) and/or T1317 (T) (1 µmol/L) for 48 or 72 h. Because of the difference in signal intensities the upper and lower panels show high and low exposures respectively of the filters blotted with the Arg1 antibody.

B. BMDM were treated with AcLDL for (50 µg/ml) for 24 h and T1317 (T) (1 µmol/L) in the absence or presence of IL-4 (10ng/ml) for 48 h. Arg1 and Hsp90 protein levels were analyzed by immunoblotting.
Online Figure III: LXR ligand-induced Arg1 and IRF8 expression in LPS-activated macrophages. RAW-LXRα cells were treated as in Figure 2D. The indicated transcripts were analyzed by qRT-PCR. Values are mean ± SD (n=3) and indicate expression normalized to Cyclophilin A and presented relative to the expression in +LPS+DMSO-treated cells, which was set as 1.
Online Figure IV: Both LXRα and LXRβ activate ARG1 promoter activity. RAW264.7 cells were co-transfected with pCMV-β galactosidase, reporter vector pXP2 -4.8 ARG1prom and pcDNA3.1-hLXRα (A) or pcDNA3.1-hLXRβ (B) expression vectors or pcDNA3.1(∅) as control. After 24 hours, transfected cells were treated as described in the methods section. Luciferase and β-galactosidase activities were measured and their ratio was compared to that in the DMSO/∅ condition, which was set as 1. Each bar is the mean value ± SD of triplicate determinations.
Online Figure V: LXRα does not bind to the Arg1 promoter.
RAW-VO and RAW-LXRα cells were treated with vehicle or T1317 (T) (1 µmol/L) for 2 hours. ChIP assays were performed with antibodies against LXRα. Immunoprecipitated DNA was analyzed by qPCR using primers spanning the ABCA1 promoter LXRE (A) as positive control or the LXR ligand responsive -0.7 kb region (B) on the Arg1 promoter. Chromatin isolated from RAW-VO was used as a negative control for specificity of the LXRα antibody. Data were normalized to input chromatin and reported as fold induction over background signal from RAW-VO cells which was set as 1. Shown is a representative experiment of n=3.
Online Figure VI: PU.1 and IRF8 effects on gene expression.
RAW-LXRα cells were transfected with siRNA control or siRNA PU.1 (A), IRF8 (B) or both (C, D) RNA oligos and treated as in Figure 2. Arg1 and ABCA1 mRNA expression were analyzed by qRT-PCR. Values are mean ± SD (n=3) and indicate expression normalized to Cyclophilin A and presented relative to the expression in siRNA-control vehicle-treated cells, which was set as 1. *p< 0.01. PU.1, IRF8 and Hsp90 expression were analyzed by immunoblotting.
Online Figure VII: Regulation of PU.1 and IRF8 expression by LXR.
Bone marrow-derived macrophages (A) and the indicated RAW cell lines (B) were treated as in Figure 2. PU.1 expression was measured by qRT-PCR and normalized to Cyclophilin A mRNA levels. Each bar is the mean value ± SD of triplicate determinations. Shown are fold inductions relative to DMSO-treated cells. C. RAW-LXRα cells were pre-incubated with vehicle or LXR agonist T1317 (T) for 18h and thereafter activated with IL-4 for 6h. Transcripts were analyzed by qRT-PCR. Values are mean ± SD (n=3) and indicate expression of IRF8 normalized to Cyclophilin A mRNA levels and are presented relative to the expression in vehicle-treated cells, which was set as 1.
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**Online Table I**: Sequences of primers used in this study