The macrophage LBP gene is an LXR target that promotes macrophage survival and atherosclerosis

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Abstract  The liver X receptors (LXRs) are members of the nuclear receptor superfamily that regulate sterol metabolism and inflammation. We sought to identify previously unknown genes regulated by LXRs in macrophages and to determine their contribution to atherogenesis. Here we characterize a novel LXR target gene, the lipopolysaccharide binding protein (LBP) gene. Surprisingly, the ability of LXRs to control LBP expression is cell-type specific, occurring in macrophages but not liver. Treatment of macrophages with oxysterols or loading with modified LDL induces LBP in an LXR-dependent manner, suggesting a potential role for LBP in the cellular response to cholesterol overload. To investigate this further, we performed bone marrow transplant studies. After 18 weeks of Western diet feeding, atherosclerotic lesion burden was assessed revealing markedly smaller lesions in the LBP−/− recipients. Furthermore, loss of bone marrow LBP expression increased apoptosis in atherosclerotic lesions as determined by terminal deoxynucleotidyl transferase dUTP nick end labeling staining. Supporting in vitro studies with isolated macrophages showed that LBP expression does not affect cholesterol efflux but promotes the survival of macrophages in the setting of cholesterol loading. The LBP gene is a macrophage-specific LXR target that promotes foam cell survival and atherogenesis. — Sallam, T., A. Ito, X. Rong, J. Kim, C. van Stijn, B. T. Chamberlain, M. E. Jung, L. C. Chao, M. Jones, T. Gilliland, X. Wu, G. L. Su, R. K. Tangirala, P. Tontonoz, and C. Hong. The macrophage LBP gene is an LXR target that promotes macrophage survival and atherosclerosis. J. Lipid Res. 2014. 55: 1120–1130.

Despite recent advances in our treatment and understanding of its biology, CVD contributes to one in every three deaths (1, 2). Additionally, CVD costs more than any other condition with an estimated annual burden of $312 billion, an astonishing figure in light of the growing interest in healthcare utilization and costs (1). This unacceptably high disease burden galvanizes efforts to better understand mechanisms contributing to CVD and developing novel diagnostic and therapeutic strategies.

At the epicenter of the most devastating forms of CVD, including myocardial infarction, peripheral vascular disease, and stroke, is atherosclerosis. Atherosclerosis is a chronic condition of the arterial lining characterized by a prolonged asymptomatic phase, making it difficult to study in humans. Thus, mouse models have been invaluable in understanding disease mechanisms (3, 4). A hallmark feature of atherosclerosis is the accumulation of cholesterol-loaded macrophages within the vessel wall (5). At the early stages of atherosclerosis, macrophages ingest modified lipoproteins to form “foam cells,” and in turn release various substances that recruit smooth muscle cells and other immune cells, ultimately leading to advanced plaque formation (6). Macrophages, as well as other immune cells, have a powerful impact on disease progression (7). Therefore, macrophages are a pivotal cell type in the pathogenesis of atherosclerosis and potential targets for therapy.

Supplementary key words  nuclear receptor • atherogenesis • liver X receptor • lipopolysaccharide binding protein

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Acknowledgments: AIM, apoptosis inhibitor of macrophage; CETP, cholesteryl ester transfer protein; EMSA, electrophoretic mobility shift assay; LBP, lipopolysaccharide binding protein; LDLR, LDL receptor; LPS, lipopolysaccharide; LXR, liver X receptor; LXRE, liver X receptor response element; PLTP, phospholipid transfer protein; RXR, retinoid X receptor; SREBP, sterol response element binding protein; TLR4, toll receptor-4; TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labeling.

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At the crossroads of linking metabolic and inflammatory responses within macrophages are the liver X receptors (LXRs) (8, 9). LXRs are ligand-dependent transcription factors that form permissive heterodimers with the retinoid X receptor (RXR) on DNA to regulate gene expression (10). The activation of LXR by its oxysterol ligands induces the expression of genes involved in cholesterol efflux (ABCA1), LDL receptor (LDLR) degradation [inducible degrader of the LDL receptor (IDOL)], and inhibition of gut cholesterol absorption (ABCG5 and ABCG8) (11–14). Thus, the sterol-sensing LXRs act as the “yin” to the sterol response element binding protein’s (SREBP’s) “yang” maintaining cholesterol homeostasis.

In addition to their metabolic functions, LXRs have been found to modulate immune and inflammatory responses in macrophages (15, 16). Ligand activation of LXRs inhibits transcription of pro-inflammatory cytokines (9). Furthermore, induction of LXR target genes supports the survival of immune cells against pro-apoptotic stimuli, such as oxidized LDL, and enhances the clearance of apoptotic cells (17, 18). The diverse biologic effects of LXRs make them attractive targets for pharmacologic manipulations. In murine models, administration of LXR agonists has consistently shown potent atheroprotective effects and reversal of established disease; hence LXR and its downstream pathways have generated interest as therapeutic targets (19, 20).

At least in part, the atheroprotective properties of LXR agonists are derived from effects on hematopoietic cells (21–23). LXRs prevent foam cell formation in macrophages by enhancing cholesterol efflux and reverse cholesterol transport in addition to their potent anti-inflammatory effects. A number of LXR target genes have been proposed to modulate atherogenesis through macrophage-specific effects. For example, atherogenic mouse models where macrophage ABCA1 was ablated through bone marrow transplants, showed increases in atherosclerotic plaque burden due to impairment of reverse cholesterol transport and inflammatory signaling (24).

Fig. 1. LXR regulates LBP expression in macrophages. A: Primary mouse peritoneal macrophages were treated with GW3965 (GW, 1 μM) and/or the RXR ligand LG268 (LG, 100 nM). Gene expression in this and all subsequent figures was analyzed by real-time PCR. Results are representative of three independent experiments. Values are mean ± SD. B: Primary bone marrow-derived macrophages were treated with GW3965 (GW, 1 μM), oxidized LDL (oxLDL, 50 μg/ml), or acetylated LDL (AcLDL, 50 μg/ml) for 60 h. Results are representative of four independent experiments. Values are mean ± SD. C: Primary peritoneal mouse macrophages were treated with GW3965 (GW, 1 μM) and/or the protein synthesis inhibitor emetine (E, 5 μg/ml). Results are representative of three independent experiments. Values are mean ± SD. D: Primary mouse peritoneal macrophages were treated with GW3965 (GW, 1 μM), endogenous ligand 22(R)-hydroxycholesterol (22R, 2.5 μM), or 25-hydroxycholesterol (25OH, 2.5 μM). E: Primary mouse peritoneal macrophages were treated with GW3965 (GW, 1 μM) overnight. Results are representative of two independent experiments. Values are mean ± SD. F: Primary mouse peritoneal macrophages were treated with GW3965 (GW, 0.5 μM). Results are representative of two independent experiments. Values are mean ± SD. G: Primary mouse peritoneal macrophages were treated with the LXR ligands GW3965 (GW, 0.5 μM) or T0901317 (T, 1 μM). Results are representative of three independent experiments. Values are mean ± SD. DKO, double knockout; Conc, concentration.
assays were performed by adding increasing concentrations of non-
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(31–34). On the other hand, studies have shown that LBP
the toll receptor-4 (TLR4)-lymphocyte antigen 96 (MD2)
the lipid A moiety of LPS, facilitating its transfer via CD14 to
saccharide (LPS) and secreted into the blood. LBP binds
sized in hepatocytes, where it is highly inducible by lipopoly-
properties known to modulate the innate immune response
was associated with decreased lesion progression (27, 28).
In this setting, apoptotic cell death of foam cells at the initial stages of atherosclerosis
marrow transplantation experiments owing to an increase
in macrophage apoptosis (25, 26). In this setting, apoptotic
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Lipoplysaccharide binding protein (LBP) is a glyco-
protein with dual pro-inflammatory and anti-inflammatory properties known to modulate the innate immune response (29, 30). As an acute phase response protein, LBP is synthe-
sized in hepatocytes, where it is highly inducible by lipoplysaccharide (LPS) and secreted into the blood. LBP binds the lipid A moiety of LPS, facilitating its transfer via CD14 to the toll receptor-4 (TLR4)-lymphocyte antigen 96 (MD2) complex and triggering downstream signal transduction (31–34). On the other hand, studies have shown that LBP can neutralize the biologic effects of LPS by facilitating its binding to LDL, HDL, or the scavenger receptor on the surface of target cells, thus detoxifying its effects (35–38). Alternatively, LBP may inhibit the cellular responses to LPS by acting as a buffer, preventing its transfer from membranous CD14 to the TLR4-MD2 signaling receptor (39, 40). In vivo models have shown that LBP prevents LPS-induced TNFα release in a murine septic shock model and enhances survival in this setting (41, 42). In fact, LBP knockout mice are dramatically more susceptible to gram-negative infections compared with controls (43). Clinical studies have demonstrated that LBP is a useful biomarker for various infectious and inflammatory states (44–47).

Although previous studies have suggested that LBP in-
duction in hepatocytes is involved in inflammatory responses, its precise role and regulation in extrahepatic tissues is poorly understood. Here we demonstrate that the gene encoding LBP is a direct target of LXRs in macrophages, but not in hepatocytes. Intriguingly, LBP has high structural homology to cholesteryl ester transfer protein (CETP) and phospholipid transfer protein (PLTP), two additional LXR target genes in peripheral tissues (48–50). Moreover, we demonstrate that deletion of LBP from the bone marrow is atheroprotective and that this effect correlates with enhanced macrophage apoptosis. Our study outlines an unexpected role for bone marrow-derived LBP in macrophage survival and the pathogenesis of atherosclerosis.

METHODS

Reagents, plasmids, and gene expression
GW3965 and T0901317 were synthesized as described (51–53). LG268 was a gift of R. Heyman (Ligand Pharmaceuticals). Oxys-
terols were purchased from Sigma and used as described (54).
Emetine was a gift from D. Black (Howard Hughes Medical Institu-
tute, University of California, Los Angeles). Ligands were dis-
solved in dimethyl sulfoxide before use in cell culture. pBABE-LBP retrovirus was generated using pBABE-puro (Invitrogen Gate-
way adapted) as a backbone. For gene expression analysis, RNA
was isolated using TRIzol reagent (Invitrogen) and analyzed by
real-time PCR using an Applied Biosystems 7900HT. Results are
normalized to 36B4. The primer sequences are available upon
request.

Animals and diets
All animals (C57Bl/6, greater than 10 generations back-
crossed) were housed in a temperature-controlled room under a
12 h light/12 h dark cycle and under pathogen-free conditions.
LXRα−/−, LXRβ−/−, and LXRAβ−/− mice were originally pro-
vided by David Mangelsdorf, University of Texas Southwestern
Medical Center, Dallas, TX. LBP−/− mice (C57Bl/6 background)
were obtained from Grace Su University of Michigan. Mice were
fed either standard chow or Western diet as indicated (21% fat,
0.21% cholesterol; D12079B; Research Diets Inc.). For bone mar-
row transplantation studies, recipient LDLR−/− mice (11 weeks
of age) were lethally irradiated with 900 rads and transplanted
with 3 × 106 bone marrow cells from 8-week-old or older donors
(WT or LBP−/−) via tail vein injection as previously described
(22). Mice were gavaged with either vehicle or 40 mg/kg of
GW3965 once a day for 3 days. Livers were harvested 4 h after the
last gavage. Cholesterol and triglyceride levels were measured as
previously described (55). All animal experiments were approved
by the Institutional Animal Care and Research Advisory Commit-
tee at University of California, Los Angeles.

Cell culture
Primary peritoneal macrophages were isolated 4 days after
thioglycollate injection and prepared as described (19). Bone
marrow cells were harvested as described and cultured in L929
cell-conditioned media for 7 days to induce differentiation into
macrophages (22). Peritoneal macrophages were incubated in
0.5% FBS in DMEM, with 5 μM simvastatin and 100 μM me-
valonic acid. Five to eight hours later, cells were pretreated with
DMSO or an appropriate ligand overnight. For inflammation
studies, cells were treated with 10 or 100 ng/ml LPS (Axxora,
Cholesterol efflux

Assays were performed as previously described (55). Briefly, bone marrow-derived macrophages from WT or LBP−/− mice were labeled with [3H]cholesterol (1.0 Ci/ml) in the presence of acyl-CoA:cholesterol O-acyltransferase inhibitor (2 g/ml) either with DMSO or with ligand for LXR and RXR (1 μM GW3965, 100 nM LG268). After equilibrating the cholesterol pools, cells were washed with PBS and incubated in DMEM containing 0.2% BSA in the absence or presence of apoA-I (15 μg/ml) or HDL (50 μg/ml) for 6 h. The data are presented as percent apoA-I- or HDL-specific efflux.

Histological and lesion analysis

Immunohistochemistry of sections and preparation and staining of frozen and paraffin-embedded sections from aortas were performed as described previously (19). Atherosclerosis in the aortic roots and the descending aortas (en face) were quantified by computer-assisted image analysis as described (58). The presence of apoptotic cells was assessed by terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay of paraffin-embedded tissue sections as previously described using a Promega DeadEnd fluorometric TUNEL system (26). Thirty high power fields from at least seven mice from each genotype were analyzed for apoptotic TUNEL, positive and DAPI-stained cells.
To investigate the induction of LBP mRNA by physiologic LXR agonists, we treated bone marrow-derived macrophages from WT and LXRα−/− macrophages with oxidized LDL or acetylated LDL. We observed an LXR-dependent induction of LBP expression in response to both forms of modified LDL, consistent with a potential role for LBP in the cellular response to cholesterol overload (Fig. 1B). LXR regulation of LBP was not sensitive to the protein synthesis inhibitor emetine, suggesting a direct transcriptional effect (Fig. 1C). To further investigate the regulation of LBP, peritoneal macrophages were treated with known endogenous LXR ligands, the oxysterols 22(R) hydroxycholesterol and 25-hydroxycholesterol. As expected, both oxysterols increased expression of LBP. The oxysterol 25-hydroxycholesterol is known to be a modest LXR activator, but also an inhibitor of SREBP processing. The observed expression pattern of LBP underscores the dependency and specificity of LXR (Fig. 1D). Furthermore, in Fig. 1E, LBP was induced by LXR ligand in WT, LXRα−/−, and LXRβ−/− macrophages, indicating that each LXR isoform is capable of regulating LBP. However, genetic ablation of both LXRα resulted in the clear loss of LBP regulation. LXR ligand-dependent regulation of LBP was both time and concentration dependent as shown in Fig. 1F, G. We next screened the LBP promoter sequence for DR-4 LXR

Statistical analysis

Non-paired Student’s t-test was used to determine statistical significance, defined at P < 0.05. Unless otherwise noted, error bars represent standard deviations. For real-time PCR analysis of macrophages, each condition represents averages of two independent samples.

RESULTS

It has been well-established that LXRs induce the expression of genes involved in reverse cholesterol transport, anti-inflammatory signaling, and cell survival in macrophage cell lines. Previously published transcriptional profiling studies identified LBP as a potential LXR target (60). Thioglycollate-elicited peritoneal macrophages from WT and LXRα−/− animals were administered GW3965, a known LXR agonist, confirming LXR-dependent activation of LBP. Gene expression analysis demonstrated that LBP expression was induced in a parallel manner to the established LXR target, ABCA1, by GW3965. Importantly, the increased expression of LBP was not observed in LXRα−/− macrophages demonstrating an LXR-dependent effect (Fig. 1A). An enhanced induction was observed when cells were treated with both an LXR and an RXR ligand (LG268, 100 nM) (Fig. 1A).
LXR, LBP, and atherosclerosis

A number of LXR target genes in macrophages have been shown to modulate atherosclerosis through various mechanisms, including alteration of cellular cholesterol balance, changes in innate and adaptive immune responses, and the promotion of cell survival (61). We hypothesized that LBP deletion in macrophages may affect the development of atherosclerosis through one of these mechanisms. To examine the impact of LBP on the development of atherosclerosis, we reconstituted the bone marrow of irradiated LDLR⁻/⁻/⁻ male mice with WT or LBP⁻/⁻/⁻ hematopoietic cells and analyzed plaque area after 18 weeks of Western diet feeding (Fig. 4A). Engraftment was confirmed by real-time PCR analysis of bone marrow from recipient mice collected at the time of euthanization (Fig. 4B). We performed en face lesion analysis and expressed the extent of atherosclerosis as the percentage of surface area of the entire aorta covered by lesion (58). LDLR⁻/⁻/⁻ recipients reconstituted with LBP⁻/⁻/⁻ bone marrow showed markedly reduced atherosclerotic burden in comparison with WT reconstituted controls (WT 17.55 ± 1.064% versus LBP⁻/⁻/⁻ 11.36 ± 0.8056%; P < 0.0001) (Fig. 4C, D). As expected, a broad distribution in lesion area was observed in both groups, but the overall effect was striking with an approximately 35% relative reduction in lesion area. Serum analysis uncovered no significant changes in cholesterol, triglyceride, cytokines, or chemokines (Fig. 4E–G).

Atherosclerosis assessed by quantification of Oil-Red O-stained aortic root sections showed consistent results. A potential binding site was identified 225 bp upstream of the transcription start site (Fig. 2A). In an EMSA, in vitro translated LXRα and RXRα protein were able to bind to a radiolabeled probe containing this putative LXRE, but not to a probe in which the LXRE had been mutated. In addition, only unlabeled WT LBP LXRE was able to compete for complex formation (Fig. 2B). These results imply that LXR/RXR heterodimers bind directly to the LBP promoter.

Because the predominant site of LBP production is the liver, we investigated the transcriptional regulation of LBP by LXR in this tissue. Unexpectedly, real-time quantitative PCR showed that LBP was not increased by LXR in primary hepatocytes, despite induction of known LXR target genes such as ABCA1 (Fig. 3A). A trend toward LBP down-regulation by LXR was observed as suggested by Fig. 3A. However, dedicated repeats of this experiment in WT hepatocytes showed no significant change from baseline (Fig. 3B). Additionally, in vivo administration of GW3965 for 3 days failed to induce LBP expression in the liver (Fig. 3C). Consistent with previous studies, we observed dramatic induction in LBP levels in primary hepatocytes in response to LPS stimulation (Fig. 3D). Surprisingly, LBP expression was not regulated by LPS in primary peritoneal macrophages, either at baseline or with administration of LXREs (direct repeats with four nucleotide spacer). A potential binding site was identified 225 bp upstream of the transcription start site (Fig. 2A). In an EMSA, in vitro translated LXRα and RXRα protein were able to bind to a radiolabeled probe containing this putative LXRE, but not to a probe in which the LXRE had been mutated. In addition, only unlabeled WT LBP LXRE was able to compete for complex formation (Fig. 2B). These results imply that LXR/RXR heterodimers bind directly to the LBP promoter.
expected, we found that LBP−/− macrophages retained the capacity to efflux cholesterol to apoA1 or HDL acceptors (Fig. 6D). These findings indicate that the loss of LBP in macrophages does not significantly alter reverse cholesterol transport or inflammatory signaling upon LXR activation or LPS stimulation.

We have previously reported that LXR signaling promotes the survival of macrophages through activation of target genes such as AIM (26). We investigated whether LBP may play a role in the pathogenesis of atherosclerosis by supporting the survival of lesion macrophages. After 18 weeks on Western diet, the aortic lesions from LBP−/− bone marrow transplant recipients contained more TUNEL-positive cells (Fig. 7A). Quantitative analysis showed significantly more apoptotic burden by percent TUNEL-positive area, as well as percent DAPI-stained cells that were TUNEL positive (Fig. 7B, C).

These data suggested that cells lacking LBP may have altered expression of genes involved in apoptosis. Gene expression analysis of apoptotic markers from bone marrow-derived macrophages (isolated at the time of aorta harvest then differentiated) showed enhanced expression of Bok, a proapoptotic marker (Fig. 8A). Bok is a member of the bcl-2 family of genes and has previously been implicated in LXR signaling.
LBP belongs to a family of rapidly evolving proteins (65). Interestingly, two members of this family, PLTP and CETP, are established LXR targets with roles in lipoprotein remodeling and reverse cholesterol transport (48–50, 66, 67). All three proteins show variable tissue distribution and species conservation. Both PLTP and CETP function as lipid transfer proteins (68–70). Mice lacking PLTP expression showed marked dysregulation of lipid homeostasis (71, 72). LBP shares their ability to bind and transfer lipids, and we have shown here that it is induced by lipid loading of macrophages. However, LBP does not appear to function directly in plasma lipid homeostasis. Previous studies of LBP knockout mice found no alterations in lipoprotein profiles, even in the setting of a high-fat diet (43). Thus, there is currently no evidence for an essential nonredundant role for LBP in plasma lipoprotein metabolism in mice (43).

Although it has long been known that hematopoietic cells are key players in atherosclerotic plaque development, it has been widely believed that foam cell formation is dependent on macrophage recruitment within atherosclerotic lesions. Recent evidence suggests that macrophage proliferation may contribute to the accumulation of hematopoietic cells in lesions (73, 74). These data highlight the important role of macrophage survival pathways in influencing disease progression. The balance of pro- and anti-apoptotic factors within the local environment is critical to disease progression. A better understanding of pathways and atherosclerosis (17).

DISCUSSION

Previous studies have established LXRs as important regulators of atherosclerotic lesion progression (19, 61–64). LXRs influence a variety of cellular functions, including lipid homeostasis, inflammatory signaling, and cell survival. We have shown here that LXRs regulate the expression of the LBP gene in macrophages but not in the liver, the predominant site for LBP production in vivo. Additionally, LBP is not induced in response to pro-inflammatory stimulation in macrophages. This contrasts with its behavior as an acute phase response protein with LPS neutralizing properties (39, 40, 30). Our findings suggest a context-specific and physiologically distinct role for LBP in macrophages compared with liver.

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models have many limitations (76). Several clinical studies have observed a correlation between plasma LBP and atherosclerosis, with the presumption that LBP is a nonspecific marker of chronic inflammation similar to other acute phase proteins such as CRP (46, 77). Unlike CRP however, which showed no effect on atherosclerosis progression in independent transgenic mouse models (78, 80), we show that LBP possesses distinct atherogenic actions. Thus, LBP’s role as a “maker” rather than a “marker” could potentially have utility in predicting patients at risk or with subclinical atherosclerosis, given the prolonged asymptomatic phase of the disease. Whether LBP would offer additional benefit than traditional risk factor screening remains to be explored.

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