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.**

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

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.

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Abbreviations: 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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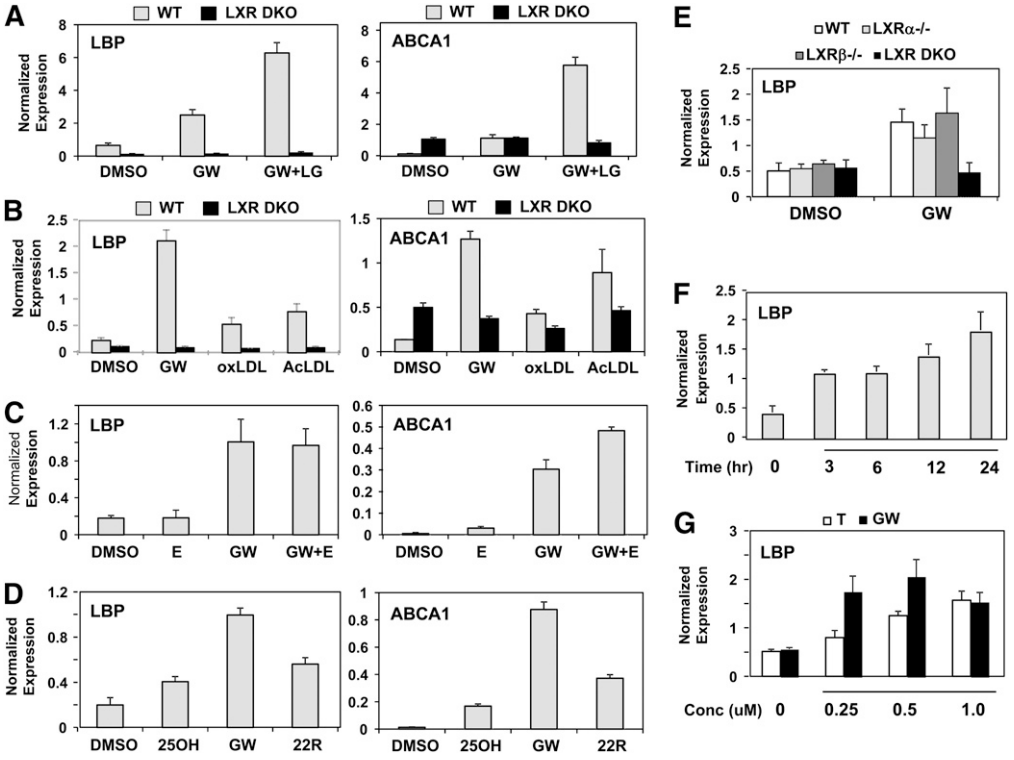

Fig. 1. LXR regulates LBP expression in macrophages. A: Primary mouse peritoneal macrophages were treated with GW3965 (GW, 1 μM) and/or the RXR ligand LG268 (LG, 100 nM). Gene expression in this and all subsequent figures was analyzed by real-time PCR. Results are representative of three independent 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.

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). Paradoxically, knockout studies of the LXR targets ABCG1 and apoptosis inhibitor of

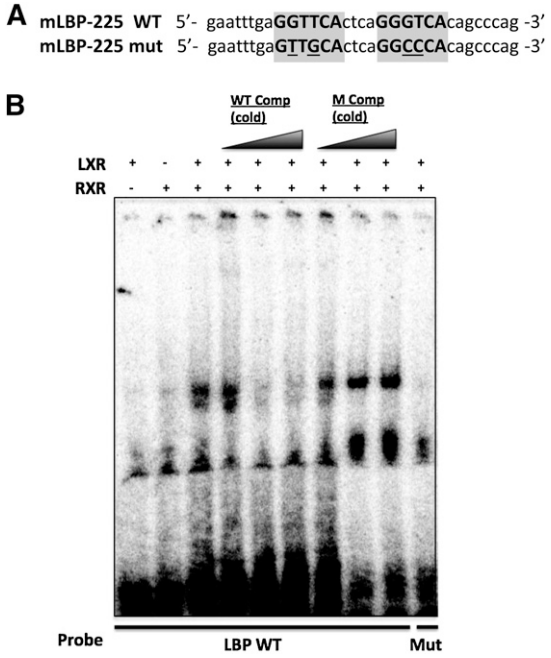

Fig. 2. LXR/RXR heterodimers bind to a DR4 site on the LBP promoter. A: WT and mutant (mut) LXR DR4 binding sites 225 bp upstream of the LBP transcription start site. B: EMSA using labeled oligonucleotides (32-P) to either the WT or the mutant LXR DR4 sites, in vitro translated LXRα or RXRα protein. Competition (Comp) assays were performed by adding increasing concentrations of non-labeled WT or mutant (M) LBP probe.

macrophage (AIM) decreased lesion formation in bone marrow transplantation experiments owing to an increase in macrophage apoptosis (25, 26). In this setting, apoptotic cell death of foam cells at the initial stages of atherosclerosis was associated with decreased lesion progression (27, 28).

Lipopolysaccharide binding protein (LBP) is a glycoprotein 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 synthesized in hepatocytes, where it is highly inducible by lipopolysaccharide (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 induction 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). Oxysterols were purchased from Sigma and used as described (54). Emetine was a gift from D. Black (Howard Hughes Medical Institute, University of California, Los Angeles). Ligands were dissolved in dimethyl sulfoxide before use in cell culture. pBABE-LBP retrovirus was generated using pBABE-puro (Invitrogen Gateway 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 backcrossed) were housed in a temperature-controlled room under a 12 h light/12 h dark cycle and under pathogen-free conditions. LXRα$^{-/-}$, LXRβ$^{-/-}$, and LXRαβ$^{-/-}$ mice were originally provided 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 marrow transplantation studies, recipient LDLR$^{-/-}$ mice (11 weeks of age) were lethally irradiated with 900 rads and transplanted with 3×10^6 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 Committee 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 mevalonic 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,

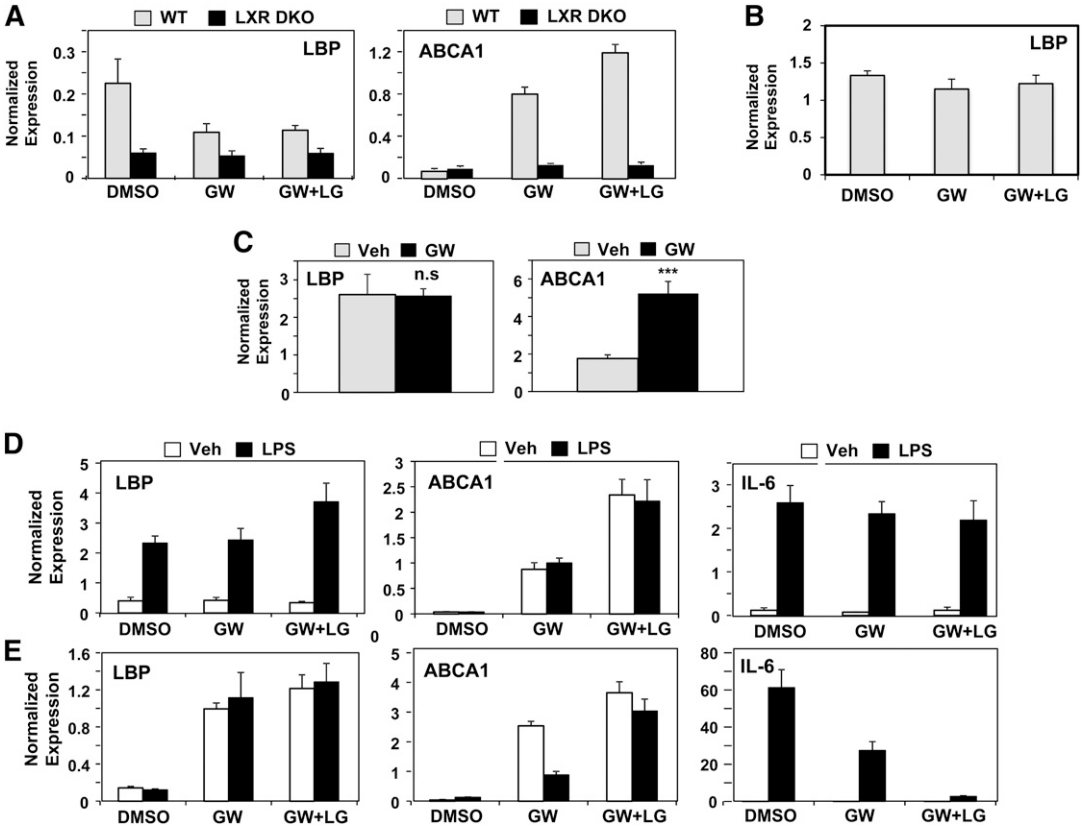

Fig. 3. LBP regulation by LXRs and LPS is cell-type selective. A: Primary mouse hepatocytes were treated with GW3965 (GW, 1 μM) overnight. Results are representative of three independent experiments. Values are mean ± SD. B: Primary mouse hepatocytes were treated with GW3965 (GW, 1 μM) overnight. Results are representative of four independent experiments. Values are mean ± SD. C: LBP expression in livers of WT mice treated with 40 mg/kg/day GW3956 by oral gavage for 3 days (n = 5 per group). Values are mean ± SEM. D: Primary mouse hepatocytes were treated with GW3965 (GW, 1 μM) and/or the RXR ligand LG268 (LG, 50 nM) in the absence or presence of LPS stimulation (100 ng/ml). Results are representative of three independent experiments. Values are mean ± SD. E: Primary mouse peritoneal macrophages were treated with GW3965 (GW, 1 μM) and/or the RXR ligand LG268 (LG, 50 nM) in the absence or presence of LPS stimulation (100 ng/ml). Results are representative of three independent experiments. Values are mean ± SD. DKO, double knockout; Veh, vehicle.

ALX-581-008-L002) for 4 h after ligand stimulation. Mouse primary hepatocytes were isolated as previously described and cultured in William's E medium with 5% FBS (54). Stable cell lines were made using pBABE retroviral vectors as described (56).

Electrophoretic mobility shift assay

Electrophoretic mobility shift assay (EMSA) was performed as previously described (57). Briefly, Klenow enzyme buffer system (Promega) was used for labeling and annealing oligonucleotides. TNT quick coupled transcription/translation system (Promega) was used for in vitro translation of RXRα and LXRα. The translated protein products were combined and incubated at room temperature for 10 min with labeled DNA and 100 M NaCl, 1 mM EDTA, 20 mM HEPES, 5% glycerol, 0.01% Nonidet P-40, and 2 μg/μl poly(dI:dC). Protein-DNA complexes were electrophoresed on a polyacrylamide gel and visualized by autoradiography.

Antibodies and immunohistochemistry

The following antibodies were used for immunohistochemistry: CD68 (MCA1957GA, AbD) 1:400 with secondary antibody biotin-SP-conjugated AffiniPure goat anti-rat IgG (H+L) (Jackson Laboratories) 1:1,000 and α-smooth actin rabbit monoclonal (E184) 1:100 with secondary antibody biotinylated goat anti-rabbit IgG (Vector Laboratories) 1:200. For immunoblot analysis the following antibodies were used: ABCA1 (Novus) 1:1,000, COX-2 (Santa Cruz) 1:200, LDLR (Cayman Chemicals) 1:1,000, and actin (Sigma) 1:10,000.

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 μCi/ml) in the presence of acyl-CoA:cholestrol *O*-acyltransferase inhibitor (2 μg/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). Atherosclerotic lesions at the aortic valve were analyzed as described (59). 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.

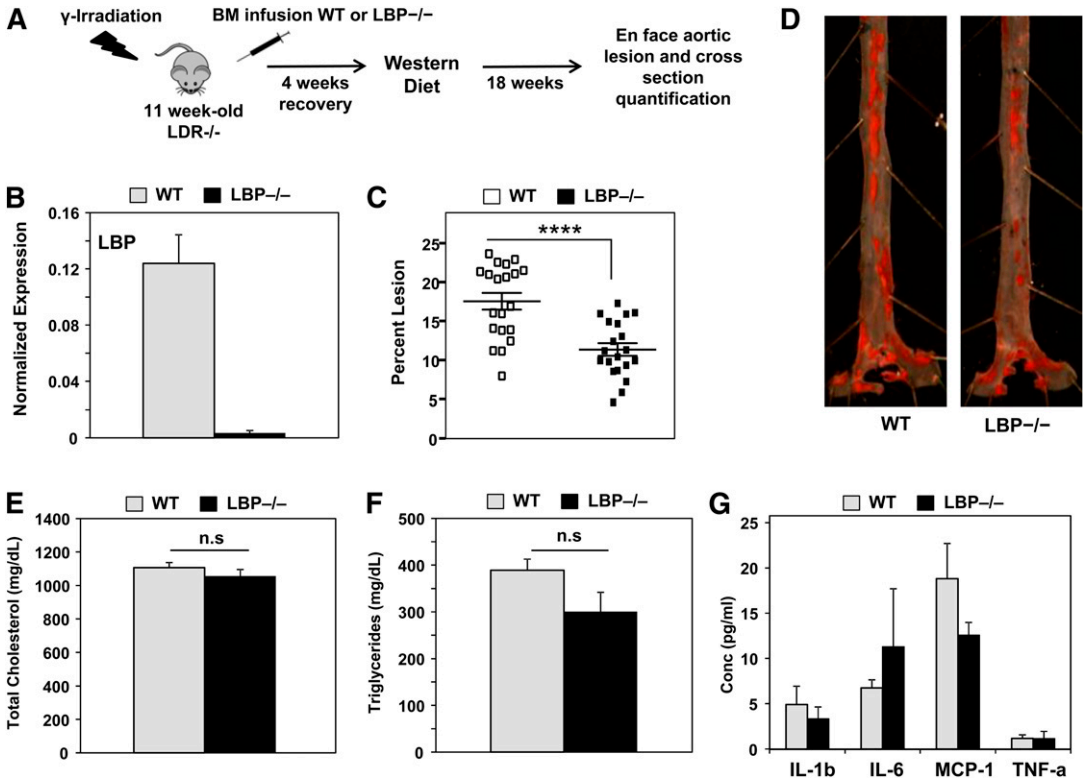

Fig. 4. Bone marrow expression of LBP promotes atherogenesis in LDLR-deficient mice. A: Schematic outline of bone marrow (BM) transplantation protocol. B: Confirmation of engraftment by gene expression analysis. Results are representative of three biological replicates. Values are mean ± SEM. C: Percentage of aorta surface area with atherosclerotic plaque in transplanted LDLR$^{-/-}$ mice (n = 20/group). Horizontal lines indicate mean ± SEM; ****$p < 0.0001$. D: Representative photographs from en face analysis of aortas from WT or LBP$^{-/-}$ transplanted mice after 18 weeks on a Western diet. Twenty mice in each group were analyzed. E: Total serum cholesterol levels were measured. Values are mean ± SEM. F: Total serum triglyceride levels were measured. Values are mean ± SEM. G: Serum cytokine levels were measured using a Milliplex mouse cytokine/chemokine panel (Millipore). Values are mean ± SEM. n.s, not significant.

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 LXRαβ$^{-/-}$ 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 LXRαβ$^{-/-}$ 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).

To investigate the induction of LBP mRNA by physiologic LXR agonists, we treated bone marrow-derived macrophages from WT and LXRαβ$^{-/-}$ mice 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 isotype is capable of regulating LBP. However, genetic ablation of both LXRs 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

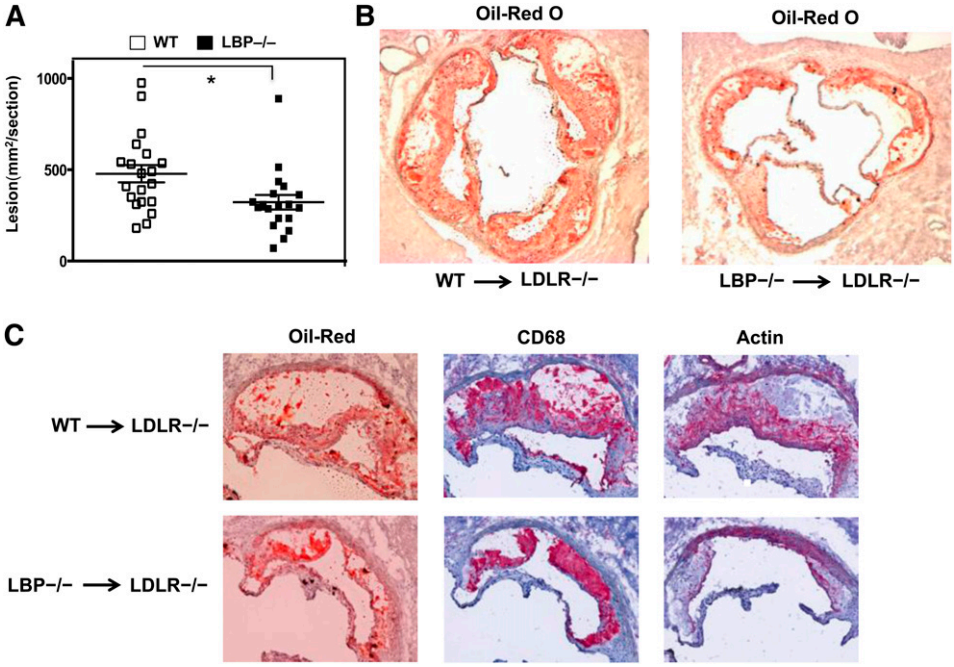

Fig. 5. LBP deletion in macrophages decreases the development of atherosclerosis. A: Quantification of aortic root lesions (n = 19–20 per group). Horizontal lines indicate mean ± SEM. B: Frozen sections from the aortic roots of bone marrow transplanted LDLR$^{-/-}$ mice with WT or LBP$^{-/-}$ maintained on a Western diet for 19 weeks. Lesions were stained with Oil-Red O (objective magnification: ×5). C: Representative histological data of the aortic valve area stained with Oil-Red O, the macrophage marker CD68, and α-smooth muscle actin (objective magnification: ×10).

response elements (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.

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 downregulation 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 LXR and RXR ligands (Fig. 3E). As expected, LPS induced the expression of interleukin (IL)-6 in primary hepatocytes and macrophages (Fig. 3D, E). These results suggest that the regulation of LBP by LXR is highly context specific and are suggestive of a different physiologic function for LBP in macrophages and liver.

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.

$LBP^{-/-}$ bone marrow transplant recipients showed significantly reduced atherosclerotic burden by cross-sectional area, an approximately 32% relative reduction in comparison with control mice (478,225 ± 47,099 μm^2/section versus 322,158 ± 39,936 μm^2/section; $P < 0.01$) (**Fig. 5A, B**). Histological analysis uncovered a marked reduction in lesions in $LBP^{-/-}$ transplanted mice, with decreased staining for the macrophage-specific marker CD68, as well as α-smooth muscle actin (Fig. 5C). Taken together, these results indicate that macrophage LBP expression is a determinant of susceptibility to atherosclerosis in $LDLR^{-/-}$ mice.

To further explore the role of LBP in atherogenesis, we analyzed bone marrow-derived macrophages from WT and $LBP^{-/-}$ mice. Previous studies have shown that LBP may bind lipids directly; suggesting loss of LBP may impact LXR-dependent regulation of cholesterol homeostasis or inflammation. mRNA analysis of $LBP^{-/-}$ macrophages showed no alteration in the baseline expression or activation of LXR target genes such as ABCA1, ABCG1, and AIM The capacity to repress LPS-induced inflammatory mediators, such as TNFα and COX-2, was not affected at the transcriptional or protein level (**Fig. 6A, B**). Loss of LBP did not affect sensitivity to LPS stimulation as measured by gene expression of inflammatory markers (Fig. 6C). As 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

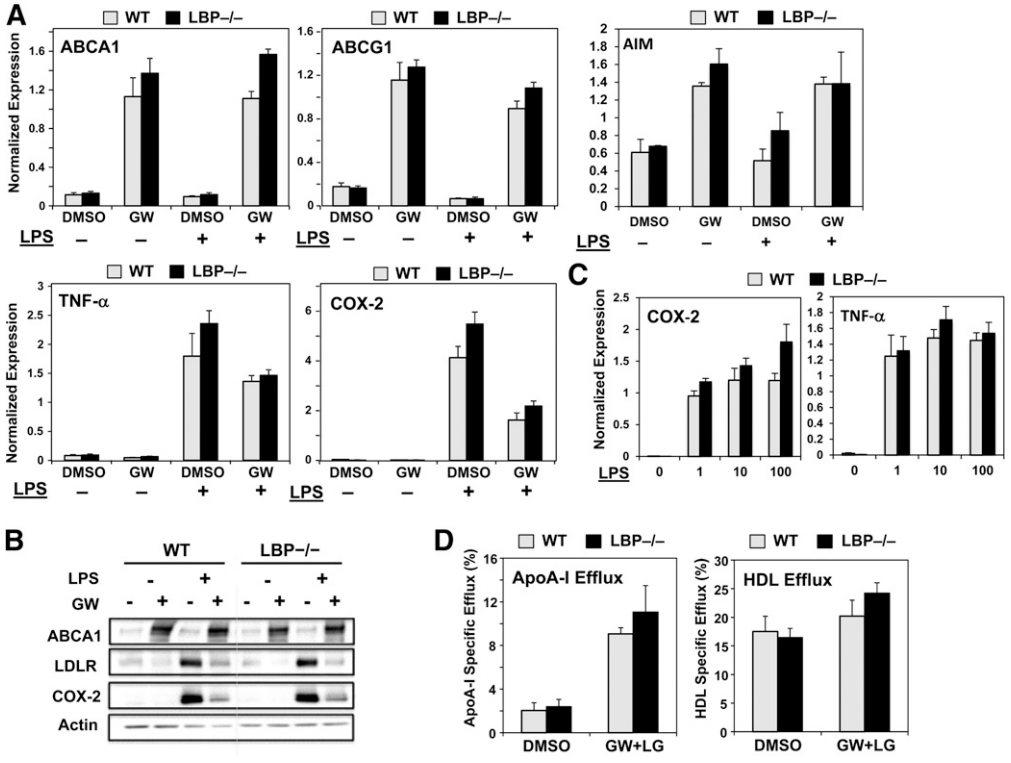

Fig. 6. LBP expression does not affect cholesterol efflux or inflammatory responses. A: Primary bone marrow-derived macrophages were treated with GW3965 (GW) overnight with (+) or without (−) LPS (10 ng/ml). Results are representative of two experiments. Values are mean ± SD. B: Western blot analysis of bone marrow-derived macrophages treated with GW3965 overnight with or without LPS (10 ng/ml). C: Bone marrow-derived macrophages treated with increasing concentration of LPS (ng/ml). Results are representative of two experiments. Values are mean ± SD. D: Cholesterol efflux assays. WT or $LBP^{-/-}$ macrophages were loaded with [3H]cholesterol (1.0 μCi/ml) in the presence of acyl-CoA:cholestrol *O*-acyltransferase inhibitor (2 μg/ml) either with DMSO or with ligand for LXR and RXR [1 μM GW3965, 50 nM LG268 (LG)]. Efflux was measured in the presence of apoA-I or HDL. Experiments were conducted in triplicate. Data are expressed as mean ± SD.

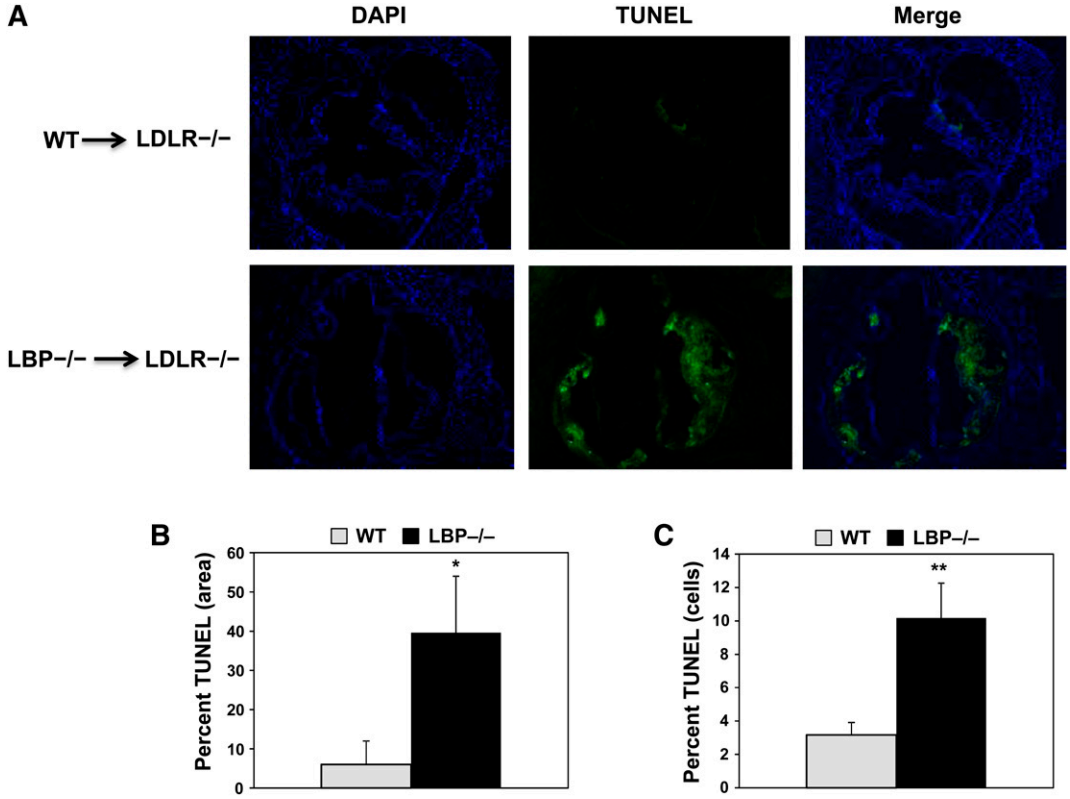

Fig. 7. Loss of LBP from hematopoietic cells enhances apoptosis in atherosclerotic lesions. A: Frozen sections from the aortic roots were stained for 4',6-diamidino-2-phenylindole (DAPI) and TUNEL (blue and green respectively). Representative samples are shown (objective magnification: ×5). B: Percent TUNEL-positive cells were quantified by TUNEL-positive area; *$p < 0.05$. C: Percent DAPI-stained cells that were TUNEL positive (n = 7 mice per group). Values are mean ± SEM; **$p < 0.005$.

pathways and atherosclerosis (17). Conversely, expression of the anti-apoptotic regulator BIRC5 (also known as Survivin) was reduced (Fig. 8A). To further explore this finding, we generated murine RAW264.7 macrophage cell lines stably expressing LBP (Fig. 8B). Ectopic expression of LBP led to a reduction in Bok expression and a reciprocal increase in BIRC5 in cells loaded with acetylated LDL (Fig. 8B). These results demonstrate that loss of LBP in hematopoietic cells affects macrophage survival in the context of atherosclerosis. In turn, upregulation of LBP expression in macrophages may potentially contribute to the previously documented pro-survival effects of LXR activation.

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.

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

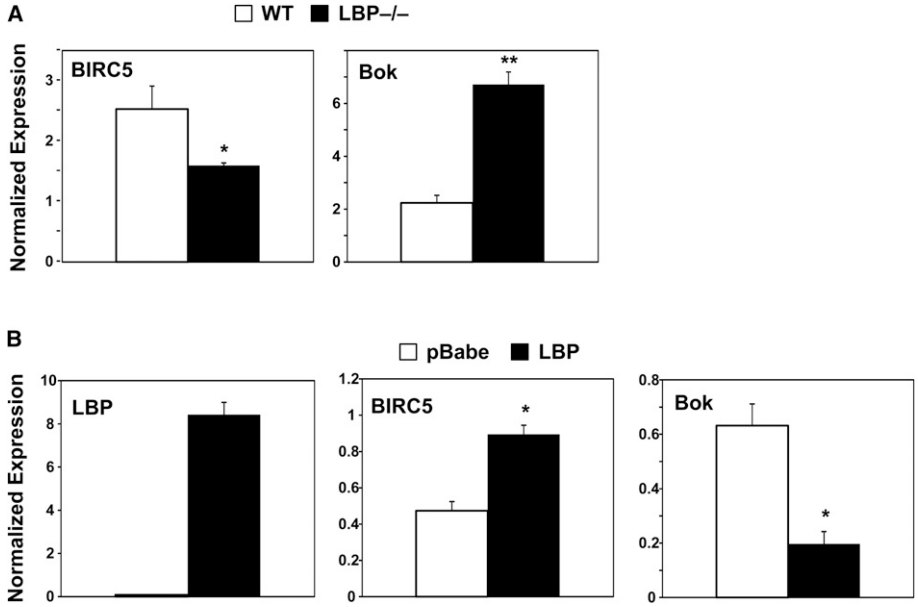

Fig. 8. LBP expression modulates apoptotic signaling in macrophages. A: Bone marrow-derived macrophages were obtained at time of harvest from transplanted LDLR$^{-/-}$ mice placed on a Western diet. Bone marrow from four individual mice was analyzed. Values are mean ± SD. B: RAW264.7 cells stably expressing LBP were treated with acetylated LDL for 3 days. Gene expression was analyzed by real-time PCR. Results are representative of three independent experiments. Values are mean ± SD; *$p < 0.05$, **$p < 0.005$.

factors that regulate macrophage proliferation and survival could therefore offer novel therapeutic strategies.

We have shown here that the loss of LBP expression in macrophages is atheroprotective, stemming from increased apoptotic death of lesion macrophages. Modified LDL is an established pro-apoptotic stimulus for macrophages. A protective role for macrophage apoptosis in early lesion development in mice is supported by multiple prior studies, including the observation of accelerated plaque development in LDLR$^{-/-}$ mice reconstituted with marrow lacking the pro-apoptotic element Bax (75). Our results imply that the LBP is another important LXR-responsive factor that supports the survival of macrophages within lesions against the apoptotic effects of modified LDL.

It is important to note that LBP deficiency by itself does not cause excessive cell death (32). This suggests that LBP-mediated pro-survival mechanisms function under a specific context where pro-apoptotic stimuli are enriched, such as the atherosclerotic lesion. The exact mechanisms by which LXR target genes regulate macrophage survival remain unclear. Intriguingly, both AIM and LBP are secreted into the blood, so it is conceivable that they could potentially trigger anti-apoptotic signaling cascade(s) in an autocrine or paracrine fashion. Interestingly, previous studies have shown that activation of LXR antagonizes the apoptotic program induced by engagement of TLR4 (17). The ability of LBP to bind and neutralize LPS could be one potential protective mechanism against LPS-induced apoptosis. Alternatively, LBP may mediate innate survival responses by binding another lipid moiety or intermediate. Further studies will be needed to address these possibilities.

Recent guidelines have placed a strong emphasis on cardiovascular risk reduction, but current risk stratification 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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