# Fatty acid binding protein 4/aP2-dependent BLT1R expression and signaling

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**Abstract** **Previous studies have shown that reduced levels of the adipocyte fatty acid binding protein (FABP)4 (AFABP/aP2), result in metabolic improvement including potentiated insulin sensitivity and attenuated atherosclerosis. Mechanistically, pharmacologic or genetic inhibition of FABP4 in macrophages upregulates UCP2, attenuates reactive oxygen species (ROS) production, polarizes cells toward the anti-inflammatory M2 state, and reduces leukotriene (LT) secretion. At the protein level, FABP4 stabilizes $LTA_4$ toward chemical hydrolysis, thereby potentiating inflammatory $LTC_4$ synthesis. Herein, we extend the FABP4-LT axis and demonstrate that genetic knockout of FABP4 reduces expression of the major macrophage LT receptor, $LTB_4$ receptor 1 (BLT1R), via a ROS-dependent mechanism. Consistent with inflammation driving BLT1R expression, M1 polarized macrophages express increased levels of BLT1R relative to M2 polarized macrophages and treatment with proinflammatory lipopolysaccharide increased BLT1R mRNA and protein expression. In FABP4 knockout macrophages, silencing of UCP2, increased ROS levels and led to increased expression of BLT1R mRNA. Similarly, addition of exogenous $H_2O_2$ upregulated BLT1R expression, whereas the addition of a ROS scavenger, *N*-acetyl cysteine, decreased BLT1R levels. As compared with WT macrophages, $LTB_4$-BLT1R-dependent JAK2-phosphorylation was reduced in FABP4 knockout macrophages. In summary, these results indicate that FABP4 regulates the expression of BLT1R and its downstream signaling via control of oxidative stress in macrophages.**—Hertzel, A. V., H. Xu, M. Downey, N. Kvalheim, and D. A. Bernlohr. **Fatty acid binding protein 4/aP2-dependent BLT1R expression and signaling. *J. Lipid Res.* 2017.** 58: **1354–1361.**

**Supplementary key words** macrophages • leukotrienes • inflammation • lipids • lipid mediators • signal transduction • leukotriene $B_4$ receptor 1 • adipocyte myelin P2

The prevalence of obesity and its associated pathologies, including insulin resistance, have increased in the past several decades (1–4). Numerous studies have implicated adipose tissue-recruited immune cells as a critical component of insulin resistance and significant attention has been focused on macrophages, whose malleable nature allows for alternative states of activation dependent on the local environment (5, 6). With the development of obesity, adipose tissue acquires a chronic low-grade proinflammatory state, along with increased reactive oxygen species (ROS), dysfunctional mitochondria, and whole body insulin resistance (7–9).

Leukotrienes (LTs) are lipid mediators derived from arachidonic acid via the actions of the 5-lipoxygenase pathway (10–13). $LTA_4$ hydrolase activity results in the production of $LTB_4$, whereas $LTC_4$ synthase initiates the glutathione conjugation of $LTA_4$ to produce $LTC_4$, $LTD_4$, and $LTE_4$ (14, 15). These lipids bind with high affinity to a subfamily of G protein-coupled receptors, thus initiating signal cascades to direct functional consequences, typically of a proinflammatory nature (16–18). Lipopolysaccharide (LPS), zymosan, and calcium ionophores have all been demonstrated to increase $LTB_4$ production and secretion in multiple cell types, including neutrophils and macrophages (19, 20). $LTB_4$ binds with high affinity to the $LTB_4$ receptor 1 (BLT1R), a member of the G protein-coupled receptor family (16, 21, 22). A second $LTB_4$ receptor, BLT2R, binds $LTB_4$ with a 20-fold higher $K_d$ (21, 22). Several other lipoxygenase products have been demonstrated to bind to BLT2R, with the thromboxane synthase product, 12-HHT, having a 10-fold higher affinity than $LTB_4$; thus 12-HHT is thought to be an endogenous ligand (22, 23). $LTB_4$ is involved in host defense following infection in a number of ways, including increasing the recruitment of immune cells via increased chemotaxis, calcium mobilization, and

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Abbreviations: AKO, FABP4/aP2 knockout; BLT1R, leukotriene $B_4$ receptor 1; BMDM, bone marrow-derived macrophage; FABP, fatty acid binding protein; LPS, lipopolysaccharide; LT, leukotriene; ROS, reactive oxygen species; TFIIE, transcription factor II E.

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altered gene expression, ultimately leading to an inflammatory state (13, 24, 25). Additionally, $LTB_4$ stimulates neutrophil secretion of proteins, including lysozyme and matrix metalloproteinases (26, 27). In macrophages, $LTB_4$ has been demonstrated to increase proliferation and plays a role in phagocytosis (28–30). Chronic $LTB_4$ proinflammatory signaling has been implicated in several pathological states including atherosclerosis, asthma, arthritis and cancer (16, 24, 31–33).

On a high-fat diet, murine adipose tissue accumulates M1-activated macrophages, thereby producing an inflammatory state (34–36). However, BLT1R knockout mice demonstrate an anti-inflammatory adipose phenotype and are resistant to pathologies associated with a high-fat diet. Without BLT1R, adipose tissue accumulates M2 macrophages with a corresponding decrease in M1 macrophages. This ultimately results in decreased circulating proinflammatory chemokines and cytokines and reduced insulin resistance (37, 38). Additionally, BLT1R has been implicated in the insulin resistance derived by adipose tissue B2 cells (39). BLT1R deletion also protects from the progression of atherosclerosis in an ApoE-deficient mouse (37). In addition to the BLT1R, Olefsky and colleagues have demonstrated that high-fat diet-induced obesity results in increased $LTB_4$ production in metabolic tissues, including adipose, liver, and muscle (38).

Fatty acid binding proteins (FABPs) are small soluble fatty acid carriers and comprise a family of nine members (40). FABP4 (aP2/AFABP) plays a significant role in lipid metabolism; in the absence of FABP4, lipolysis is reduced (41–43). Furthermore in the obese state, genetic deletion of FABP4 improves metabolism, resulting in reduced insulin resistance, atherosclerosis, and asthma (44–49). More specifically, FABP4-null macrophages have been shown to exert an anti-inflammatory phenotype, most closely resembling an M2 state. Interestingly, members of the FABP family, including FABP4, have been implicated in stabilizing short-lived LT intermediates, by binding the unstable epoxide containing $LTA_4$, functionally protecting it from water hydrolysis to the inactive 5,6- or 5,12-diHETEs (50, 51). Herein, we report that in macrophages, the inflammatory stimuli LPSs, as well as ROS, upregulate BLT1R expression in a FABP4-dependent manner. Furthermore, we demonstrate that in macrophages lacking FABP4, the $LTB_4$-induced signal cascade is dramatically reduced.

## RESEARCH DESIGN AND METHODS

### Cell culture

RAW264.7 macrophages were maintained in DMEM (Invitrogen) with 10% FBS. WT, FABP4/aP2 knockout (AKO), shGFP control, and shUCP2 knockdown AKO macrophages were maintained in RPMI 1640 (Invitrogen) with 5% FBS. Concentrations and times of treatment of LPS, $H_2O_2$, *N*-acetyl cysteine, or $LTB_4$ are listed in each figure legend.

### Bone marrow-derived macrophage isolation

Bone marrow-derived macrophages (BMDMs) were isolated from 10-week-old female C57BL/6J mice as indicated in Ying et al. (52). Cells were plated and maintained in Iscove's modified Dulbecco's medium (Invitrogen) with 10% FBS and 10 ng/ml macrophage colony-stimulating factor (M-CSF) for 1 week to allow maturation of the cells into naïve macrophages. Polarization of cells to either M1 or M2 activation states occurred by treatment with either LPS (1 μg/ml)/IFNγ (10 ng/ml) or IL-4 (10 ng/ml)/IL-13 (10 ng/ml) for 48 h, respectively. All experimental procedures using animals were reviewed and approved by the University of Minnesota Institutional Animal Care and Use Committee.

### Quantitative RT-PCR

Total RNA was isolated using TRIzol reagent (Invitrogen) and cDNA synthesis was performed using iScript according to the manufacturer's protocol (Bio-Rad). Quantitative (q)RT-PCR amplification utilized a Bio-Rad CFX 96 real-time system with a SYBR green Supermix (Bio-Rad). Transcription factor II E (TFIIE) was used as the internal control to normalize expression. Primer sequences used are (forward; reverse): Arginase, (AACACGGCAGTGGCTTTAACC; GGTTTTCATCTGGCGCATTC); BLT1R, (GGCATCTGGGTGGTGTCTTTTC; TGCTCTTTGTTGGGATAGTTCG); CD206, (CTCGTGGATCTCCGTGACAC; GCAAATGGAGCCGTCTGTGC); CXCL10, (CGTGTTGAGATCATTGCCAC; TTAAGGAGCCCTTTTAGACC); IL-1β, (AAATACCTGTGGCCTTGGGC; CTTGGGATCCACACTCTCCAG); iNOS, (AGCGAGTTGTGGATTGTCC; TCTCTGCCTATCCGTCTCG); LCN2, (GGGAGTGCTGGCCAAATAAG; TGCCACTCCATCTTTCCTGTT); PPARγ, (GCCATTGAGTGCCGAGTC; TGTGGATCCGGCAGTTAA); TFIIE, (CAAGGCTTTAGGGGACCAGATAC; CATCCATTGACTCCACAGTGACAC).

### Immunoblot analysis

Cells were lysed with RIPA buffer supplemented with protease inhibitors (Calbiochem). Fifty micrograms of protein from each sample were separated by SDS-PAGE and transferred to a PVDF membrane. After blocking with Odyssey blocking buffer (Li-Cor Biosciences), membranes were incubated with primary antibody overnight at 4°C. Membranes were washed and incubated with secondary antibody conjugated to Li-Cor IRDye for 1 h and visualized using Odyssey infrared imaging (Li-Cor Biosciences). The primary antibodies used were anti-BLT1R (Cayman Chemical) and anti-actin (Sigma).

### Immunoprecipitation

For JAK2 phosphorylation experiments, following $LTB_4$ treatments, JAK2 antibody (Cell Signaling) was added to cell lysates and incubated overnight at 4°C. Subsequently protein A-agarose was added and after 2 h of rocking at 4°C, centrifuged and the pellet was washed three times. The immunoprecipitants were denatured by boiling and run on SDS-PAGE. Immunoblots utilized antibodies to phospho-tyrosine (4G10; Millipore) and total JAK2 (Cell Signaling).

### Statistical analysis

All data in this work are expressed as ±SEM. Statistical significance was determined using an unpaired two-tailed Student *t*-test ($*P < 0.05$).

## RESULTS

### Genetic or pharmacologic depletion of FABP4 reduces BLT1R expression

Previously, genetic knockout or pharmacologic inhibition of FABP4 has been shown to reduce inflammation and

improve insulin sensitivity (44, 53). This inhibition of FABP4 results in accumulation of intracellular fatty acids in both adipocytes and macrophages (41, 42, 54). Recently we demonstrated that monounsaturated fatty acids, particularly C16:1 palmitoleic acid and C18:1 oleic acid, drive the upregulation of UCP2 in macrophages resulting in reduced ROS and proinflammatory cytokine production (54). Because the $LTB_4$ pathway is intimately involved in promoting inflammation, we evaluated the expression of the receptor, BLT1R, in macrophages generated from C57BL/6J (WT) or FABP4 knockout mice (AKO). FABP4 knockout macrophages expressed approximately 50% the BLT1R mRNA levels of WT (**Fig. 1A**) with BLT1R protein levels similarly reduced in FABP4 knockout macrophages (Fig. 1B, C).

### Signaling by $LTB_4$ is compromised in FABP4 knockout macrophages

Because the expression of BLT1R is reduced in FABP4 knockout macrophages, we next determined whether this translated into a functional effect on signaling. $LTB_4$ has been demonstrated to activate the JAK2 pathway in macrophages via coupling to GαI, which inhibits the known JAK2 inhibitor, SOCS1 (55). Therefore, we tested whether the $LTB_4$-dependent activation of JAK2 differed in FABP4 knockout as compared with control macrophages. As expected, a time course of $LTB_4$ treatment resulted in phosphorylation of JAK2 in WT macrophages; however this was dramatically blunted in FABP4 knockout macrophages (Fig. 1D).

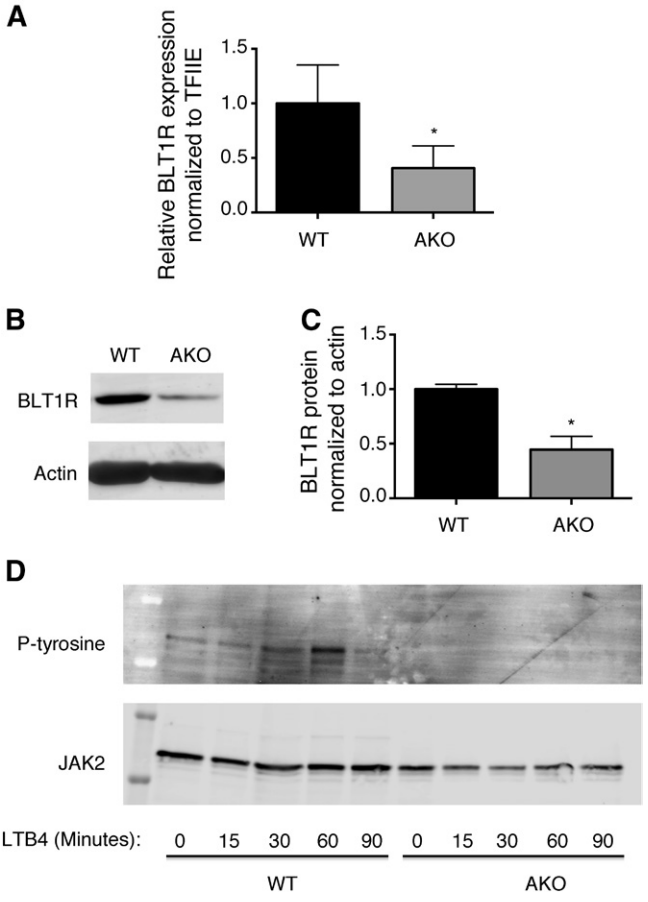


**Fig. 1.** FABP4 regulates BLT1R expression and activity. A: BLT1R mRNA expression normalized to TFIIE in stable macrophage cell lines from WT C57BL/6J (black bars) and genetic knockout of FABP4/aP2 (AKO) (gray bars) mice. B: BLT1R and actin protein expression in WT and AKO macrophages. C: Quantification of BLT1R protein levels normalized to actin. D: WT and FABP4/aP2 (AKO) macrophages were treated with 100 nM $LTB_4$ for the indicated times. Following JAK2 immunoprecipitation, immunoblot analysis was conducted using phospho-tyrosine and total JAK2 antibodies. Data were analyzed by Student's *t*-test; $^*P < 0.05$.

### Macrophage polarization affects BLT1R expression

Macrophages can be activated by a variety of autocrine or paracrine factors to yield either a classical inflammatory M1 state or an alternatively activated anti-inflammatory M2 state (35, 56, 57). To determine whether expression of BLT1R is altered depending on the activation state, we utilized BMDMs activated to the M1 proinflammatory state using LPS and IFNγ, or to the M2 anti-inflammatory state using IL-4 and IL-13. To evaluate macrophage polarization, we quantified the expression of several genes known to be markers of M1 or M2 macrophages (58). As shown in **Fig. 2A**, LPS and IFNγ resulted in robust expression of the M1 markers, iNOS, CXCL10, IL-1β, and LCN2, while reducing expression of the M2 markers, arginase, PPARγ, and CD206 (Fig. 2B). In contrast, IL-4 and IL-13 increased the M2 markers while reducing the expression of the M1 markers. Consistent with an inflammatory state regulating the expression of BLT1R, M1 macrophages expressed higher BLT1R mRNA levels than macrophages in the M2 state (Fig. 2C).

### BLT1R expression is regulated by LPS in macrophages

The regulatory effect of inflammatory signals such as LPS on BLT1R expression varies greatly dependent on the specific cell type (59–61). Therefore, we tested the effect of LPS on BLT1R in the murine macrophage cell line, RAW264.7. LPS exerted a time- and dose-dependent increase in BLT1R mRNA (**Fig. 3A**, B) in RAW264.7 cells. Interestingly, short-term exposure to LPS increased BLT1R protein levels (Fig. 3C). However, longer LPS exposure resulted in a decrease in BLT1R protein (Fig. 3D). This result might be due to receptor desensitization that includes internalization and degradation or perhaps some other mechanism. Because AKO macrophages express lower basal levels of BLT1R, we evaluated the effect of LPS treatment on BLT1R expression in AKO cells. Figure 3E demonstrates that although LPS increased BLT1R mRNA expression in WT macrophages, LPS had no effect on AKO macrophages, consistent with the reduced levels of ROS and inflammation in this cell line (54, 62, 63).

### Regulation of BLT1R expression by UCP2 and ROS

We have previously demonstrated that knockout or inhibition of FABP4 leads to decreased inflammation and ROS due to the upregulation of UCP2 (54). To determine whether downregulation of BLT1R in AKO cells was UCP2-dependent, we silenced UCP2 expression (approximately 80%) in AKO macrophages using stable shRNA (54). As

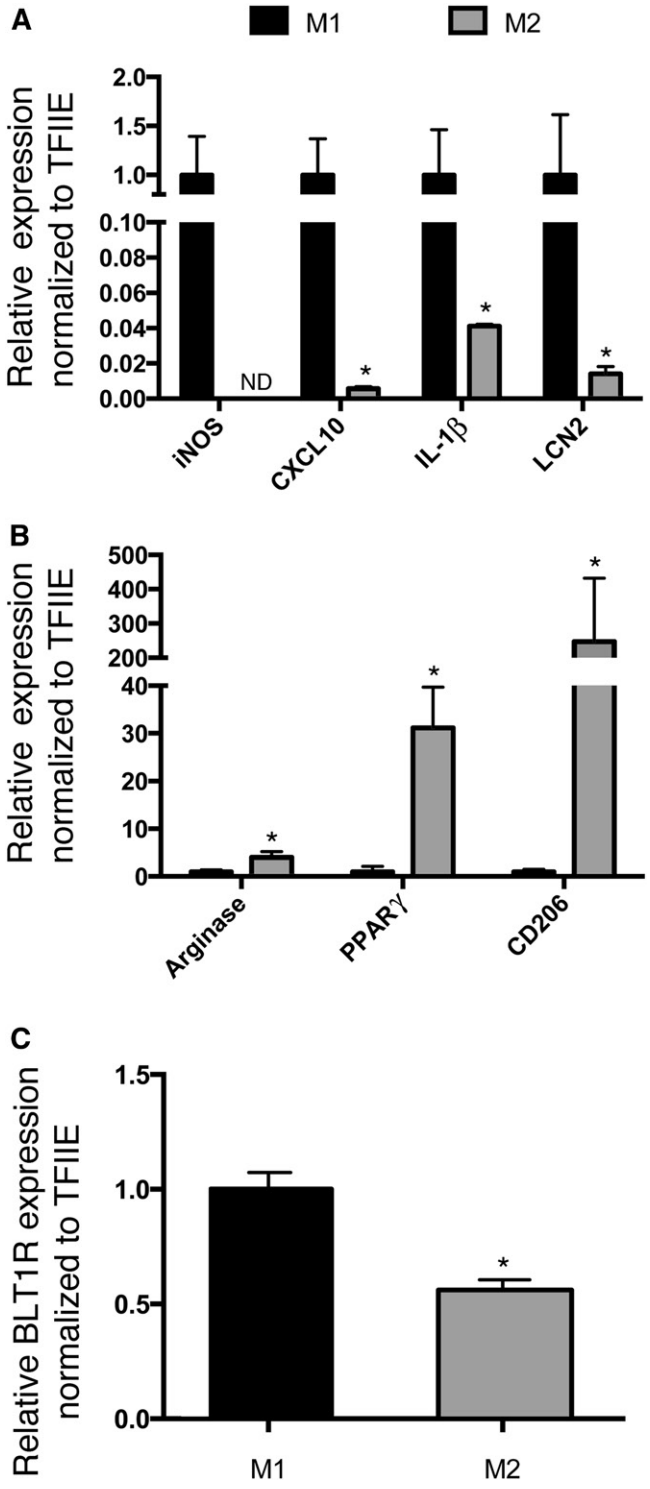


**Fig. 2.** Macrophage polarization regulates BLT1R expression. Cultured BMDMs from C57BL/6J mice were activated to the M1 state with LPS (1 μg/ml)/IFNγ (10 ng/ml) (black bars) or the M2 state with IL-4 (10 ng/ml)/IL-13 (10 ng/ml) (gray bars) for 48 h and gene expression evaluated by quantitative RT-PCR. A: Expression of genes representing M1-polarized macrophage markers. B: Expression of genes representing M2-polarized macrophage markers. C: Relative expression of BLT1R in the M1- and M2-activated macrophages. All expression results were normalized to TFIIE. Data were analyzed by Student's *t*-test; $^*P < 0.05$.

shown in **Fig. 4A**, loss of UCP2 in AKO cells resulted in increased expression of BLT1R mRNA. Although there is some controversy as to whether UCP2 has uncoupling activity, it is generally agreed that UCP2 expression is negatively correlated with ROS (64, 65). We too have provided evidence supporting this correlation, because we measured increased ROS levels when UCP2 was silenced in RAW264.7 macrophages (52). To determine whether ROS could independently regulate BLT1R, Raw264.7 macrophages were treated with hydrogen peroxide and BLT1R mRNA levels were evaluated. As shown in Fig. 4B, BLT1R mRNA levels were robustly increased following $H_2O_2$ treatment of RAW264.7 macrophages. Furthermore, if reduced oxidative stress in AKO macrophages was responsible for the decreased BLT1R expression, exogenous $H_2O_2$ should increase BLT1R in both WT and AKO macrophages. For this, we isolated and treated BMDMs from C57BL/6J (WT) and AKO mice, and treated both with exogenous $H_2O_2$. Figure 3C shows that basal expression of BLT1R in AKO is trending down, similar to our stable macrophage cell lines, but exogenous $H_2O_2$ results in a similar increase in BLT1R expression in both cell types. Finally, we tested whether the general ROS scavenger, *N*-acetyl cysteine, could decrease expression of BLT1R in WT and AKO macrophages. Consistent with the model of ROS being a primary regulator of BLT1R, mRNA levels of BLT1R decreased in both WT and AKO following treatment with *N*-acetyl cysteine (Fig. 4D).

## DISCUSSION

Herein, we demonstrate that the expression of macrophage BLT1R is upregulated by LPS and ROS, and that FABP4 knockout (AKO) macrophages have significantly blunted BLT1R expression and activity. Previous studies have demonstrated that the reduced proinflammatory activity in the FABP4 knockout macrophages is due in large part to increased UCP2 expression and, in the present study, we extend that analysis to link UCP2 expression and ROS to the expression of BLT1R (40, 62, 63). Increased expression of UCP2 in FABP4 knockout macrophages reduces inflammatory cytokine synthesis and secretion (54). Moreover, FABP4 protein stabilizes $LTA_4$ to chemical degradation and stimulates LT synthesis and secretion. These findings are consistent with our current data demonstrating the inverse relationship of elevated BLT1R expression and reduced UCP2 expression.

FABP4 knockout mice are protected from chronic inflammation, insulin resistance, atherosclerosis, and experimental autoimmune encephalomyelitis (44–49, 66). The $LTB_4$-BLT1R axis has been demonstrated to play a significant role in promoting each of these conditions (16, 24, 37, 67, 68) and has been identified as a potential therapeutic target (69). Furthermore, the phenotype of the BLT1R knockout mice shares similarities to that of the FABP4 knockout mice. These include increased alternative activation (M2) of macrophages, decreased MCP-1 expression, and JNK activation, as well as decreased liver triglycerides (37). Additionally, inhibition of BLT1R in the

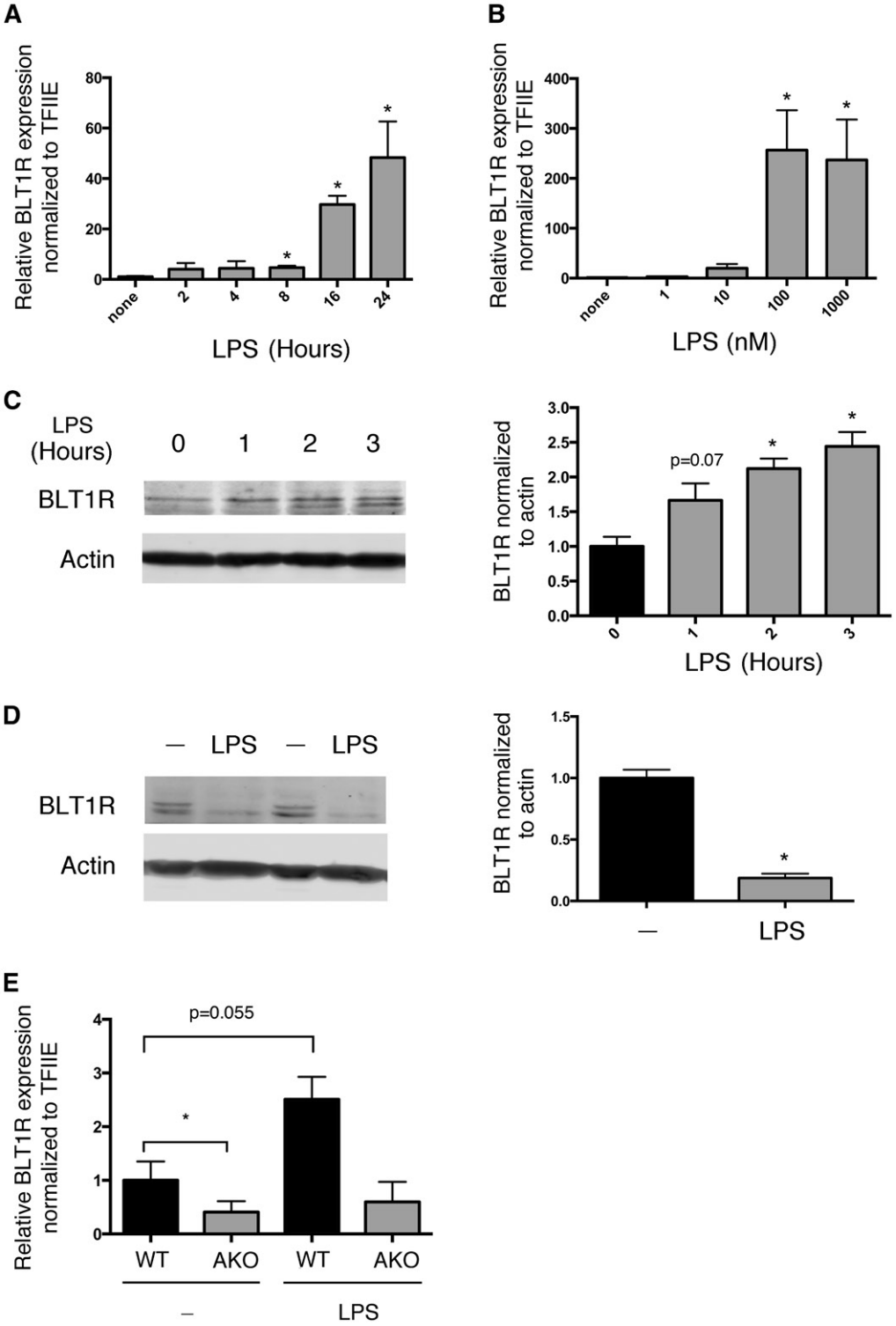


**Fig. 3.** LPS regulates BLT1R expression. A: Time course (2–24 h) of BLT1R mRNA expression in RAW264.7 macrophages treated with LPS (100 ng/ml). B: Expression of BLT1R mRNA in response to a 24 h concentration course of LPS. C: Expression of BLT1R protein in response to short-term LPS (100 ng/ml) treatment (0–3 h). D: Expression of BLT1R protein in response to 24 h of LPS (100 ng/ml) treatment. E: BLT1R mRNA expression in WT and AKO macrophages under basal conditions and in response to LPS (100 ng/ml) treatment for 24 h. Data were analyzed by Student's *t*-test. *$P < 0.05$ as compared with no treatment (A–D). *$P < 0.05$ AKO compared with WT (E).

leptin-deficient obesity model, ob/ob, increased the amount of M2 adipose tissue macrophages and decreased M1 macrophages (38). Consistent with this, inhibition of BLT1R also led to decreased expression and circulation of proinflammatory cytokines (37, 55). Therefore, the reduction of BLT1R in the FABP4-null macrophages may underlie the reduced susceptibility to metabolic diseases in the FABP4-null mice.

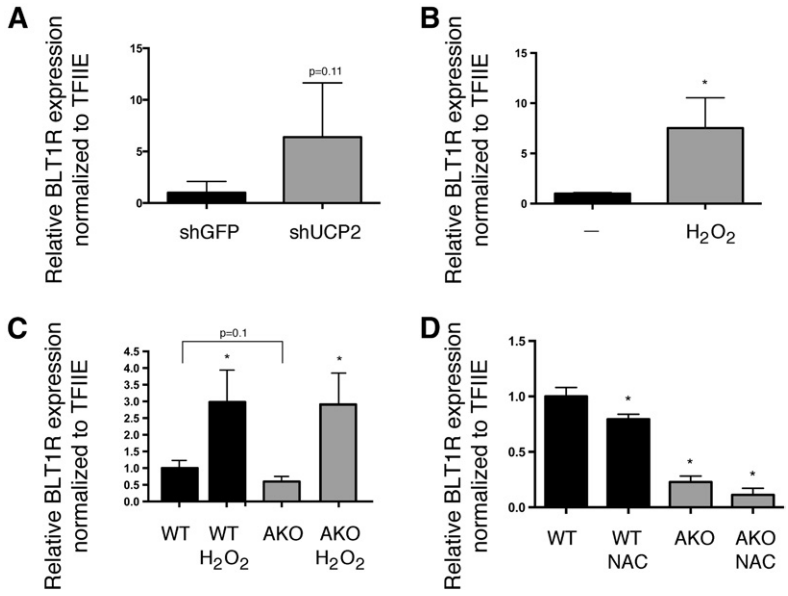


**Fig. 4.** UCP2 and ROS regulate BLT1R expression. Macrophage expression of BLT1R mRNA normalized to TFIIE mRNA. A: shRNA knockdown of GFP (control) and UCP2 in AKO macrophages. B: RAW264.7 macrophages treated with hydrogen peroxide (100 μM, 6 h). C: BMDMs from WT and AKO mice treated with hydrogen peroxide (100 μM, 6 h). D: WT and FABP4/aP2-null (AKO) macrophages treated with or without *N*-acetyl cysteine (10 mM) for 24 h. Data were analyzed by Student's *t*-test (*$P < 0.05$). *$P < 0.05$ as compared with WT (C, D).

Upon ligand binding, G protein-coupled receptors typically become desensitized, internalized, and degraded (70, 71) and this regulatory mechanism has been demonstrated for $LTB_4$-BLT1R in an atypical phosphorylation-independent manner via β-arrestin association (72). In this study, LPS treatment resulted in a short-term increase in protein expression of BLT1R; however, chronic LPS treatment resulted in decreased BLT1R protein levels (Fig. 3C, D). The apparent discordance between BLT1R mRNA and protein expression may be linked to desensitization and subsequent degradation of the receptor protein. However, preliminary work thus far has not supported this assertion (data not shown). Thus the mechanism that leads to the dramatic decrease in $LTB_4$ signaling in the FABP4-null macrophages, in addition to the reduction of the receptor, is not currently known. Additional work is necessary to determine the mechanism underlying this observation.

The $LTB_4$ precursor, $LTA_4$, has been shown to be highly unstable due to the water hydrolysis of its epoxide ring, eliminating its biological activity (73). The half-life of $LTA_4$ is markedly increased by members of the FABP family, including FABP4 (50), and pharmacologic inhibition of FABP4 in macrophages markedly reduces $LTC_4$ secretion (74). As such, FABP4 regulates LT signaling on multiple levels. First, it directly affects the stability and secretion of LTs and, second, it indirectly regulates BLT1R via an UCP2-dependent ROS-based mechanism. Together these effects significantly influence the activity of $LTB_4$/BLT1R proinflammatory signaling and suggest that decreased $LTB_4$ signaling in FABP4 knockout mice would contribute to the anti-inflammatory phenotype of the animals.

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