Leukotriene B₄ Enhances the Generation of Proinflammatory MicroRNAs To Promote MyD88-Dependent Macrophage Activation


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activation of pattern recognition receptors (PRRs) by either pathogen-associated molecular pattern molecules (PAMPs) or by endogenous damage-associated molecular pattern molecules (DAMPs) elicits proinflammatory programs characterized by the production of inflammatory mediators that are important for the control of microbial infection (1). However, excessive production of such mediators also results in tissue injury (2). Among the PRRs, TLRs are the best studied. TLR family members and the IL-1βR share a conserved cytoplasmic Toll/IL-1R (TIR) domain that binds adaptor proteins, including myeloid differentiation factor 88 (MyD88) (3). MyD88 mediates signaling through all of the known TLRs except TLR3. MyD88 is necessary for host defense against a variety of experimental infections (4, 5); it also promotes development of atherosclerosis, autoimmune responses, diabetes, colitis, and familial Mediterranean fever (4, 5). MyD88 directs a variety of signaling programs that culminate in the activation of the master transcription factor NF-κB, leading to the expression of proinflammatory genes (6). Among the mediators produced by activated macrophages, the 5-lipoxygenase (5-LO)-derived bioactive lipid leukotriene (LT) B₄ (LTB₄) is conventionally known as a potent phagocyte chemoattractant, and its role in eliciting acute inflammatory responses and maintaining chronic inflammation is well established (7). We and others have shown that LTB₄ signaling via its cognate receptor B leukotriene receptor 1 (BLT1) through a Gαi-mediated decrease in cAMP levels enhances phagocytosis and killing of both opsonized and nonopsonized (8–12) microbes. LTB₄ activates NF-κB (13) and stimulates production of cytokines including TNF-α (14), and it enhances the production of microbicidal molecules including ROIs (9), RNIs (15, 16), and antimicrobial peptides. We have also shown that LTB₄ has a permissive role in TLR activation by mediating both basal and inducible MyD88 expression and NF-κB activation in macrophages in vitro and in vivo (13). Suppressor of cytokine signaling (SOCS-1) is an important inhibitor of JAK2 and STAT1 (17)—a transcription factor for MyD88 (13)—and it impairs TLR activation by increasing degradation of MyD88-like adaptor and IRAKs (18, 19). A key mechanism of LTB₄/BLT1-mediated effects is enhanced degradation of mRNA encoding SOCS-1 (13). Reducing the SOCS-1 level represents an important step in macrophage activation (19). SOCS-1 mRNA levels can be controlled both at the levels of transcription and via microRNA-mediated inhibition. MicroRNAs are small oligonucleotides (~22 nt) that bind complementary sequences in mRNAs, usually resulting in gene silencing via translational repression or target degradation (20). MicroRNAs are also potent regulators of homeostasis and of pathologic conditions. Recent studies have shown that microRNAs are key determinants of macrophage activation, controlling the expression of a variety of molecules involved in PRR signaling and NF-κB activation (20). There are several reports showing that SOCS-1 is targeted by microRNAs to control TLR activation in phagocytes. We have shown that leukotriene (LT) B₄ (LTB₄) positively regulates macrophage MyD88 expression by decreasing suppressor of cytokine signaling-1 (SOCS-1) mRNA stability. In this study, we investigated the possibility that LTB₄ control of MyD88 expression involves the generation of microRNAs. Our data show that LTB₄, via its receptor B leukotriene receptor 1 (BLT1) and Gαi signaling, increased macrophage expression of inflammatory microRNAs, including miR-155, miR-146b, and miR-125b. LTB₄-mediated miR-155 generation was attributable to activating protein-1 activation. Furthermore, macrophage transfection with antagomirs against miR-155 and miR-146b prevented both the decrease in SOCS-1 and increase in MyD88. Transfection with miR-155 and miR-146b mimics decreased SOCS-1 levels, increased MyD88 expression, and restored TLR4 responsiveness in both wild type and LT-deficient macrophages. To our knowledge, our data unveil a heretofore unrecognized role for the GPCR BLT1 in controlling expression of microRNAs that regulate MyD88-dependent activation of macrophages.

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different microRNAs, particularly miR-155, but also miR-30b and miR-150 (21–23). The mechanisms underlying expression of microRNAs in macrophages are poorly understood. It remains to be determined whether microRNA expression is modulated by G protein–coupled receptors. We hypothesized that LTB4/BLT1/Gαi signaling enhances the expression of microRNAs that will in turn control macrophage TLR activation. In this study, we report on the role of the GPCR BLT1 in the generation of microRNAs involved in SOCS-1 mRNA degradation, resulting in increased MyD88 expression and TLR-mediated NF-κB activation. We also identified miR-125b as a novel regulator of MyD88 degradation.

Materials and Methods

Animals

Eight-week-old female 5-LO<sup>−/−</sup> [B6.129-Alox<sup>em1Tam</sup> (24)], BLT1<sup>−/−</sup> [B6.129A-Ltb4r<sup>tm1maka</sup>/J (25)], and strain-matched wild type (WT) C57BL/6 mice (The Jackson Laboratory) were maintained according to National Institutes of Health guidelines for the use of experimental animals with the approval of the University of Michigan and Indiana University Committees for the Use and Care of Animals.

Cell harvest

Elicited macrophages were harvested from the peritoneal cavities of mice by lavage with PBS 4 d after the injection of 2 ml 3% thiglycollate as described previously (11).

Immunoblotting

Western blots were performed as described previously (11, 13). Protein samples were resolved by SDS-PAGE, transferred to a nitrocellulose membrane, and probed with commercially available primary Abs against MyD88, SOCS-1 (both 1:500; Abcam), or β-actin (1:10,000; Sigma-Aldrich). Denitrometric analysis was performed as described previously (11, 13).

Measurement of nitrite and TNF-α

Levels of TNF-α were determined by ELISA (R&D Dusset; R&D Systems) as suggested by the manufacturer. Nitrite, the stable oxidized derivative of NO, was determined using the Griess reaction (13).

RNA isolation and semiquantitative real-time RT-PCR

Total RNA from cultured cells was isolated using the miRNeasy Mini Kit (Qiagen) according to the manufacturer’s instructions. Real-time RT-PCR was performed as described previously (11, 13).

Activating protein-1 activity assay

WT and 5-LO<sup>−/−</sup> macrophages were stimulated for 24 h with 100 nM LTB4 in the presence or absence of the activating protein-1 (AP-1) inhibitor SR11302 (10 μM), and DNA binding activity in nuclear extracts (10 μg of protein) was assayed using a transcription factor ELISA for AP-1 (Panomics), according to the manufacturer’s instructions.

Targeted miRNA inhibition and overexpression

For inhibition of miR-155, -125b, and -146b, macrophages were transfected using lipofectamine siRNA max transfection agent with appropriate target antagonists or anti-miR-negative control 1 (anti-miR-control). For overexpression, macrophages were transfected with premiR-155, -125b, or premiR-negative control 1 (premiR-control). Transfection reagents, antagonists, and control miRNAs were purchased from Invitrogen. Knockdown efficiency and overexpression efficiency was assessed by determining mature miR-155 and -125b levels in transfected cells. Transfected cells were treated with or without 30 nM microRNA mimic or antagonist for 48 h and, where indicated, treated for indicated times with LTB4 (100 nM) or LPS (100 ng/ml) before collecting supernatants or preparing cell lysates for isolation of RNA (RNeasy Kit; Qiagen) or proteins.

Luciferase assays

The plasmids containing 3′UTR of murine MyD88 were purchased from Genecopeia, and those containing the 3′UTR of murine SOCS1 were provided by Christos Tsatsanis (University of Crete, Heraklion, Greece). Plasmids were packaged in the pEZX-MT01 Vector (for MyD88) or pMIR-REPORT-luciferase vector (for SOCS1; Ambion) as described previously (21). Constructs were transfected in Raw 264.7 cells in six-well plates using lipofectamine siRNA max (Invitrogen). Firefly luciferase reporter gene constructs (0.1 μg per well) were cotransfected together with 30 nM of microRNA (miR-155, miR-125b and miR-146b or scrambled-miR control), cells were lysed 24 h after transfection, and luciferase activity was measured. Each sample was assayed in triplicate.

RNA analysis

Quantitative RT-PCR analyses for miR-125b, miR-146a, miR-155, and RNU6 (used as normalization control) were performed using TaqMan miRNA assays with reagents, primers, and probes obtained from Qiagen. In brief, a stem loop primer was used for reverse transcription (30 min, 16°C; 30 min, 42°C; 5 min, 85°C) followed by quantitative PCR (qPCR) using TaqMan probes and primers in a Bio-Rad CFX96 Mastercycler. For assessing expression of SOCS-1, MyD88, b-actin, and the primary microRNAs (pri-miRs) miRNAs, cDNA was synthesized using a reverse transcription system (miScript II; Qiagen). qPCR was performed using the CFX96 Real-Time PCR Detection System (Bio-Rad Laboratories) as described previously (13). Primers and pri-miRs were purchased from Qiagen. Relative expression was calculated using the comparative threshold cycle (Ct) and expressed relative to control or WT (ΔΔCt method).

Focused miRNA arrays

To determine the expression of inflammatory microRNAs, WT and 5-LO<sup>−/−</sup> macrophages were treated with or without LTB4 for 24 h. RNA was then extracted from the cells using a Qiagen RNeasy mini kit according to the manufacturer’s instructions. cDNA was synthesized using the RT2 microRNA First Strand Kit (Qiagen) and applied to PCR-focused immunopathology microRNA array plates (Qiagen). Plates were processed in a BioRad CFX96 Connect Real-Time PCR System using automated baseline and threshold cycle detection.

Array normalization and statistical analysis

Normalization and statistical analysis of miRNA expression were conducted using the SABiosciences Online PCR Array Data Analysis Web Portal. miRNA expression was compared between WT and 5-LO<sup>−/−</sup> cells or between WT cells treated or not with LTB4. The ΔΔCt method was used to calculate fold change (FC). The endogenous control was derived from the average FC of four control genes in the PCR arrays, snorRNA251, snorRNA202, snorRNA142, and U6. The vehicle control group was used as the external control to normalize each sample. The formula

\[ FC = 2^{\Delta C_{\text{t untreated}} - \Delta C_{\text{t treated}}} \]

was used to calculate FC for up-regulated genes, and

\[ FC = -2^{\Delta C_{\text{t treated}} - \Delta C_{\text{t untreated}}} \]

was used to calculate FC for downregulated genes. The t tests were used to calculate significance of differences in microRNA expression between the control and the treatment groups for each miRNA at each time point; p < 0.01 was considered significant. miRNAs that displayed threshold cycles (Ct) > 35 were excluded from the analysis. Data were interpreted using the SABiosciences Web-based PCR array data analysis tool (http://pcrdatanalysis. sabiosciences.com/pcr/arrayanalysis.php).

Statistics

Data are presented as mean ± SEM. Comparisons among groups were assessed with ANOVA followed by Bonferroni analysis. A p value < 0.05 was considered significant.

Results

LTB4 controls the expression of proinflammatory microRNAs in macrophages

We have previously shown that both 5-LO- and BLT1-deficient macrophages manifested a selective decrease in expression of MyD88 mRNA and protein (13), but not of other adaptors, and this deficiency was reversed by LTB4 in 5-LO<sup>−/−</sup> but not in BLT1<sup>−/−</sup> cells. Decreased MyD88 was a consequence of higher SOCS-1 expression in LTB4- and BLT1-deficient animals (13). The increased SOCS-1 mRNA expression in 5-LO<sup>−/−</sup> macrophages was associated with an increase in its mRNA stability, whereas exogenous LTB4 enhanced degradation of its mRNA (13). In this
study, we sought to determine the molecular mechanism responsible for LTB4-mediated degradation of SOCS-1 miRNA. We specifically addressed the capacity of 5-LO metabolites to enhance the expression of microRNAs known to be involved in control of expression of both SOCS-1 and of TLRs and/or their adaptors. We performed a focused microRNA qPCR array (Immunopathogenesis kit; Qiagen) in WT and 5-LO−/− macrophages and found that of 88 microRNAs studied, 22 were downregulated at least 2.5-fold in LT-deficient cells, including the following microRNAs that amplify TLRs: Let-7g, miR-155, miR-125b, miR-19a, miR-146a, and miR-146b. Eleven microRNAs were upregulated at least 2.5-fold in 5-LO−/− macrophages, and 54 microRNAs were not changed (Fig. 1A). To confirm these changes identified in the array, we analyzed the expression of individual microRNAs by qPCR (Fig. 1B). Compared with WT cells, 5-LO−/− macrophages exhibited lower basal expression of miR-155, miR-125b, and miR-146ab, but unchanged expression of Let-7g.

Because the 5-LO enzyme generates a variety of metabolic products, including both LTB4 and cysteinyl LTs (cysLTs), we investigated which class of LTs was involved in the regulation of expression of specific microRNAs. We used pharmacologic means to inhibit 5-LO activation and to selectively antagonize BLT1 and the cysteinyl LT receptor 1 (cysLT1), and treated cells with these agents for 24 h prior to microRNA determination. 5-LO inhibition markedly and significantly decreased the expression of miR-146b and miR-155, and modestly but significantly decreased that of miR-19a and miR-125b (Fig. 1C). As was also true for 5-LO−/− cells, no differences were seen in Let-7g expression. BLT1 antagonism followed the same pattern as observed in 5-LO−/−-inhibited cells (Fig. 1C). The cysLT1 antagonist MK571 was less effective than BLT1 in decreasing expression of all of the microRNAs studied with the exception of miR125b. Because endogenous LTB4 appeared to exhibit a greater effect on microRNA expression than did endogenous cysLTs, we directly investigated the ability of LTB4 to modulate expression of inflammatory microRNAs known to target SOCS-1 as well as the resulting consequences regarding TLR activation.

PCR array analysis of WT macrophages treated with LTB4 for 24 h revealed enhanced expression of those microRNAs that were downregulated in 5-LO−/− cells, as well as of additional microRNAs involved in TLR responses (Fig. 2A). We validated the enhancement by LTB4 of the levels of miR-155 and miR-146b, but not of Let-7g, in both WT and 5-LO−/− macrophages (Fig. 2B). We also assessed the expression of inflammatory microRNAs in macrophages from BLT1−/− mice, and we found markedly lower expression of miR-155, miR-146b, and miR-125b than in WT mice (Fig. 2C). Because BLT1 can be coupled to both Goi and Goq in macrophages (8), we sought to determine which G protein accounted for BLT1 effects on microRNA expression. Basal expression of all the microRNAs studied except Let-7g was diminished by treatment with the Goi inhibitor pertussis toxin (PTX; Fig. 2D). These data show that Goi signaling controls basal expression of microRNAs. To determine whether Goi actions were also involved in the enhancing effects of LTB4/BLT1 signaling, cells were pretreated with PTX for 18 h, followed by treatment with LTB4 for an additional 24 h. PTX treatment substantially decreased LTB4-induced expression of miR-155 and miR-146b, but only partially attenuated LTB4-induced expression of miR-125b, suggesting a contribution of Goq signaling in BLT1-mediated expression of this microRNA (Fig. 2D).

AP-1 mediates LTB4-induced miR-155 expression

Expression of ~25% of the microRNAs was lower in LT-deficient cells than in WT cells; therefore, we speculated that LTs could be involved in the expression or activation of enzymes responsible for the generation of microRNAs, such as Dicer and Drosha (26). We observed an insignificant decrease in Dicer miRNA expression; LTB4 enhanced the expression of Dicer in 5-LO−/− cells, and no changes in Drosha levels were detected in cells of either genotype, treated or not with LTB4 (Fig. 3A).

MicroRNAs are initially expressed as long primary transcripts termed “primary microRNAs” (pri-miRNAs) that undergo sequential processing by Drosha and Dicer (26). In the absence of any change in the expression of enzymes involved in microRNA biosynthesis, we speculated that LTB4 could be involved in the generation of pri-miRNAs. Therefore, we measured the amounts of pri-miR-155, pri-miR-146b, pri-miR-125b, and pri-let-7g using qPCR. Indeed, expression of all pri-miRNAs other than pri-Let-7g was lower in both 5-LO−/− and BLT1−/− than in WT macrophages (Fig. 3B). Lower expression of these pri-miRNAs suggested a role for an LT in the control of the transcriptional machinery involved in microRNA expression. AP-1 is a key transcription factor involved in the expression of different primary microRNAs including miR-155, miR-125b, miR-146a, and miR-146b (27, 28). We tested the ability of LTB4 to enhance the activation of AP-1 and the subsequent expression of miR-155. Initially, we performed AP-1 DNA binding assays in WT and 5-LO−/− macrophages incubated with or without LTB4 for 4 h. LT-deficient cells exhibited lower basal AP-1 activation than WT macrophages did, and LTB4 greatly enhanced AP-1 DNA binding activity in 5-LO−/− cells (Fig. 3C).

FIGURE 1. Role of 5-LO metabolites in the expression of proinflammatory microRNAs in macrophages. (A) Thioglycollate-elicited macrophages from WT and 5-LO−/− mice were harvested, and total RNA was isolated. MicroRNAs were isolated and subjected to miScript cDNA synthesis and to real-time PCR array as described in Materials and Methods. Data are representative of five individual experiments. (B) Detection of individual microRNAs in WT and 5-LO−/− macrophages. Detection of miR-155, Let-7g, and miR-146b was performed as described in Materials and Methods. (C) WT macrophages treated with the 5-LO inhibitor AA-861, the BLT1 antagonist U7532 and the cysLT1 antagonist MK571 (all at 1 μM) for 24 h, followed by determination of the indicated microRNAs. Data are mean ± SEM from three individual experiments, each performed in triplicate. *p < 0.05 versus untreated WT.
RNAs were isolated from WT and BLT1 three to five independent experiments performed in triplicate. Detection of miR-155, Let-7g, and miR-146b was performed as described in Materials and Methods. Data represent three to five independent experiments performed in triplicate. MicroRNAs were isolated from WT and BLT1−/− macrophages stimulated with 100 nM LTB4 for 24 h and in unstimulated WT controls. Detection of miR-155, Let-7g, and miR-146b was performed as described in Materials and Methods. Data represent three to five independent experiments performed in triplicate. (C) MicroRNAs were isolated from WT and BLT1−/− macrophages, and the expression of Let-7g, miR-155, miR-146b, miR-19a, and miR-125b was determined by real time RT-PCR. Dashed line shows the level of microRNAs detected in WT macrophages normalized to 100%. Data represent three to five independent experiments performed in triplicate. (D) WT macrophages were pretreated for 24 h with or without the Gsi inhibitor PTX (600 ng/ml), followed by 30 min of treatment with or without LTB4. The expression of the indicated microRNAs was detected in (B). In (B–D), data are mean ± SEM from three individual experiments, each performed in triplicate. *p < 0.05 versus untreated WT, †p < 0.05 versus WT control or LTB4 alone.

To determine whether AP-1 is involved in LTB4-induced miR-155 expression, we pretreated WT macrophages with the AP-1 inhibitor SR11302, followed by LTB4 challenge for 24 h. AP-1 inhibition decreased basal miR-155 levels and attenuated LTB4-enhanced miR-155 expression (Fig. 3D). That the AP-1 inhibitor prevents AP-1 DNA binding activity was confirmed in LTB4-treated cells (Fig. 3C). Together, these findings show that LTB4-mediated AP-1 activation accounts for the expression of inflammatory microRNAs, which control expression of SOCS-1 and activation of TLR (Fig. 3D).

**LTB4-mediated miR-155 and miR-146b expression accounts for increased SOCS-1 mRNA turnover, MyD88 expression, and TLR4 responsiveness**

We previously reported that SOCS-1 acts as a brake on expression of MyD88 by inhibiting the MyD88 transcription factor STAT-1 (13). SOCS-1 gene silencing allows STAT-1 to be active, and it enhances MyD88 expression and improves TLR responsiveness in macrophages (13). We next sought to determine whether LTB4-enhanced microRNA expression leads to changes in SOCS-1 and MyD88 levels, and therefore macrophage function. We transfected antagonirs known to directly inhibit expression of SOCS-1 (anti–miR-155 and anti–miR-146b) and MyD88 (anti–miR-125b) into macrophages stimulated or not with LTB4 for 24 h. Our data show that miR-155 inhibition decreased basal MyD88 expression and prevented LTB4-enhanced MyD88 expression. By contrast, inhibition of miR-125b and miR-146b enhanced MyD88 above baseline levels, but did not alter levels in the presence of LTB4. Furthermore, miR-125b increased MyD88 to a greater extent than did miR-146b (Fig. 4A). As expected (13), LTB4 decreased basal SOCS-1 expression, and miR-155 antagonism increased both basal and LTB4-induced SOCS-1 expression (Fig. 4B). However, miR-125b did not influence SOCS-1 levels, indicating that it instead exerted a direct effect on MyD88 mRNA expression (Fig. 4B). The miR-146b antagonist enhanced basal SOCS-1 levels and prevented LTB4 inhibitory effects on SOCS-1 expression (Fig. 4B). These findings were confirmed by immunoblot (Fig. 4D). 5-LO−/− cells exhibited lower microRNA expression; therefore, we performed complementary “add-back” experiments, in which miR-155, miR-146b, and miR-125b precursors (miRNA mimic) were transfected into both WT and 5-LO-deficient macrophages, and the expression of both MyD88 and SOCS-1 were determined with qPCR. MyD88 expression was slightly enhanced in WT cells transfected with miR-155 mimic, and the levels of MyD88 expression were elevated...
in 5-LO−/− cells transfected with miR-155 mimic. MyD88 expression was increased by miR-146b mimic and decreased by miR-125b mimic in both strains studied, again showing that these microRNAs control MyD88 expression (Fig. 4E). We observed higher
was also investigated in LTB4-stimulated cells. We observed that miR-146b and miR-155 directly target SOCS-1. The temporal relationship between microRNAs, SOCS-1 and MyD88 expression was also investigated in LTB4-stimulated cells. We observed that LTB4 rapidly enhanced miR-155 expression within 1 h, whereas the decrease in SOCS-1 expression required 4 h. We observed only significant differences in LTB4-induced MyD88 expression after 24 h, further suggesting that decreased SOCS-1 accounts for the enhanced MyD88. Our data show that LTB4 enhances MyD88 expression by increasing the expression of microRNAs that directly target SOCS-1.

We next sought to determine whether changes in the expression of MyD88 and SOCS-1 influenced TLR signaling and whether increased microRNA expression restored 5-LO-dependent macrophage responsiveness to LPS, which we have previously reported to be impaired (13). WT and 5-LO−/− macrophages were transfected with mimics of miR-155, miR-125b, or miR-146b for 24 h, followed by LPS stimulation for another 24 h. As expected, 5-LO−/− macrophages generated less TNF-α and nitrite than WT cells did in response to LPS. Transfection of both miR-155 and miR-146b mimics enhanced LPS-induced TNF-α (Fig. 5A) and NO (Fig. 5B) production in WT and especially 5-LO−/− cells. In contrast, miR-125b mimic inhibited LPS-enhanced TNF-α levels exclusively in WT macrophages, which is consistent with the findings in Fig. 4B.

**Discussion**

This study highlights a novel regulatory mechanism of microRNA expression induced after GPCR activation. We have previously shown that BLT1 activation inhibits SOCS-1 expression, leading to enhanced MyD88 expression and consequently increased TLR activation. In this study, we extended these findings by investigating the involvement of BLT1 in microRNA generation that influences the molecular programs involved in SOCS-1/MyD88 expression. To our knowledge, this is the first demonstration of an essential role for a GPCR in microRNA generation. More specifically, we found that: 1) LTB4/BLT1/Goi activation enhances the expression of inflammatory microRNAs, 2) LTB4/BLT1 activation of AP-1 accounts for the enhanced microRNA generation in macrophages, 3) preventing miR-155 and miR-146b actions abolished LTB4-induced SOCS-1 mRNA degradation, 4) adding back miR-155 and miR-146b restored MyD88 levels and responsiveness in LT-deficient mice, and 5) miR-125b degrades MyD88 in a manner dependent on LTB4 synthesis.

TLR activation is dictated by fine-tuning events that culminate in important changes in inflammatory response and host defense (20). Among the “fine-tuners”, microRNAs are known to inhibit the expression of many key components of the TLR signaling program, including MyD88 (29, 30), IRAKs (31–33), and TRAF-6 (33–35). MicroRNAs can be produced constitutively or can be upregulated by a variety of inflammatory stimuli, including LPS (21, 36–38), poly-IC (37, 38), and TNF-α (36). However, whether GPCRs also participate in the generation of microRNAs is unknown. We have shown that the other major class of 5-LO metabolite, the CysLTs, did not exert effects on most microRNAs, excluding miR-125b, emphasizing the specific and nonredundant role of LTβR in regulating microRNAs that control SOCS-1 expression in macrophages. Also striking was the fact that the regulatory influence on microRNA expression was observed not only following the addition of exogenous LTβR, but also under basal conditions, in which levels of constitutive endogenous LTβR generation would be expected to be low. Basal release of LTβR by elicited peritoneal macrophages from WT animals over 90 min was ~100 pg/ml, which is equivalent to 0.5 nM (13). This concentration is indeed sufficient to activate BLT1, because we have shown that LTβR amplifies alveolar macrophage antimicrobial functions at concentrations as low as 0.01 nM (8). These findings support the conclusion that LTβR is a homeostatic regulator of microRNA expression that controls numerous aspects of macrophage biology.

We also identified Goi-independent microRNA expression, such as that of miR-125b and miR19a (not shown). The relative role of other G proteins, such as Gq and Gαs, in modulating expression of microRNAs that regulate macrophage biology is an active area of research in our laboratory.

The fact that LT deficiency decreased the expression of a myriad of microRNAs suggested the possibility that LTs enhance the expression or activity of the enzymes involved in microRNA biogenesis, such as Dicer and Drosha (20, 39). Although we did not observe any effect of LTs on the expression of these enzymes, we cannot exclude possible actions of LTβR on Dicer or Drosha activation. It remains to be determined whether GPCRs also influence the expression or activation of enzymes involved in the microRNA machinery. A variety of transcription factors have been implicated in microRNA generation, such as NF-κB (40), Egr (41, 42), and AP-1 (27, 43–46). We further investigated whether LTβR/BLT1 could enhance the generation of microRNAs by controlling their expression. This study provides evidence for a novel regulatory mechanism of microRNA expression, which could be exploited for therapeutic purposes.
activation of AP-1, because this transcription factor is responsible for the expression of miR-155 and miR-146b (27, 28). AP-1 regulates gene expression in cells activated by a myriad of stimuli, including proinflammatory cytokines, TLR ligands, growth factors, and stress (45). Upon cell activation, regulation of gene expression by AP-1 is dependent mainly on the activation of MAP kinases, including ERK1/2 and p38 (45). We and others have shown that LTβ2 activates ERK1/2 and p38 in macrophages (47, 48); however, whether LTβ2 enhances AP-1 activation is controversial. Stanková et al. (49) have shown that LTβ4 enhances expression and DNA binding activity of the AP-1 member c-fos, whereas Brach et al. (50) showed that AP-1 is not affected by LTβ4 in stimulated human monocytes. In this study, we showed that LTβ4 activates AP-1 to enhance miR-155 expression. AP-1 binding to the BIC promoter of these microRNAs has been reported previously in both human and mouse cells (27, 44, 51, 52). Furthermore, McCoy et al. (53) have shown that Ets3 activation inhibits miR-155 expression. Whether LTβ4 enhances miR-155 expression by activating transcription factors other than AP-1, such as NF-kB, or by inhibiting Ets3 actions, remains to be determined.

We further investigated whether changes in microRNA levels account for changes in the expression of SOCS-1 and MyD88 in macrophages from LT-deficient mice, and we found that miR-155 and miR-146b decrease SOCS-1 levels and inhibit MyD88 only. We recognize that these microRNAs could be modulating the expression of SOCS-1 and MyD88 indirectly. Therefore, we used different complementary approaches such as antagonim treatment of LTβ2-treated cells and 3′-UTR luciferase assay to confirm our findings further. Our findings confirm that miR-155 inhibits SOCS-1 expression and newly identify that miR-125b targets MyD88 and miR-146b targets SOCS-1. A caveat of our studies is that we cannot exclude a potential indirect effect of the microRNA mimics in the degradation of SOCS-1 and MyD88 3′UTR constructs in RAW 264.7 macrophages, because we did not transfect macrophages with the mutants lacking the 3′UTR microRNA binding sites. The temporal relationship in the expression of these molecules is consistent with a causal role for miR-155 in the degradation of SOCS-1 and MyD88, as the increase in miR-155 expression (noted 1 h after LTβ4 treatment) preceded the decrease in SOCS-1 levels (noted after 4 h), which in turn preceded the increase in MyD88 levels (noted after 24 h). Although we did not measure the temporal expression of the other microRNAs, the microRNA mimic and antagonist data supporting the roles of miR-146b in control of SOCS-1 and of miR-125b in the control of MyD88 strongly imply that these microRNAs too would be expected to precede changes in levels of their target genes. Furthermore, we cannot definitively exclude the possibility that the microRNAs studied here might target other genes that also influence TLR activation. The fact that miR-125b does not further inhibit MyD88 expression and TNF-α levels in 5-LO−/− cells is intriguing, and the molecular programs involved in these events will be studied in the future. Another intriguing fact is that miR-125b suppresses LTβ2-induced SOCS1 mRNA without affecting its UTR. We speculate that the effect of miR-125b on SOCS-1 is indirect, by targeting either transcription factors involved in SOCS-1 mRNA or by changing the levels of other microRNAs that further degrade SOCS-1 in LTβ2-treated cells.

In summary, our findings show that LTβ4, via its cognate receptor BLT1 and G protein activation, enhances the expression of microRNAs known to degrade SOCS-1 levels and thereby enhances MyD88 protein expression and TLR responsiveness. We also showed for the first time that miR-125b targets MyD88 directly. These data demonstrate that GPCRs amplify innate immune and inflammatory responses by modulating the expression of important microRNAs that further control macrophage activation. It will be of interest to dissect the interplay between GPCR signaling and microRNAs in vivo host defense and inflammatory responses. Our findings have direct translational importance, as unveiling the LTβ2/BLT1/microRNA axis to control TIR-dependent macrophage activation would be expected to attenuate excessive NF-kB activation, which contributes to tissue injury (4, 6), or unchecked IL-1βR activation in autoinflammatory conditions (54). Moreover, states of immunosuppression might be overcome by the administration of exogenous LTβ4 plus microRNA mimics, such as miR-155 and miR-146b.

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Disclosures
The authors have no financial conflicts of interest.

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