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Macrophage Dectin-1 Expression Is Controlled by Leukotriene B4 via a GM-CSF/PU.1 Axis

C. Henrique Serezani,* Steve Kane,* Latima Collins,* Mariana Morato-Marques, † John J. Osterholzer,*,‡ and Marc Peters-Golden*

Pattern recognition receptors for fungi include dectin-1 and mannose receptor, and these mediate phagocytosis, as well as production of cytokines, reactive oxygen species, and the lipid mediator leukotriene B4 (LTB4). The influence of G protein-coupled receptor ligands such as LTB4 on fungal pattern recognition receptor expression is unknown. In this study, we investigated the role of LTB4 signaling in dectin-1 expression and responsiveness in macrophages. Genetic and pharmacologic approaches showed that LTB4 production and signaling through its high-affinity G protein-coupled receptor leukotriene B4 receptor 1 (BLT1) direct dectin-1–dependent binding, ingestion, and cytokine production both in vitro and in vivo. Impaired responses to fungal glucans correlated with lower dectin-1 expression in macrophages from leukotriene (LT)- and BLT1-deficient mice than their wild-type counterparts. LTB4 increased the expression of the transcription factor responsible for dectin-1 expression, PU.1, and PU.1 that is required for fungi-protective host responses. In addition, LTB4 effects on dectin-1, PU.1, and cytokine production were blunted in GM-CSF–deficient macrophages. Our results identify LTB4-BLT1 signaling as an unrecognized controller of dectin-1 transcription via GM-CSF and PU.1 that is required for fungi-protective host responses.

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Abbreviations used in this article: AM, alveolar macrophage; BAL, bronchoalveolar lavage; BLT1, leukotriene B4 receptor 1; 5-LO, 5-lipoxygenase; LT, leukotriene; LTB4, leukotriene B4; PKC, protein kinase C; PRR, pattern recognition receptor; PTX, pertussis toxin; sRNA, small interfering RNA; Syk, spleen tyrosine kinase; WT, wild-type.
dectin-1 agonist curdlan from Alcaligenes faealis and zymosan depleted of TLR agonists (by treatment with chloroform/methanol) (14) were from Invivogen. U75302 (BLT1 antagonist) was from Cayman Chemicals. CsA and CXCL1 were from R&D. Compounds requiring reconstitution were dissolved in either ethanol or DMSO. Required dilutions of all compounds were prepared immediately before use, and equivalent quantities of vehicle were added to the appropriate controls.

**Animals**

Eight-week-old female 5-LO−/− mice (15) were bred in-house, and strain-matched wild-type (WT) sv/129 mice were purchased from The Jackson Laboratory. GM-CSF−/− mice (16) were originally a gift from J. Whitsett (Children’s Hospital, Cincinnati, OH) and were bred in-house; BLT1−/− mice (17) and strain-matched WT C57BL/6 mice were obtained from The Jackson Laboratory.

**Ethics statement**

Mice were treated according to National Institutes of Health guidelines for the use of experimental animals, with the approval of the University of Michigan Committee for the Use and Care of Animals. All surgery was performed under sodium pentobarbital anesthesia, and all efforts were made by the attending veterinarian to minimize suffering.

**Cell isolation and culture**

Elicited peritoneal macrophages were harvested from the peritoneal cavities of mice by lavage with PBS 4 d after the injection of 2 ml of 3% thiglycollate, as described previously (18). Resident murine alveolar macrophages (AMs) were obtained by bronchoalveolar lavage (BAL) as described previously (18). Cells were cultured overnight in RPMI 1640 containing 10% FBS and antibiotics, and washed twice the next day with warm medium to remove nonadherent cells.

**C. albicans culture**

*C. albicans* strain CHN1 (a human pulmonary clinical isolate) was grown on Sabouraud dextrose agar plates and maintained at 4°C. Seventy-two hours before the experiment, yeast were grown to stationary phase at 37°C in Sabouraud dextrose broth (Difco; 1% neopeptone, 2% dextrose) with shaking. The cultures were washed in sterile nonpyrogenic PBS, counted with a hemocytometer, and diluted to 2 × 10^7 CFU/ml in sterile nonpyrogenic PBS. *C. albicans* was killed through heating for 30 min at 56°C and FITC labeled as described previously (8).

**In vitro binding assay**

In vitro *C. albicans* binding assays were performed as previously described (19). In brief, overnight cultures of macrophages were cooled to 4°C and washed three times with prechilled serum-containing medium. FITC-*C. albicans* was added to the macrophages at a ratio of 10 particles/cell for 1 h on ice, and cells were washed three times to remove unbound FITC-yeast and then lysed with 3% Triton X-100. FITC-*C. albicans* in lysates was quantified using a Spectranax Gemini EM fluorometer (Molecular Devices) at settings of 485 excitation/535 emission.

**In vivo injection with curdlan**

Curdlan (100 μg/kg) was reconstituted in PBS with 1% BSA and administered to the lungs of mice via oropharyngeal injection as described previously (20). BAL was performed by three successive instillations of 1 ml PBS, followed by gentle suction. BAL fluid from WT and 5-LO−/− mice was harvested after 24 h, and levels of LTB4, cytokines, and chemokines were measured by ELISA or by Ab-based cytokine array. The pelleted cells were subjected to cytospin, and cell counts and differentials for evaluation of neutrophil recruitment were determined by light microscopy.

**Semi quantitative cytokine array**

WT and 5-LO−/− mice underwent intrapulmonary challenge with curdlan as described earlier and the BAL fluid was harvested 24 h later. Protein content was quantified by Bradford assay, and 50 μg protein was used for qualitative measurement of cytokine expression using the Mouse Cytokine Ab Array, Panel A (CAS−/− mice) was recommended by the manufacturer (R&D Systems, Wiesbaden, Germany).

**Measurement of LTB4**

Levels of LTB4 in the BAL fluid obtained from WT mice 24 h after intrapulmonary challenge with curdlan were determined using enzyme immunoassay kits (Cayman Chemical) as described previously (8).

**Measurement of cytokine and chemokine levels**

Levels of IL-12p40, IL-17A, GM-CSF, M-CSF, KC, IL-1β, and TNF-α were determined by ELISA (R&D Duoset; R&D Systems) by the University of Michigan Cancer Center Cellular Immunology Core.

**Flow cytometry**

For flow cytometric analysis, cells were resuspended in PBS containing 2 nM EDTA and 0.5% FCS. Fc receptor-mediated and nonspecific Ab binding was blocked by addition of excess CD16/CD32 (BD Biosciences Pharmingen). For staining, macrophages were incubated with anti-dectin-1 conjugated to FITC (1:200; BD Biosciences Pharmingen) at 4°C in the dark for 15 min. Samples were stabilized with 1% paraformaldehyde and analyzed on the same day. A FACSCalibur flow cytometer (BD Biosciences) was used for flow cytometric characterization of cell populations, and data were analyzed with WinMDI and FlowJo Version 7.6.4 software (Tree Star).

**In vivo phagocytosis assay**

WT and 5-LO−/− mice were subjected to intrapulmonary administration of 1 μg/ml zymosan as described earlier for curdlan, and 24 h later, cells were harvested by BAL and subjected to cytospin and stained with Diff-Quick. The number of intracellular zymosan particles was determined microscopically. The phagocytic index was generated by counting the number of macrophages containing intracellular zymosan multiplied by the number of intracellular zymosan particles.

**RNA isolation and semiquantitative real-time RT-PCR**

RNA from cultured cells was isolated using the RNeasy Mini Kit (Qiagen) according to the manufacturer’s instructions, and real-time RT-PCR was performed as previously described (18). Dectin-1 (Clec7A), dectin-2 (Clec3f/0), GM-CSF (Gmcsf), or PU.1 (Spi1) mRNAs were normalized to β-actin and GAPDH, and the respective WT control was set at 100%. WT and 5-LO−/− macrophages were treated with or without 2.5 μg/ml antinomycin D (Sigma-Aldrich), and the amount of mRNA was determined after harvesting at different time points, to determine the decay of Clec7A mRNA. Clec7A mRNA was normalized to β-actin, and the respective WT control was set to 100%. Percentages were plotted against time, and decay curves were calculated.

**Western blotting**

A total of 2 × 10^6 macrophages were plated in 6-well tissue culture dishes and were incubated in the presence or absence of 100 nM LTB4 for 24 h and then lysed in buffer (50 mM Tris-HCl [pH 7.4], 25 mM KCl, 5 mM MgCl2, and 0.2% Nonidet P-40) supplemented with protease inhibitors (Roche Diagnostics). For immunoblot analysis, protein samples (30 μg) were mixed with loading buffer (50 mM Tris HCl [pH 6.8], 2% SDS, 100 mM DTT, 10% glycerol, and 0.1% bromphenol blue), boiled, applied to 10% SDS-polyacrylamide gels, and subjected to electrophoresis. Immunoblot analysis was performed as previously described (21), using primary Abs against dectin-1 (1:1,000; Biovision), Dectin-2 (Clec3f/0), GM-CSF (Gmcsf), or PU.1 (Spi1) mRNAs were normalized to β-actin and GAPDH, and the respective WT control was set to 100%. Percentages were plotted against time, and decay curves were calculated.

**RNA interference**

RNA interference was performed according to a protocol provided by Drmaracon and as we have previously reported (18). WT and 5-LO−/− macrophages were transfected using DharmaFECT 1 reagent with 30 nM nonspecific control or specific ON-TARGETSMARTpool Pu.1 small interfering RNAs (siRNAs). After 48 h of transfection, macrophages were incubated with or without 100 nM LTB4 for 24 h, and the cells were harvested for mRNA or protein analysis.

**Statistics**

All experiments were performed at least three times unless otherwise specified, and data are presented as the mean ± SE of the values from all experiments. Within each experiment, triplicate values were used for each condition. Comparisons among three or more experimental groups were performed with ANOVA followed by the Bonferroni analysis. Differences were considered significant if p < 0.05.
**Results**

**LTB4 regulates macrophage expression of dectin-1**

LTB4 can enhance PRR-mediated responses by regulating expression of MyD88 (18) and can also enhance expression of certain macrophage receptors, such as CD11b and CD11c (23). However, it is not known whether LTB4 can influence the expression of PRRs, including dectin-1. This possibility was first examined in elicited peritoneal macrophages from WT and 5-LO−/− mice, because this cell population is known to express high levels of dectin-1 (3). LT-deficient macrophages exhibited reduced baseline expression of dectin-1 mRNA, as determined by real-time RT-PCR (Fig. 1A), and protein, as determined by both FACS (Fig. 1B) and immunoblotting (Fig. 1C). Reduced dectin-1 mRNA was confirmed in WT cells treated for 24 h with a 5-LO inhibitor (Fig. 1D). Twenty-four-hour treatment with 100 ng/ml LTB4 fully rescued dectin-1 mRNA (Fig. 1A) and protein (Fig. 1B, 1C) expression in 5-LO−/− macrophages back to the levels observed in WT cells. By contrast with its effects on dectin-1, neither endogenously produced nor exogenously added LTB4 modulated expression of dectin-2 mRNA or protein (Fig. 1E and inset). Reduced expression of dectin-1 was also observed in AMs (Fig. 1F) and bone marrow-derived macrophages (not shown) from LT-deficient mice, and again, levels were significantly increased with 24 h treatment with LTB4. We next determined whether the decreased dectin-1 expression correlated with lower macrophage binding of *C. albicans*. AMs from 5-LO−/− mice bound substantially less *C. albicans* than did WT macrophages, but LTB4 treatment for 24 h restored yeast binding to levels exhibited by WT cells (Fig. 1G). The importance of dectin-1 in mediating yeast binding was confirmed by showing that treatment of WT cells with the dectin-1 receptor antagonist laminarin, but not the mannose receptor antagonist mannan, decreased *C. albicans* binding to levels approximating that observed in 5-LO−/− cells (Fig. 1H). The specific role of BLT1 in controlling dectin-1 expression was confirmed by demonstrating reduced baseline dectin-1 mRNA in elicited macrophages from BLT1−/− mice (Fig. 1I) and from WT mice treated overnight with a BLT1 antagonist. However, because of lack of BLT1, LTB4 was unable to restore deficient dectin-1 expression (Fig. 1I), in contrast with 5-LO−/− cells. Together, these results show that LTB4 enhances basal expression of dectin-1 mRNA and protein in various macrophage populations.

**FIGURE 1.** LTB4 is necessary for basal dectin-1 expression in macrophages. (A) WT and 5-LO−/− macrophages were treated ± 100 nM LTB4 for 24 h, and dectin-1 mRNA was determined by real-time RT-PCR. (B) WT- and 5-LO−/−-elicited peritoneal macrophages were probed with anti-dectin-1 Ab, and the cells were subjected to FACS analysis as described in Materials and Methods. Mean fluorescence intensity (MFI) is expressed as the mean ± SEM from three individual experiments. (C) Elicited macrophages were incubated ± LTB4 for 24 h, and the expression of dectin-1 and GAPDH was determined by immunoblot analysis. Numbers under lanes indicate the relative density of dectin-1, determined from densitometric analysis and expressed as the mean ± SEM from three individual experiments, with the values of the WT control group set as 100%. (D) WT macrophages were pretreated with the 5-LO inhibitor AA-861 (10 μM) or the BLT1 antagonist U7532 (1 μM) for 24 h, and dectin-1 mRNA levels were determined by real-time RT-PCR. (E) WT and 5-LO−/− macrophages were incubated ± LTB4 for 24 h, and the expression of dectin-2 was determined by real-time RT-PCR. (Inset) Dectin-2 protein abundance in WT and 5-LO−/− macrophages determined by immunoblotting. (F) AMs were incubated ± LTB4 for 24 h, and the expression of dectin-1 and GAPDH was determined by immunoblot analysis. Data are expressed and analyzed as in (C). (G) AMs from WT and 5-LO−/− mice were incubated ± 100 nM LTB4 for 24 h, and the binding capacity for 10:1 heat-killed FITC *C. albicans* was determined as described in Materials and Methods. (H) AMs from WT mice were pretreated with mannan or laminarin (both at 100 μg/ml) for 30 min before the addition of 10:1 heat-killed FITC *C. albicans*, and yeast binding capacity was determined as described in (G). (I) WT- and BLT1−/−-elicited peritoneal macrophages were incubated ± LTB4 for 24 h, and dectin-1 mRNA was determined by real-time RT-PCR. In all circumstances, data represent the mean ± SEM from three individual experiments, each performed in triplicate. *p < 0.05 versus WT control or untreated control, **p < 0.001 versus untreated 5-LO−/− macrophages by ANOVA.
LTB₄ regulates macrophage responses via dectin-1 in vitro and in vivo

Because LTB₄ and BLT1 signaling control dectin-1 expression, we reasoned that LTB₄ would also control host responses to dectin-1 engagement. This was tested both in vitro and in vivo. In the in vitro experiments, we used the dectin-1–selective agonist curdlan or zymosan, which is able to ligate dectin-1 but whose TLR ligands were removed by treatment with chloroform/methanol (14). Initially, we performed dose–response experiments in which elicited peritoneal macrophages from WT and 5-LO⁻/⁻ mice were stimulated with concentrations of curdlan or treated zymosan. TNF-α production increased in dose-dependent fashion and peaked at a dose of 100 μg/ml for both agonists. In all circumstances, 5-LO⁻/⁻ cells exhibited lower responsiveness to both agonists than WT macrophages (Supplemental Fig. 1A, 1B). To determine whether LTB₄ is the 5-LO product involved in dectin-1 responsiveness, 5-LO⁻/⁻ cells were pretreated in the presence or absence of LTB₄ for 24 h and stimulated with curdlan for another 24 h. Curdlan stimulation induced IL-12p40, TNF-α, and GM-CSF in WT macrophages, as expected, but the response to curdlan in 5-LO⁻/⁻ cells was significantly reduced (Fig. 2A–C). Pharmacologic inhibition of 5-LO by 24-h pretreatment with AA-861 likewise resulted in attenuation of curdlan-induced cytokine generation in WT macrophages (Fig. 2D–F). The specific role of endogenous LTB₄ in regulating responses to curdlan was evidenced by the facts that overnight LTB₄ pretreatment restored the ability of curdlan to induce IL-12p40, GM-CSF, and TNF-α levels in 5-LO⁻/⁻ macrophages (Fig. 2A–C), and that overnight pretreatment with the selective BLT1 antagonist U7532 prevented curdlan-induced cytokine generation to the same extent as did pharmacologic inhibition of 5-LO (Fig. 2D–F). Because curdlan preparations could be contaminated with endotoxins, we pretreated WT macrophages with the LPS inhibitor polymyxin B sulfate (10 μg/ml) before curdlan stimulation. Polymyxin B did not alter curdlan-induced TNF-α production (data not shown), which excludes a possible role for contaminating endotoxin in our curdlan preparations. Also, the primary role of dectin-1 in mediating curdlan effects was demonstrated by showing that the dectin-1–selective antagonist laminarin, which blocks dectin-1, but not complement receptor 3 and mannose receptor (19, 24), impaired curdlan-induced TNF-α secretion by ∼70% (Supplemental Fig. 2).

The dectin-1–dependent production of proinflammatory mediators in the lung in vivo was determined by oropharyngeal injection of curdlan in WT and 5-LO⁻/⁻ mice. Twenty-four hours after curdlan injection, high levels of LTB₄ were measured in BAL fluid of WT mice (Fig. 3A), verifying that generation of this lipid mediator is a component of the host response to dectin-1 ligation. Next, we determined the pattern of cytokine/chemokine secretion in the BAL fluid of WT and 5-LO⁻/⁻ mice using an Ab-based array. 5-LO⁻/⁻ mice were globally less responsive to curdlan than were WT mice (Fig. 3B). This finding was confirmed by ELISA determination of individual mediators in BAL fluid. Levels of TNF-α, KC, and M-CSF were decreased by at least 70% in fluid from 5-LO⁻/⁻ mice, whereas levels of IL-12p40, IL-1β, IL-17A, and IL-23 were decreased by ∼30–50% (Fig. 3C–I). The recruitment of neutrophils to the lung of 5-LO⁻/⁻ mice in response to curdlan challenge was also lower than in WT animals (Fig. 3J). After intrapulmonary challenge with zymosan, LT-deficient mice also manifested significantly lower in vivo ingestion of the yeast particles by macrophages (Fig. 3K), but not by neutrophils (data not shown). These findings indicate that LTB₄ produced in response to dectin-1 engagement amplifies macrophage phagocytosis, cytokine secretion, and neutrophil recruitment.

**FIGURE 2.** LTB₄ is necessary for dectin-1 responses in macrophages. (A–C) WT and 5-LO⁻/⁻ macrophages were pretreated ± LTB₄ for 24 h, then incubated with the dectin-1 selective agonist curdlan (100 μg/ml) for another 24 h before determination of IL-12 p40 (A), TNF-α (B), and GM-CSF (C) levels by ELISA. (D–F) WT macrophages were incubated with the 5-LO inhibitor AA-861 (10 μM) or the BLT1 antagonist U7532 (1 μM) for 24 h, followed by curdlan stimulation for 24 h, and IL-12 p40 (D), TNF-α (E), and GM-CSF (F) levels were determined by ELISA. Data represent the mean ± SEM from three individual experiments, each performed in triplicate. *p < 0.05 versus WT control or untreated control, #p < 0.01 versus untreated 5-LO⁻/⁻ macrophages or untreated WT and stimulated with curdlan by ANOVA.

**LTB₄ enhances dectin-1 expression by a transcriptional mechanism involving PU.1**

Because LTB₄ can modulate the mRNA turnover rate of SOCS-1 in macrophages (18), we considered the possibility that it may increase dectin-1 mRNA expression by enhancing message stability. This was examined by comparing its decay in WT and 5-LO⁻/⁻ macrophages. At various time points after addition of actinomycin D to block the formation of new transcripts, cells were processed for real-time RT-PCR analysis. No difference in mRNA stability was observed between 5-LO⁻/⁻ and WT macrophages (Fig. 4A), which suggests instead that reduced dectin-1 mRNA in 5-LO⁻/⁻ cells reflects a transcriptional defect.
Transcription factors for dectin-1 include PU.1 (25), Sp1 (25), and peroxisome proliferator-activated receptor-γ (4). We examined the effects of LT deficiency and exogenous LTB₄ on levels of these transcription factors. Elicited peritoneal (Fig. 4B) and resident alveolar (Fig. 4C) macrophages from 5-LO²/² mice both exhibited less PU.1 than did cells from WT mice. Overnight treatment of 5-LO²/² cells with LTB₄ largely restored PU.1 protein expression to WT levels in both populations of macrophages (Fig. 4B, 4C) and drove PU.1 mRNA in elicited peritoneal macrophages to levels that far exceeded WT (Fig. 4D). By contrast, neither Sp1 protein (Fig. 4B) nor mRNA (data not shown) levels were reduced compared with WT cells. Likewise, no reduction in peroxisome proliferator-activated receptor-γ expression was observed in 5-LO²/² macrophages (data not shown). To investigate the importance of PU.1 for BLT1-mediated dectin-1 expression, we used siRNA to knock down this transcription factor in elicited WT macrophages. We achieved ∼75% knockdown of PU.1 mRNA (Fig. 4E) and ∼55% knockdown of protein (Fig. 4G), when compared with control siRNA. PU.1 silencing decreased dectin-1 mRNA (Fig. 4F) and protein (Fig. 4G) expression by ∼55%. In addition, PU.1 siRNA abolished LTB₄ enhancement of dectin-1 expression (Fig. 4F, 4G). These data show that LTB₄ enhancement of dectin-1 involves upregulated expression of its transcription factor, PU.1.
BLT1/Gαi plays a nonredundant role in enhancing dectin-1 and PU.1 expression

Although BLT1 can couple to both Gαi and Gαq in macrophages (26), numerous activation responses in macrophages preferentially involve Gαi signaling (18). To determine the importance of Gαi in LTβ-/-BLT1 control of dectin-1 expression, we tested the ability of pretreatment with the Gαi inhibitor PTX to interfere with basal and LTβ-/-enhanced dectin-1 mRNA. PTX treatment for 24 h decreased basal dectin-1 expression and also prevented the enhancement in dectin-1 expression elicited by LTB4 (Fig. 5A), suggesting that constitutive Gαi signaling is required for dectin-1 expression and that LTB4/BLT1 signaling requires Gαi.

Because other Gαi-coupled receptors besides BLT1 are expressed and promote activation responses in macrophages, we tested whether other selected ligands could also enhance dectin-1 expression. Neither C5a nor CXCL1 was capable of increasing dectin-1 mRNA expression (Fig. 5B), suggesting a nonredundant role for BLT1/Gαi signaling in controlling the expression of this PRR. The importance of Gαi signaling in controlling PU.1 expression was also studied. PTX treatment of elicited macrophages revealed that Gαi signaling is necessary for baseline PU.1 expression and for LTβ4/BLT1 enhancement of PU.1 expression (Fig. 5C), as it was for dectin-1 expression (Fig. 5A).

GM-CSF is a critical mediator of LTβ4-enhanced PU.1 and dectin-1 expression

GM-CSF upregulates PU.1 (27) and dectin-1 (3) expression in macrophages. However, it is unknown whether endogenously produced GM-CSF is also required for dectin-1 expression. It is also unknown whether GM-CSF participates in the LTβ4 amplification of dectin-1 and PU.1. To evaluate this possibility, we initially determined the levels of GM-CSF in LTβ4-/- and WT-elicited peritoneal macrophage cultures. Expression of both mRNA (Fig. 6A) and protein (Fig. 6B) was decreased in LTβ4-/- cells when compared with WT macrophages. Overnight treatment of LTβ4-/- cells with LTB4 restored GM-CSF mRNA and protein levels beyond the WT range (Fig. 6A, 6B). To determine whether the lower GM-CSF expression in LTβ4-/- cells was responsible for their decreased PU.1 and dectin-1 expression, we pretreated LT-deficient cells with GM-CSF for 24 h and determined dectin-1 and PU.1 mRNA expression. GM-CSF treatment (10 ng/ml) significantly augmented baseline expression of both dectin-1 (Fig. 6C) and PU.1 (Fig. 6D) in WT cells and also overcame their deficient expression in 5-LO-/- macrophages. As expected, the restored dectin-1 expression achieved by GM-CSF treatment also rescued responsiveness to curdlan in LT-deficient cells, as shown by the production of IL-12p40 (Fig. 6E) and TNF-α (Fig. 6F).
To determine whether endogenous GM-CSF is required for PU.1 and dectin-1 expression, we measured the expression of these mRNAs in elicited macrophages from GM-CSF−/− and WT mice by real-time RT-PCR. As expected (28), PU.1 (Fig. 6G) expression was lower in GM-CSF−/− than WT macrophages. Accordingly, dectin-1 (Fig. 6H) expression was also markedly lower in GM-CSF−/− than WT cells. Importantly, overnight treatment with LTB4 was unable to enhance either PU.1 (Fig. 6G) or dectin-1 (Fig. 6H) expression in GM-CSF−/− macrophages, as it was in WT cells. As predicted on the basis of decreased dectin-1 expression, GM-CSF−/− macrophages exhibited lower cytokine generation in response to curdlan than did WT macrophages (Fig. 6I, 6J). Moreover, overnight LTB4 treatment was unable to potentiate curdlan-induced cytokine production in GM-CSF−/− cells as it was in WT cells (Fig. 6I, 6J). These results indicate that LTB4/BLT1 regulation of dectin-1 expression and responsiveness in macrophages depends on an autocrine loop involving GM-CSF potentiation of PU.1 (Fig. 7).

**Discussion**

We provide evidence in this article that the GPCR BLT1 is a central determinant of dectin-1 expression and of host responses to fungi recognized by this PRR, and perhaps others that recognize the β-glucan moiety. Our findings also reveal a previously unrecognized interplay between the cytokine GM-CSF and LTB4 that mediates this effect. More specifically, we have shown that: 1) homeostatic LTB4 production is required for dectin-1 responsiveness in vivo and in vitro; 2) LTB4/BLT1/Gaia signaling is necessary for basal dectin-1 expression; 3) LTB4 enhances the expression of the transcription factor PU.1, which, in turn, controls dectin-1 expression; and 4) GM-CSF is a key mediator of LTB4-induced PU.1 and dectin-1 expression in macrophages.
scheme illustrating the relevant events is depicted in Fig. 7. Because LTB4 production is a component of the host response to fungal infections (8) and because dectin-1 is a major recognition receptor for numerous fungi, including species of Candida, Aspergillus, Histoplasma, Cryptococcus, Coccidioides, and Pneumocystis carinii (29), our findings suggest a potentially broad role for LTB4 in antifungal defense. Indeed, a protective role for LTB4 has been described in a murine model of pulmonary histoplasmosis (30). Moreover, dectin-1 may also participate in recognition of other microbes, including mycobacteria (31), and it is interesting to note that potential roles for LTB4 have been identified in defense against mycobacterial infections in both humans (32) and mouse models (33).

We used both genetic and pharmacologic approaches to interrupt either LTB4 synthesis or signaling via its GPCR, BLT1. Together, these establish that basal elaboration of LTB4 and ligation of BLT1 was necessary for optimal dectin-1–dependent responses, including in vitro macrophage binding of C. albicans and cytokine production in response to curdlin, as well as in vivo phagocytosis, cytokine generation, and neutrophil recruitment. Indeed, the concentration of LTB4 elaborated constitutively by elicited peritoneal macrophages (100 pg/ml, equivalent to 0.5 nM) (18) substantially exceeds that necessary to amplify macrophage antiparasitic functions via BLT1 (0.01 nM) (26).

In addition to modulating the expression of dectin-1, LTB4 could also potentiate the signals derived from this and other fungal PRRs. Dectin-1 signaling is mediated mainly by Syk, which is responsible for optimal TNF-α secretion, and Raf/MAPK, which is responsible for IL-12 production (6). The fact that TNF-α production in the lungs of 5-LO−/− mice was more impaired relative to WT mice than was IL-12p40 production suggests that LTB4 may exert an additional potentiating effect directed at activation of Syk. Such a potentiating effect by LTB4/BLT1 on Syk activation has been previously noted in the context of macrophage phagocytosis via the Fcy receptor (21), which, like dectin-1, also signals via an ITAM-Syk mechanism (6). In any case, our findings demonstrate that LTB4 is essential for fungal engagement of dectin-1 to elicit Th1- and Th17-type responses that participate in antifungal immunity (34). These effects on dectin-1 are not the only mechanism by which LTB4 can potentiate PRR pathways. We have recently reported that by enhancing expression of the adaptor protein MyD88, BLT1 signaling increases MyD88-dependent NF-κB activation that is an integral component of the host responses to various TLR and cytokine receptors (18). This effect on MyD88 expression would be expected to have broad implications for enhancing innate immunity, and it is possible that other PRRs or their downstream partners might also be targets for modulation by LTB4. Although LTB4 has also been reported to enhance the expression of certain leukocyte cell surface receptors, including CD11b and CD11c in human monocytes and IL-2Rβ in human lymphocytes (23), we are not aware of any previous reports indicating its capacity to specifically regulate expression of a PRR. Among the transcription factors that control dectin-1 expression, only PU.1 expression is downregulated in 5-LO−/− macrophages, and LTB4 was capable of enhancing its expression. PU.1 is an ets-family transcription factor that regulates myeloid lineage development (35). PU.1 gene disruption abolishes macrophage and B lymphocyte production, and delays neutrophil and T lymphocyte production (36). PU.1 also participates in the transcriptional control of various genes involved in macrophage activation, such as TLR4 (37), CD14 (38), mannose receptor (39), CLEC5A (40), and FcRI-III (41). Our finding that LTB4 controls PU.1 expression represents a means by which this lipid mediator might similarly promote the transcription of other PRRs and functionally related receptors. This will be the subject of future studies.

Goi signaling and GM-CSF production elicited by LTB4/BLT1 were critical for its ability to enhance PU.1 expression and subsequent dectin-1 expression. Because a variety of Goi-coupled receptors are present in macrophages, one could speculate that other Goi-coupled ligands should exert similar effects as LTB4 on dectin-1 and PU.1 expression. Surprisingly, neither C5a nor CXCL1 enhanced dectin-1 expression, which supports the findings from BLT1−/− cells, in which expression of other Goi-coupled receptors are intact, that LTB4/BLT1/Goi signaling controls dectin-1 transcription in a nonredundant manner. The reasons for this nonredundant role are unknown but could reflect unique signaling programs or efficiency of BLT1, or specific spatially defined molecular interactions with dectin-1.

In addition to its ability to induce PU.1 expression, LTB4 could also potentiate its transcriptional activation. For instance, PU.1 activation is known to be controlled by protein kinase C (PKC)-δ–mediated phosphorylation (42), and LTB4 activates PKC-δ in macrophages to enhance phagocytosis (43). The possible role of PKC-δ in this axis remains to be clarified.

The regulation of dectin-1 expression is not extensively studied, but it is known that GM-CSF (3) is among the cytokines that can enhance its expression. GM-CSF exhibits a wide range of effects in macrophages, promoting maturation (44), differentiation (44), cytokine secretion (45), and phagocytosis of opsonized (41) and nonopsonized targets (46). Generation of the GM-CSF–deficient mouse was instrumental in elucidating the role of this cytokine in host defense (44). These mice exhibit reduced pulmonary clearance of various microbial pathogens, including group B Streptococcus (47), Pneumocystis carinii (48), Mycobacterium tuberculosis (49), Leishmania major (50), and Cryptococcus neoformans (51). Because both GM-CSF and LTB4 play pivotal roles in host defense, it is possible that cross talk between these two molecules contributes to their capacities to enhance macrophage function. Indeed, GM-CSF protein and mRNA levels were lower in 5-LO−/− macrophages than in WT cells, and LTB4...
challenge enhanced GM-CSF production. The molecular mechanisms by which LTβ/BLT1 controls GM-CSF mRNA expression await future investigation. However, that lower GM-CSF production is indeed responsible for lower dectin-1 and PU.1 expression was evidenced by the fact that addition of this cytokine to LT-deficient cells restored dectin-1 and PU.1 production, as well as curdural responsiveness. Although we have previously reported that GM-CSF enhances macrophage LT generation (52), its ability to enhance dectin-1 expression in 5-LO−/− macrophages indicates that this effect is independent of LTβ synthesis.

Our findings reveal a novel form of regulation in which BLT1, a GPCR ligated at sites of infection, modulates transcription of the important fungal PRR dectin-1 via a GM-CSF/Pu.1 cascade. Cross talk between BLT1 signaling and PRRs would be anticipated to participate in shaping nascent innate immune responses to infections. However, this network is likely disabled in states of immunosuppression characterized by deficient LTβ synthesis, such as malnutrition (9), infection with HIV (9), cigarette smoking (9), and bone marrow transplantation (9). These data provide important insights and new opportunities to modulate innate immune and inflammatory responses to pathogens.

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References


