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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 small interfering RNA abolished LTB4-enhanced dectin-1 expression. GM-CSF controls PU.1 expression, and this cytokine was decreased in LT-deficient macrophages. Addition of GM-CSF to LT-deficient cells restored expression of dectin-1 and PU.1, as well as dectin-1 responsiveness. In addition, LTB4 effects on dectin-1, PU.1, and cytokine production were blunted in GM-CSF−/− 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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Protective innate immune responses require host recognition of microbes through the engagement of pattern recognition receptors (PRRs), including TLRs and C-type lectins (1). PRRs recognize highly conserved microbial motifs known as pathogen-associated molecular patterns, which include carbohydrates, peptidoglycans, and LPSs (2). Dectin-1, a C-type lectin, is the major receptor on macrophages for β-1,3-glucan, a polymer of glucose present in the fungal cell wall that stimulates phagocytosis and production of inflammatory cytokines (1). This receptor is predominantly expressed on cells of the monocyte/macrophage lineage, neutrophils, dendritic cells, and a minor population of splenic T cells (1), and its expression can be enhanced by the cytokines IL-4 (3), IL-13 (4), IL-23, and GM-CSF (3), and decreased by corticosteroids and LPS (3). It is not presently known whether dectin-1 expression can be regulated by signals emanating from G-protein-coupled receptors (GPCRs).

Dectin-1 is now recognized to be the main nonopsonic receptor involved in fungal binding and uptake (5). Signal transduction after dectin-1 ligation depends on its cytoplasmic ITAM, the phosphorylation of which by Src kinase leads to the recruitment of spleen tyrosine kinase (Syk) in phagocytes (6). Dectin-1 engagement also activates phospholipase A2 with subsequent production of eicosanoid lipid mediators including cyclooxygenase-derived prostanoids and 5-lipoxygenase (5-LO)–derived leukotrienes (LTs) such as lipid mediator leukotriene B4 (LTB4) (7, 8). The latter, acting via its high-affinity GPCR leukotriene B4 receptor 1 (BLT1), is best known as a leukocyte chemotactant (9). It has been implicated in a variety of inflammatory disease states, such as atherosclerosis and ischemia-reperfusion injury (9). Importantly, LTB4 is also produced at sites of infection and participates in innate immune responses in vivo and in vitro (8). For example, we and others have previously shown that LTB4 enhances ingestion of IgG-opsonized targets (10), as well as unopsonized microbes including Leishmania amazonensis (11), Streptococcus pneumoniae (12), Candida albicans (8), and Histoplasma capsulatum (13). LTB4 can promote fungal ingestion in macrophages via both mannose and dectin-1 receptors (8). The role of specific 5-LO metabolites and receptors in modulating dectin-1-mediated responses is unknown. In this study, we demonstrate that LTB4 synthesis and signaling via BLT1 are necessary for optimal dectin-1 expression and responsiveness in macrophages in vivo and in vitro. This form of regulation involves LTB4-BLT1 control of GM-CSF production and subsequent expression of the dectin-1 transcription factor PU.1.

Materials and Methods

Reagents

RPMI 1640, LTB4, and LTD4, 5-LO inhibitors (AA861 and zileuton), and the dectin-1 antagonist laminarin prepared from Laminaria digitata were from Enzo Life Sciences. The mannose receptor antagonist mannan prepared from Saccharomyces cerevisiae, activomycin D, polymyxin B sulfate, and pertussis toxin (PTX) were purchased from Sigma. The selective

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; siRNA, small interfering RNA; Syk, spleen tyrosine kinase; WT, wild-type.

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dectin-1 agonist curdulan from Alcaligenes faecalis and zymosan depleted of TLR agonists (by treatment with chloroform/methanol) (14) were from Invivogen. U75302 (BLT1 antagonist) was from Cayman Chemicals. C5a 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 Spectramax Gemini EM fluorometer (Molecular Devices) at settings of 485 excitation/535 emission.

**In vivo injection with curdulan**

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 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.

**Semiquantitative cytokine array**

WT and 5-LO−/− mice underwent intrapulmonary challenge with curdulan 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 C (AR7306), as 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 curdulan 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 mM 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-5-LO (HuC10 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 curdulan, 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 (Clec7B), GM-CSF (Gmcsf), or PU.1 (Spl) mRNAs were normalized to β-actin or GAPDH, and the respective WT control was set at 100%. WT and 5-LO−/− macrophages were treated with or without 2.5 µg/ml actinomycin 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 × 10^7 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 described previously (21), using primary Abs against dectin-1 (1:1,000; Biovision), Dectin-2 (1:500; Abcam), PU.1 and Sp.1 (both at 1:1,000; Abcam), and GAPDH (1:10,000; Sigma). Densitometric analysis was described previously (22); the intensity of the protein band was divided by that of the GAPDH, and this ratio was then expressed relative to that of the untreated control, which was set at 100%. In all instances, density values of bands were corrected by subtraction of the background values.

**RNA interference**

RNA interference was performed according to a protocol provided by Dharmacon and as we have previously reported (18). WT and 5-LO−/− macrophages were transfected using DharmaFECT 1 reagent with 30 nM nontargeting 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 curdlin 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 curdlin 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 curdlin for another 24 h. Curdlin stimulation induced IL-12p40, TNF-α, and GM-CSF in WT macrophages, as expected, but the response to curdlin 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 curdlin was evidenced by the facts that overnight LTB4 pretreatment restored the ability of curdlin 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 curdlin-induced cytokine generation to the same extent as did pharmacologic inhibition of 5-LO (Fig. 2D–F). Because curdlin preparations could be contaminated with endotoxins, we pretreated WT macrophages with the LPS inhibitor polymyxin B sulfate (10 μg/ml) before curdlin stimulation. Polymyxin B did not alter curdlin-induced TNF-α production (data not shown), which excludes a possible role for contaminating endotoxin in our curdlin preparations. Also, the primary role of dectin-1 in mediating curdlin 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 curdlin-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 curdlin in WT and 5-LO−/− mice. Twenty-four hours after curdlin 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 curdlin 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 curdlin 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.

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.

**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 curdlin (100 μg/ml) for another 24 h before determination of IL-12p40 (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 curdlin 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 curdlin by ANOVA.
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. ELicted peritoneal (Fig. 4B) and resident alveolar (Fig. 4C) macrophages from 5-LO−/− mice both exhibited less PU.1 than did cells from WT mice. Overnight treatment of 5-LO−/− 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-LO−/− 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. 4F), 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/Ga plays a nonredundant role in enhancing dectin-1 and PU.1 expression

Although BLT1 can couple to both Ga and Goq in macrophages (26), numerous activation responses in macrophages preferentially involve Ga signaling (18). To determine the importance of Ga in LTβ/BLT1 control of dectin-1 expression, we tested the ability of pretreatment with the Ga 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 LTβ, suggesting that constitutive Ga signaling is required for dectin-1 expression and that LTβ/BLT1 signaling requires Ga. Because other Ga-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/Ga signaling in controlling the expression of this PRR. The importance of Ga signaling in controlling PU.1 expression was also studied. PTX treatment of elicited macrophages revealed that Ga signaling is necessary for baseline PU.1 expression and for LTβ/BLT1 enhancement of PU.1 expression (Fig. 5C), as it was for dectin-1 expression (Fig. 5A).

GM-CSF is a critical mediator of LTβ2-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 LTβ2 amplification of dectin-1 and PU.1. To evaluate this possibility, we initially determined the levels of GM-CSF in 5-LO−/− cells. 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).

FIGURE 4. PU.1 mediates LTβ2-enhanced dectin-1 expression in macrophages. (A) Dectin-1 mRNA decay in WT and 5-LO−/− macrophages harvested after treatment with actinomycin D (2.5 μg/ml). Data are from three experiments in triplicate; values are relative to untreated macrophages from both genotypes. (B) WT- and 5-LO−/−-elicited peritoneal macrophages or (C) resident AMs were treated ± LTβ2 for 24 h, and the expression of PU.1, Sp1, and GAPDH was determined by immunoblotting. Numbers under lanes indicate relative density of PU.1 from three independent experiments. (D) PU.1 mRNA expression was determined by real-time RT-PCR in WT- and 5-LO−/−-elicited macrophages incubated ± LTβ2 for 24 h. (E) WT macrophages were treated with siRNA to PU.1 and control siRNA, as determined by real-time RT-PCR and immunoblotting, respectively. mRNA levels are expressed relative to those in siControl-treated WT cells. Immunoblot is representative of three independent experiments. Numbers under lanes indicate relative density of dectin-1 or PU.1 from three independent experiments. Data represent the mean ± SEM from three individual experiments, each performed in triplicate. *p < 0.05 versus WT or WT siControl; #p < 0.05 versus 5-LO−/− control or LTβ2-stimulated siControl macrophages by ANOVA.
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–deficient 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–deficient 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–deficient 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/Gαi 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.

FIGURE 5. Gαi signaling is required for LTB4/BLT1-induced dectin-1 and PU.1 expression in macrophages. (A) WT-elicited macrophages were pretreated with PTX (600 ng/ml) for 24 h and incubated with or without LTB4 for another 24 h, after which RNA was isolated for dectin-1 mRNA determination by real-time RT-PCR. (B) WT macrophages were treated for 24 h with LTB4 (100 nM), C5a (50 ng/ml), or CXCL1 (20 ng/ml), and dectin-1 mRNA was determined by real-time RT-PCR. (C) WT macrophages were pretreated with PTX for 24 h and incubated with or without LTB4 for another 24 h, after which RNA was isolated for PU.1 mRNA determination by real-time RT-PCR. Data represent the mean ± SEM from three individual experiments, each performed in triplicate, and are expressed relative to untreated macrophages. *p < 0.05 versus WT control or untreated control, #p < 0.001 versus macrophages incubated with LTB4 only by ANOVA.

FIGURE 6. GM-CSF mediates the enhancement by LTB4 of PU.1 and dectin-1 in macrophages. (A) GM-CSF mRNA expression was determined by real-time RT-PCR in WT and 5-LO−/− macrophages incubated ± LTB4 for 24 h. (B) GM-CSF protein was determined by ELISA in WT and 5-LO−/− macrophages incubated ± LTB4 for 24 h. (C and D) WT and 5-LO−/− macrophages were treated with GM-CSF (10 ng/ml) for 24 h, and the expression of dectin-1 (C) or PU.1 (D) mRNA was determined. (E and F) WT and 5-LO−/− macrophages were pretreated with GM-CSF for 24 h and then stimulated with curdlan (100 μg/ml) for another 24 h, after which the supernatant levels of IL-12p40 (E) or TNF-α (F) were determined by ELISA. (G and H) WT and GM-CSF−/− macrophages were treated ± LTB4 for 24 h, and the expression of dectin-1 (G) and PU.1 (H) mRNA was determined by real-time RT-PCR. (I and J) WT and GM-CSF−/− macrophages were pretreated ± LTB4 for 24 h followed by curdlan for another 24 h, and the supernatant was harvested to determine levels of TNF-α (I) and IL-12p40 (J) by ELISA. Data represent the mean ± SEM from three individual experiments, each performed in triplicate. *p < 0.05 versus WT control; #p < 0.05 versus 5-LO−/− alone or LTB4-stimulated macrophages; #p < 0.05 versus 5-LO−/− macrophages incubated with curdlan only by ANOVA.
FIGURE 7. Proposed model of LTB4/BLT1 regulation of GM-CSF and PU.1 expression and enhancement of dectin-1 expression and responsiveness. Basal or dectin-1–activated LTB4/BLT1 signaling elicits Goi activation that results in enhanced expression of GM-CSF. This cytokine potentiates expression of PU.1, which carries out transcription of dectin-1, augmenting dectin-1 protein expression and responsiveness, as evidenced by binding, phagocytosis, cytokine secretion, and neutrophil recruitment in response to challenge with C. albicans, zymosan, or the fungal glucan curdlan. LTB4 generation in response to dectin-1 ligation represents an autocrine self-amplifying loop.

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 curdlan, 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 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 was 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 FcRf-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/LTβR controls GM-CSF mRNA expression await future investigation. However, that lower GM-CSF production is indeed responsible for lower dictin-1 and PU.1 expression was evidenced by the fact that addition of this cytokine to LT-decient cells restored dictin-1 and PU.1 production, as well as curdian responsiveness. Although we have previously reported that GM-CSF enhances macrophage LT generation (52), its ability to enhance dictin-1 expression in 5-LO−/− macrophages indicates that dictin-1 expression is independent of LTβR 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 dictin-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 LTβR 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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Disclosures

The authors have no financial conflicts of interest.

References


Supplemental Figures

Sup. Fig. 1. Dectin-1 selective agonists dose dependently increase TNF-\(\alpha\) production in macrophages. WT macrophages were treated with curdlan (A) or treated-zymosan (B) at the indicated concentrations for 24 h prior to determination of TNF-\(\alpha\) levels by ELISA. Data represent the mean ± SEM from 3 individual experiments, each performed in triplicate. *\(p < 0.05\) versus untreated control or #\(p < 0.05\) versus WT macrophages stimulated with curdlan by ANOVA.

Sup. Fig. 2. Dectin-1 activation is required for curdlan-induced TNF-\(\alpha\) production in macrophages. WT macrophages were pretreated ± laminarin (100 \(\mu\)g/mL) for 30 min, then incubated with the dectin-1 selective agonist curdlan (100 \(\mu\)g/ml) for another 24 h prior to determination of TNF-\(\alpha\) levels by ELISA. Data represent the mean ± SEM from 3 individual experiments, each performed in triplicate. *\(p < 0.05\) versus untreated control; #\(p < 0.01\) versus macrophages stimulated with curdlan only by ANOVA.