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Macrophage dectin-1 expression is controlled by leukotriene B_4 via a GM-CSF/PU.1 axis

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Abstract

Pathogen recognition receptors (PRRs) 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 B_4 (LTB_4). The influence of G protein-coupled receptor (GPCR) ligands such as LTB_4 on fungal PRR expression is unknown. Here we investigated the role of LTB_4 signaling in dectin-1 expression and responsiveness in macrophages. Genetic and pharmacologic approaches showed that LTB_4 production and signaling through its high-affinity GPCR 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 LT- and BLT1-deficent mice than their WT counterparts. LTB_4 increased the expression of the transcription factor responsible for dectin-1 expression, PU.1, and PU.1 siRNA abolished LTB_4-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, LTB_4 effects on dectin-1, PU.1 and cytokine production were blunted in GM-CSF−/− macrophages. Our results identify LTB_4-BLT1 signaling as an unrecognized controller of dectin-1 transcription via GM-CSF and PU.1 that is required for fungi protective host responses.

INTRODUCTION

Protective innate immune responses require host recognition of microbes through the engagement of pattern-recognition receptors (PRRs), including Toll-like receptors (TLRs) and C-type lectins (1). PRRs recognize highly conserved microbial motifs known as pathogen-associated molecular patterns (PAMPs), which include carbohydrates, peptidoglycans, and lipopolysaccharides (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,

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dendritic cells and a minor subpopulation 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 non-opsonic receptor involved in fungal binding and uptake (5). Signal transduction following dectin-1 ligation depends on its cytoplasmic immunoreceptor tyrosine-based activation motif (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 A_2 (PLA_2) with subsequent production of eicosanoid lipid mediators including cyclooxygenase-derived prostanoids and 5-lipoxygenase (5-LO)-derived leukotrienes (LTs) such as LTB_4 (7, 8). The latter, acting via its high-affinity GPCR BLT1, is best known as a leukocyte chemoattractant (9). It has been implicated in a variety of inflammatory disease states, such as atherosclerosis and ischemia-reperfusion injury (9). Importantly, LTB_4 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 LTB_4 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). LTB_4 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. Here we demonstrate that LTB_4 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 LTB_4-BLT1 control of GM-CSF production and subsequent expression of the dectin-1 transcription factor PU.1.

MATERIAL AND METHODS

Reagents

RPMI 1640, LTB_4 and LTD_4, 5-LO inhibitors (AA861 and zileuton), the dectin-1 antagonist laminarin prepared from *Laminaria digitata*, were from Enzo Life Sciences. The mannose receptor antagonist mannan prepared from *Saccharomyces cerevisiae*, Actinomycin D, Polymyxin B sulfate and pertussis toxin (PTX) were purchased from Sigma. The selective dectin-1 agonist curdlan 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 dimethyl sulfoxide (DMSO). Required dilutions of all compounds were prepared immediately before use, and equivalent quantities of vehicle were added to the appropriate controls.

Animals

8-week-old female 5-LO−/− mice (15) were bred in-house and strain-matched 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 NIH 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 to minimize suffering by the attending veterinarian.

Cell isolation and culture

Elicited peritoneal macrophages were harvested from the peritoneal cavities of mice by lavage with PBS 4 days after the injection of 2 ml of 3% thioglycollate, as described previously (18). Resident murine alveolar macrophages were obtained by bronchoalveolar lavage (BAL) as described (18). Cells were cultured overnight in RPMI containing 10% fetal bovine serum 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. 72 h 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 2×10^9 colony forming units (CFU)/ml in sterile nonpyrogenic PBS. *C. albicans* was killed through heating for 30 min at 56 °C and FITC-labeled as described (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 pre-chilled 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 curdlan

Curdlan (100 μg/kg) was reconstituted in PBS with 1% BSA and administered to the lungs of mice via oropharyngeal injection as described (20). BAL was performed by 3 successive instillations of 1 ml PBS with each, followed by gentle suction. BAL fluid from WT and 5-LO−/− mice was harvested after 24 h and levels of LTB_4, cytokines, and chemokines were measured by ELISA or by antibody-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 above and the BAL fluid was harvested 24 h later. Protein content was quantified by Bradford assay and 50 μg of protein were used for qualitative measurement of cytokine expression using the Mouse Cytokine Antibody Array, Panel A (ARY006), as recommended by the manufacturer (R&D Systems, Wiesbaden, Germany).

Measurement of LTB_4

Levels of LTB_4 in the BAL fluid obtained from WT mice 24 h after intrapulmonary challenge with curdlan were determined using EIA kits (Cayman Chemical Co.) as described (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 antibody 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 Bioscience Pharmigen) 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 (TreeStar).

In vivo phagocytosis assay

WT and 5-LO−/− mice were subjected to intrapulmonary administration of 1 μg/ml zymosan as described above 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 (*Clecsf10*), GM-CSF (*Gmcsf*) or PU.1 (*Spi1*) mRNAs were normalized to β-actin or GAPDH, and the respective WT control was set at 100%. To determine the decay of *Clec7A* mRNA, 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. *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

2×10^6 macrophages were plated in 6-well tissue culture dishes and were incubated in the presence or absence of 100 nM LTB_4 for 24 h and then lysed in buffer (50 mM Tris-HCl [pH 7.4], 25 mM KCl, 5 mM $MgCl_2$, 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 antibodies against dectin-1 (1:1000, Biovision), Dectin-2 (1:500, Abcam), PU.1 and Sp.1 (both at 1:1000, Abcam) and GAPDH (1:10,000, Sigma). Densitometric analysis was as described (22); the intensity of 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 of nonspecific control or specific ON-TARGET SMARTpool *Pu.1* siRNAs. After 48 h of transfection, macrophages were incubated with or without 100 nM LTB_4 for 24 h and the cells harvested for mRNA or protein analysis.

Statistics

All experiments were performed at least 3 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 $p \leq 0.05$.

RESULTS

LTB_4 regulates macrophage expression of dectin-1

LTB_4 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 LTB_4 can influence the expression of PRRs, including dectin-1. This possibility was first examined in elicited peritoneal macrophages from WT and 5-LO−/− mice, as 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. 1 A), and protein, as determined by both FACS (Fig. 1 B) and immunoblotting (Fig. 1 C). Reduced dectin-1 mRNA was confirmed in WT cells treated for 24 h with a 5-LO inhibitor (Fig. 1 D). 24 h treatment with 100 ng/ml LTB_4 fully rescued dectin-1 mRNA (Fig. 1 A) and protein (Fig. 1 B and C) expression in 5-LO−/− macrophages back to the levels observed in WT cells. By contrast to its effects on dectin-1, neither endogenously produced nor exogenously added LTB_4 modulated expression of dectin-2 mRNA or protein (Fig. 1 E and inset). Reduced expression of dectin-1 was also observed in alveolar macrophages (Fig. 1 F) and bone marrow-derived macrophages (not shown) from LT-deficient mice, and again, levels were significantly increased with 24 h treatment with LTB_4. We next determined if the decreased dectin-1 expression correlated with lower macrophage binding of *C. albicans*. Alveolar macrophages from 5-LO−/− mice bound substantially less *C. albicans* than did WT macrophages, but LTB_4 treatment for 24 h restored yeast binding to levels exhibited by WT cells (Fig. 1 G). 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. 1 H). 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. 1 I) and from WT mice treated overnight with a BLT1 antagonist. However, due to lack of BLT1, LTB_4 was unable to restore deficient dectin-1 expression (Fig. 1 I), in contrast to 5-LO−/− cells. Together, these results show that LTB_4 enhances basal expression of dectin-1 mRNA and protein in various macrophage populations.

LTB_4 regulates macrophage responses via dectin-1 *in vitro* and *in vivo*

Since LTB_4 and BLT1 signaling control dectin-1 expression, we reasoned that LTB_4 would also control host responses to dectin-1 engagement. This was tested both *in vitro* and *in vivo*. In the *in vitro* experiments, we employed the dectin-1-selective agonist curdlan or

zymosan which is able to ligate dectin-1 but whose TLR ligands were removed by treatment with choloform/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 (Suppl. Fig. 1 A and B). To determine if LTB_4 is the 5-LO product involved in dectin-1 responsiveness, 5-LO−/− cells were pretreated in the presence or absence of LTB_4 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. 2 A–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. 2 D–F). The specific role of endogenous LTB_4 in regulating responses to curdlan was evidenced by the facts that overnight LTB_4 pretreatment restored the ability of curdlan to induce IL-12p40, GM-CSF and TNF-α levels in 5-LO −/− macrophages (Fig. 2 A–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. 2 D–F). Since curdlan preparations could be contaminated with endotoxins, we pretreated WT macrophages with the LPS inhibitor polymyxin B sulfate (10 μg/ml) prior to 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% (Suppl. 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 h after curdlan injection, high levels of LTB_4 were measured in BAL fluid of WT mice (Fig. 3 A), 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 antibody-based array. 5-LO−/− mice were globally less responsive to curdlan than were WT mice (Fig. 3 B). 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. 3 C–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. 3 J). Following intrapulmonary challenge with zymosan, LT-deficient mice also manifested significantly lower *in vivo* ingestion of the yeast particles by macrophages (Fig. 3 K), but not by neutrophils (data not shown). These findings indicate that LTB_4 produced in response to dectin-1 engagement amplifies macrophage phagocytosis, cytokine secretion, and neutrophil recruitment.

LTB_4 enhances dectin-1 expression by a transcriptional mechanism involving PU.1

Since LTB_4 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 following 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. 4

A), 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 PPAR-γ (4). We examined the effects of LT deficiency and exogenous LTB_4 on levels of these transcription factors. Elicited peritoneal (Fig. 4 B) and resident alveolar (Fig. 4 C) macrophages from 5-LO−/− mice both exhibited less PU.1 than did cells from WT mice. Overnight treatment of 5-LO−/− cells with LTB_4 largely restored PU.1 protein expression to WT levels in both populations of macrophages (Fig. 4 B and C) and drove PU.1 mRNA in elicited peritoneal macrophages to levels that far exceeded WT (Fig. 4 D). By contrast, neither Sp1 protein (Fig. 4 B) nor mRNA (data not shown) levels were reduced compared to WT cells. Likewise, no reduction in PPAR-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 utilized siRNA to knock down this transcription factor in elicited WT macrophages. We achieved approximately 75% knockdown of PU.1 mRNA (Fig. 4 E) and ~ 55% knockdown of protein (Fig. 4 G), when compared to control siRNA. PU.1 silencing decreased dectin-1 mRNA (Fig. 4 F) and protein (Fig. 4 G) expression by approximately 55%. In addition, PU.1 siRNA abolished LTB_4 enhancement of dectin-1 expression (Fig. 4 F–G). These data show that LTB_4 enhancement of dectin-1 involves upregulated expression of its transcription factor, PU.1.

BLT1/Gαi plays a non-redundant 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 LTB_4/BLT1 control of dectin-1 expression, we tested the ability of pretreatment with the Gαi inhibitor pertussis toxin (PTX) to interfere with basal and LTB_4-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 LTB_4 (Fig. 5 A), suggesting that constitutive Gαi signaling is required for dectin-1 expression and that LTB_4/BLT1 signaling requires Gαi. Since 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. 5 B), suggesting a non-redundant 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 LTB_4/BLT1 enhancement of PU.1 expression (Fig. 5 C), as it was for dectin-1 expression (Fig. 5 A).

GM-CSF is a critical mediator of LTB_4-enhanced PU.1 and dectin-1 expression

GM-CSF upregulates PU.1 (27) and dectin-1 (3) expression in macrophages. However, it is unknown if endogenously produced GM-CSF is also required for dectin-1 expression. It is also unknown if GM-CSF participates in the LTB_4 amplification of dectin-1 and PU.1. To evaluate this possibility, we initially determined the levels of GM-CSF in 5-LO−/− and WT elicited peritoneal macrophage cultures. Expression of both mRNA (Fig. 6 A) and protein (Fig. 6 B) was decreased in LT-deficient cells when compared to WT macrophages. Overnight treatment of 5-LO−/− cells with LTB_4 restored GM-CSF mRNA and protein levels beyond the WT range (Fig. 6 A and B). To determine whether the lower GM-CSF expression in 5-LO−/− 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. 6 C) and PU.1 (Fig. 6 D) 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. 6 E) and TNF-α (Fig. 6 F).

To determine if 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. 6 G) expression was lower in GM-CSF−/− than WT macrophages. Accordingly, dectin-1 (Fig. 6 H) expression was also markedly lower in GM-CSF−/− than WT cells. Importantly, overnight treatment with LTB_4 was unable to enhance either PU.1 (Fig. 6 G) or dectin-1 (Fig. 6 H) 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. 6 I and J). Moreover, overnight LTB_4 treatment was unable to potentiate curdlan-induced cytokine production in GM-CSF-deficient cells as it was in WT cells (Fig. 6 I and J). These results indicate that LTB_4/BLT1 regulation of dectin-1 expression and responsiveness in macrophages depends on an autocrine loop involving GM-CSF potentiation of PU.1.

DISCUSSION

We provide evidence here 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 LTB_4 that mediates this effect. More specifically, we have shown that: (1) homeostatic LTB_4 production is required for dectin-1 responsiveness *in vivo* and *in vitro*; (2) LTB_4/BLT1/Gαi signaling is necessary for basal dectin-1 expression; (3) LTB_4 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 LTB_4-induced PU.1 and dectin-1 expression in macrophages. A scheme illustrating the relevant events is depicted in Fig. 7. Since LTB_4 production is a component of the host response to fungal infections (8) and since 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 LTB_4 in anti-fungal defense. Indeed, a protective role for endogenous LTB_4 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 LTB_4 have been identified in defense against mycobacterial infections in both humans (32) and mouse models (33).

We employed both genetic and pharmacologic approaches to interrupt either LTB_4 synthesis or signaling via its GPCR, BLT1. Together, these establish that basal elaboration of LTB_4 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 LTB_4 elaborated constitutively by elicited peritoneal macrophages (100 pg/ml, equivalent to 0.5 nM) (18) substantially exceeds that necessary to amplify macrophage antimicrobial functions via BLT1 (0.01 nM) (26).

In addition to modulating the expression of dectin-1, LTB_4 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 LTB_4 may exert an additional potentiating effect directed at activation of Syk. Such a potentiating effect by LTB_4/BLT1 on Syk activation has been previously noted in the context of macrophage phagocytosis via the Fcγ receptor (21), which, like dectin-1, also signals via an ITAM-Syk mechanism (6). In any case, our findings demonstrate that LTB_4 is essential in order for fungal engagement of dectin-1 to elicit Th1- and Th17-type responses that participate in anti-fungal immunity (34). These effects on dectin-1 are not the only mechanism by which LTB_4 can potentiate PRR pathways. We have recently reported that by enhancing expression of the adaptor protein MyD88, BLT1 signaling increases MyD88-dependent NFkB 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 LTB_4. Although LTB_4 has also been reported to enhance the expression of certain leukocyte cell surface receptors, including CD11b and CD11c in human monocytes and IL-2 receptor beta 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 controls dectin-1 expression, only PU.1 expression was downregulated in 5-LO−/− macrophages, and LTB_4 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 LTB_4 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.

Gαi signaling and GM-CSF production elicited by LTB_4/BLT1 were critical for its ability to enhance PU.1 expression and subsequent dectin-1 expression. Since a variety of Gαi-coupled receptors are present in macrophages, one could speculate that other Gαi-coupled ligands should exert similar effects as LTB_4 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 Gαi-coupled receptors are intact – that LTB_4/BLT1/Gαi signaling controls dectin-1 transcription in a non-redundant manner. The reasons for this non-redundant 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, LTB_4 could also potentiate its transcriptional activation. For instance, PU.1 activation is known to be controlled by PKC-δ-mediated phosphorylation (42), and LTB_4 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). Since both GM-CSF and LTB_4 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 LTB_4 challenge enhanced GM-CSF production. The molecular mechanisms by which LTB_4/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 curdlan 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 indicate that this effect is independent of LTB_4 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 LTB_4 synthesis, such as malnutrition (9), infection with human immunodeficiency virus (9), cigarrete smoking (9), and bone marrow transplantation (9). These data provide important insights and new opportunities to modulate innate immune and inflammatory responses to pathogens.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

5-LO	5-lipoxygenase
AM	alveolar macrophage
PTX	pertussis toxin
BLT1	leukotriene B4 receptor 1
cysLT1	cysteinyl leukotriene receptor 1

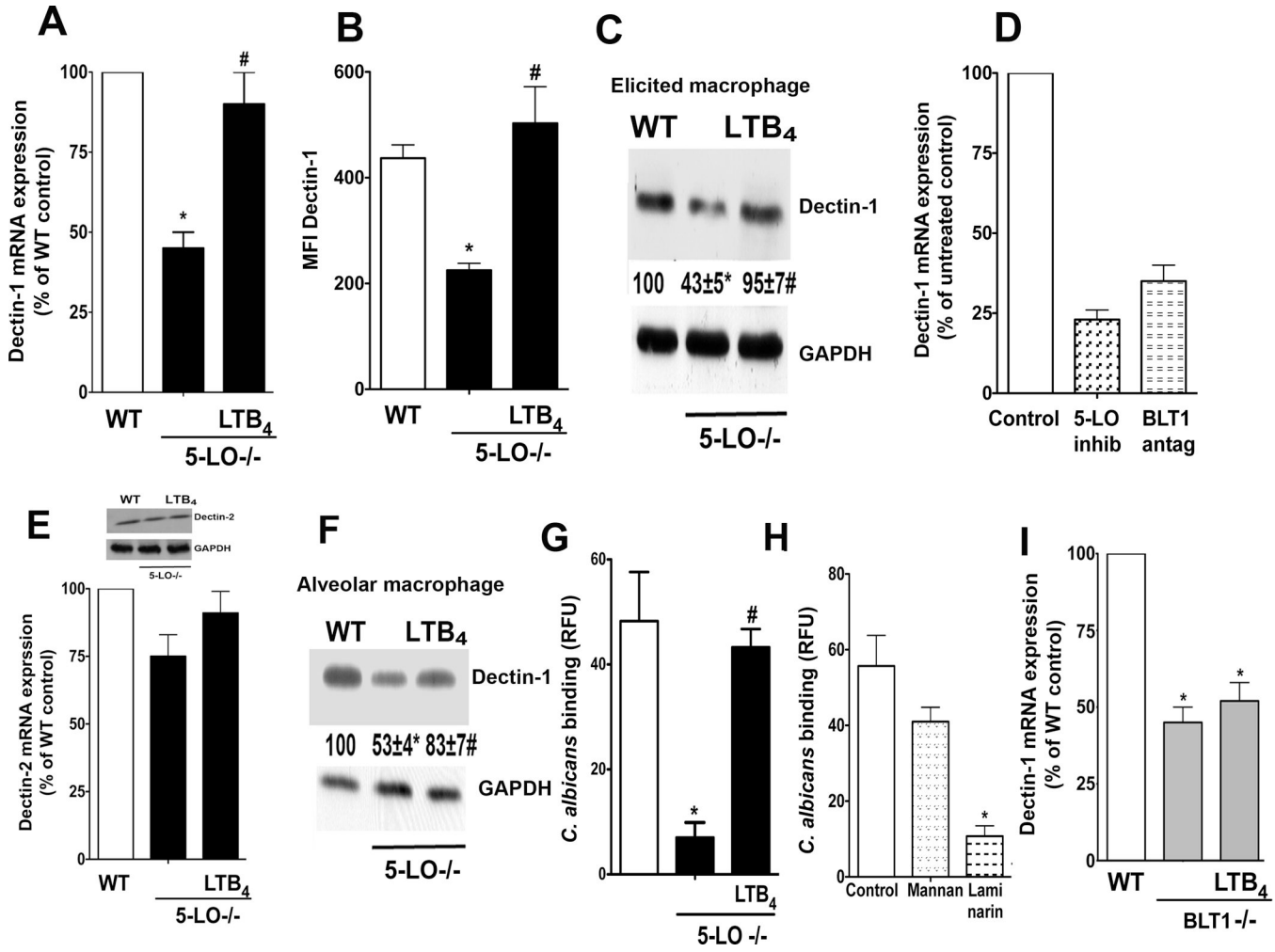

Fig. 1. LTB_4 is necessary for basal dectin-1 expression in macrophages

(**A**) WT and 5-LO−/− macrophages were treated ± 100 nM LTB_4 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 antibody and the cells subjected to FACS analysis as described in Materials and Methods. Mean fluorescence intensity (MFI) is expressed as the mean ± SEM from 3 individual experiments. (**C**) Elicited macrophages were incubated ± LTB_4 for 24 h and the expression of dectin-1 and GAPDH 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 3 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 ± LTB_4 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**) Alveolar macrophages were incubated ± LTB_4 for 24 h and the expression of dectin-1 and GAPDH determined by immunoblot analysis. Data are expressed and analyzed as in (**C**). **G**) Alveolar macrophages from WT and 5-LO−/− mice were incubated ± 100 nM LTB_4 for 24 h and the binding capacity for 10:1 heat-killed ^{FITC}*C. albicans* was determined as described in the Materials and Methods. (**H**) Alveolar macrophages 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 ± LTB_4 for 24 h and dectin-1 mRNA was determined by real-time RT-PCR. In all circumstances, data represent the mean ± SEM from 3 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.

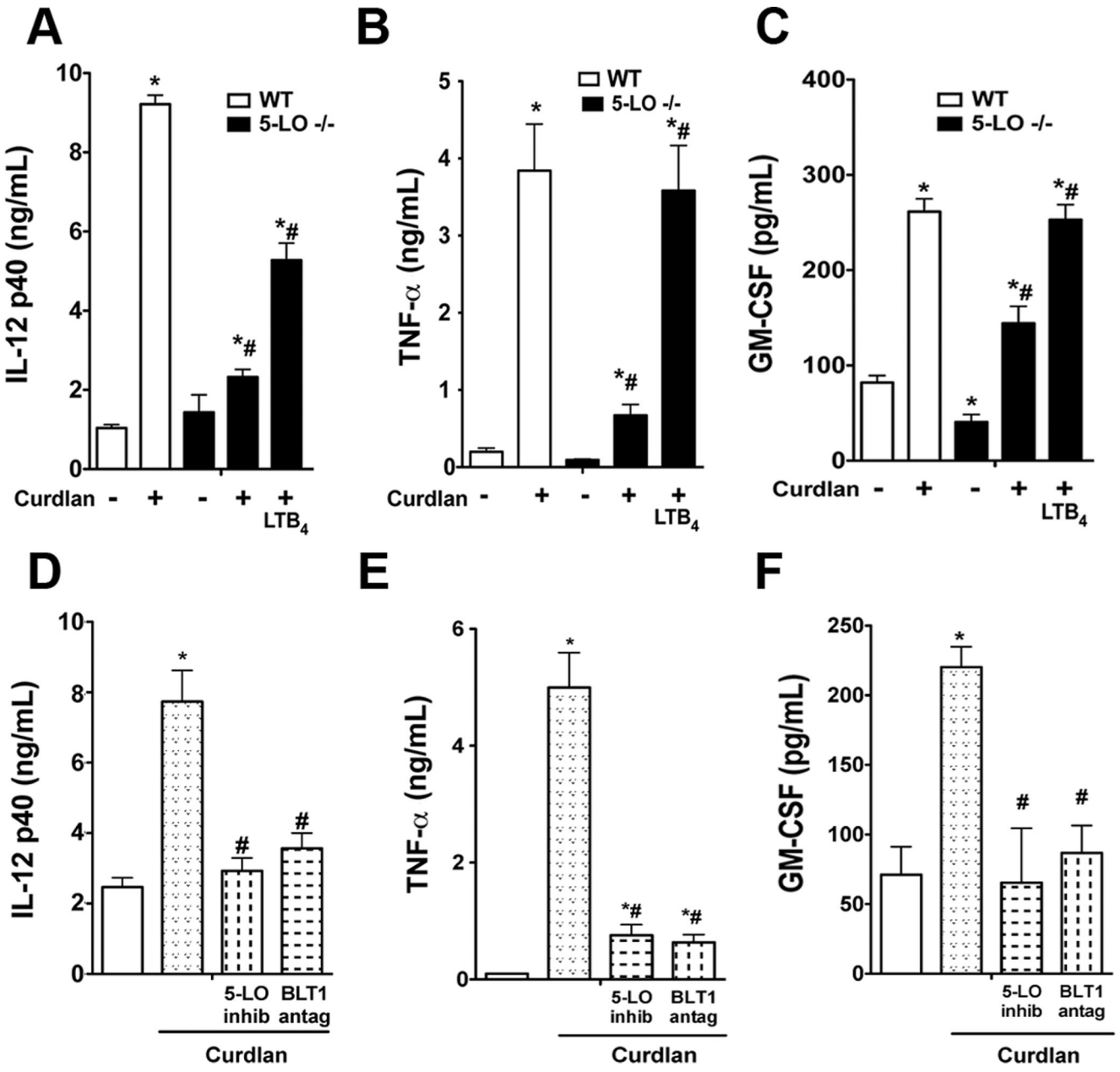

Fig. 2. LTB_4 is necessary for dectin-1 responses in macrophages

(**A–C**) WT and 5-LO−/− macrophages were pretreated ± LTB_4 for 24 h, then incubated with the dectin-1 selective agonist curdlan (100 μg/ml) for another 24 h prior to 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 determined by ELISA. Data represent the mean ± SEM from 3 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.

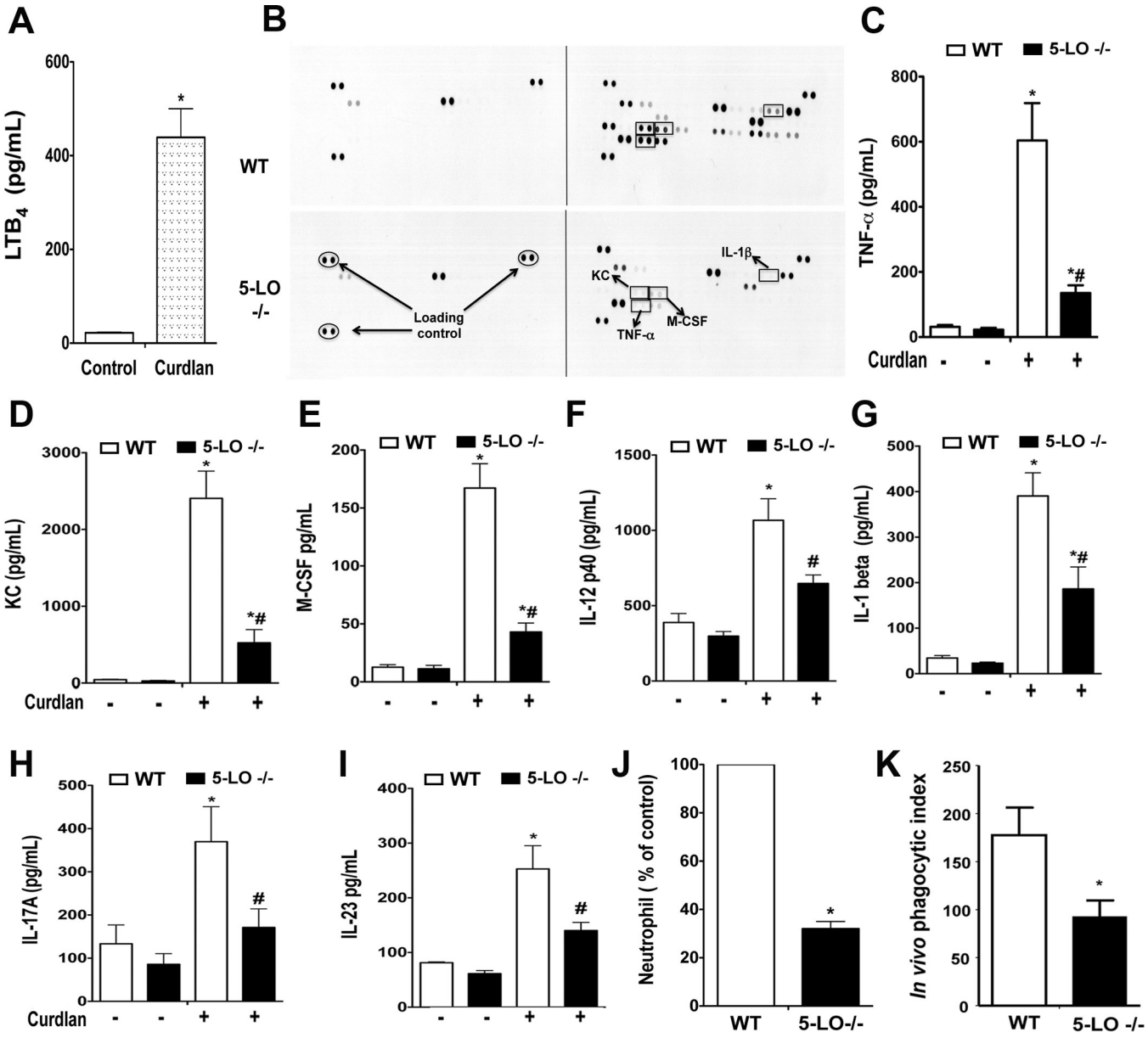

Fig. 3. LT biosynthesis is required for optimal pulmonary anti-fungal responses

(**A**) WT mice were oropharyngeally administered 100 μg/ml curdlan, and 24 h later BAL fluid was harvested and LTB_4 was measured by EIA. (**B**) BAL fluid was harvested from WT and 5-LO−/− mice 24 h after intrapulmonary administration of curdlan and subjected to cytokine/chemokine antibody array as described in Materials and Methods. Adjacent dots represent cytokine/chemokine expression from two independent experiments. Equal protein loading is demonstrated by loading controls, as indicated by the oval labels. Identities of mediators highlighted by the rectangles are as labeled in the bottom panel. Experiment representative of two independent experiments. (**C–I**) BAL fluid from WT and 5-LO−/− mice challenged with curdlan as in (**B**) was harvested and levels of TNF-α (**C**), KC (**D**), M-CSF (**E**), IL-12p40 (**F**), IL-1β (**G**), IL-17A (**H**), and IL-23 (**I**) were determined by ELISA. (**J**) BAL fluid from curdlan-challenged WT and 5-LO−/− mice was subjected to cytospin, stained with Diff-Quick and the neutrophil counts were determined microscopically; values are expressed as a percentage of the neutrophil count found in WT mice, which was 60 ± 4/

mouse when at least two hundreds cells were counted. (**K**) WT and 5-LO−/− mice were challenged with zymosan (10 μg/ml) via the oropharyngeal route and 24 h later BAL cells were subjected to cytospin and the number of intracellular zymosan particles determined microscopically from cells stained with Diff-Quick; phagocytic index was determined as described in Materials and Methods. Data represent the means ± SEM from at least three independent experiments with 6 mice per genotype. *$p < 0.05$ versus WT control; #$p < 0.01$ versus untreated 5-LO−/− macrophages by ANOVA.

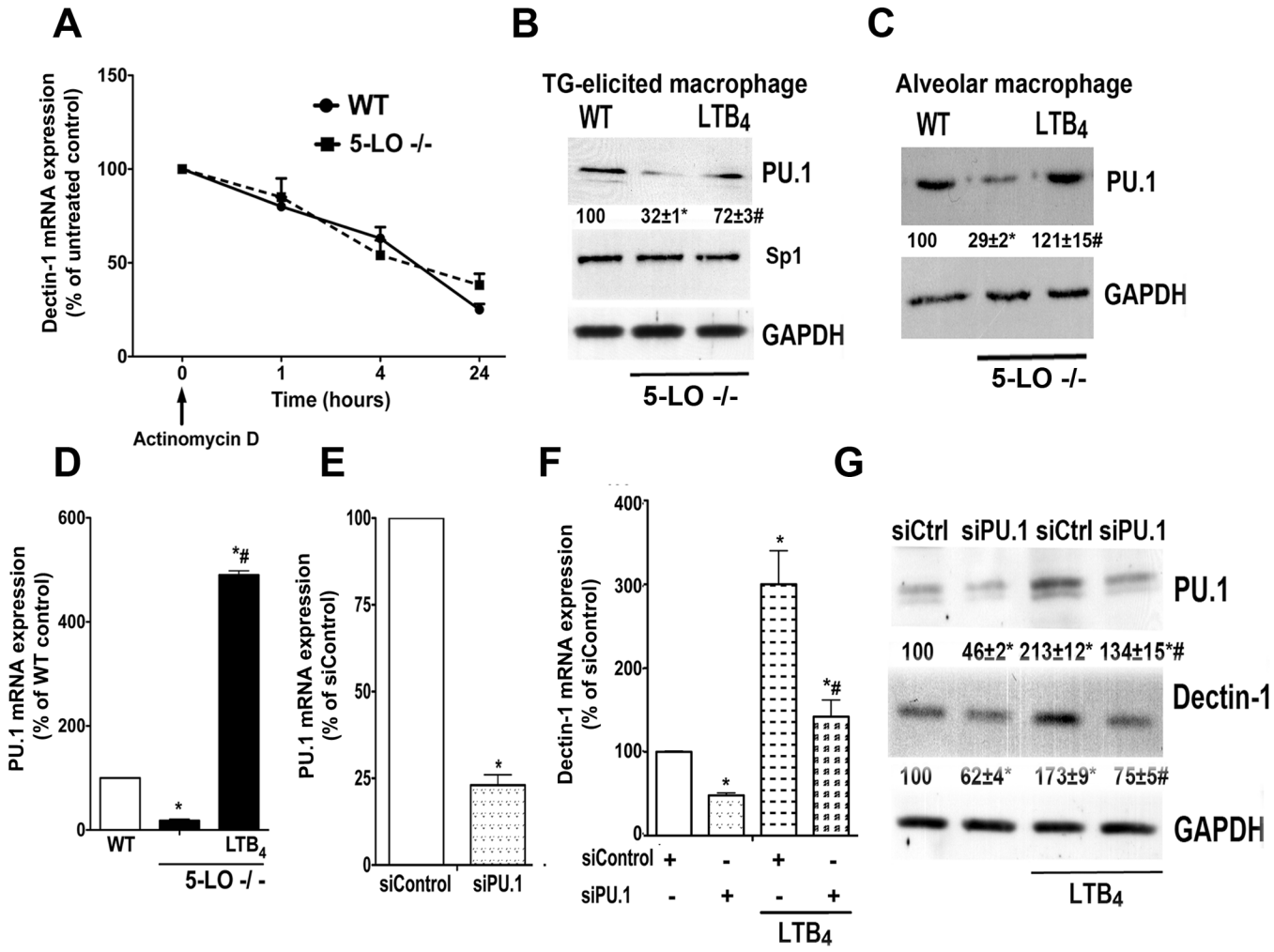

Fig. 4. PU.1 mediates LTB_4-enhanced dectin-1 expression in macrophages

(**A**) Dectin-1 mRNA decay in WT and 5-LO−/− macrophages harvested after treatment with actinomycin D (2.5 μg/ml). Data are from 3 experiments in triplicate; values are relative to untreated macrophages from both genotypes. (**B**) WT and 5-LO−/− elicited peritoneal macrophages or (**C**) resident alveolar macrophages were treated ± LTB_4 for 24 h and the expression of PU.1, Sp1 and GAPDH were 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 ± LTB_4 for 24 h. (**E**) WT macrophages were treated for 48 h in the presence of scrambled siRNA (siControl) or PU.1 siRNA, and the expression of PU.1 mRNA was determined by real-time RT-PCR and expressed relative to values in siControl cells. (**F**) Dectin-1 mRNA and (**G**) protein expression in WT macrophages treated with 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 3 independent experiments. Numbers under lanes indicate relative density of dectin-1 or PU.1 from three independent experiments. Data represent the mean ± SEM from 3 individual experiments, each performed in triplicate. *$p < 0.05$ versus WT or WT siControl; #$p < 0.05$ versus 5-LO−/− control or LTB_4-stimulated siControl macrophages by ANOVA

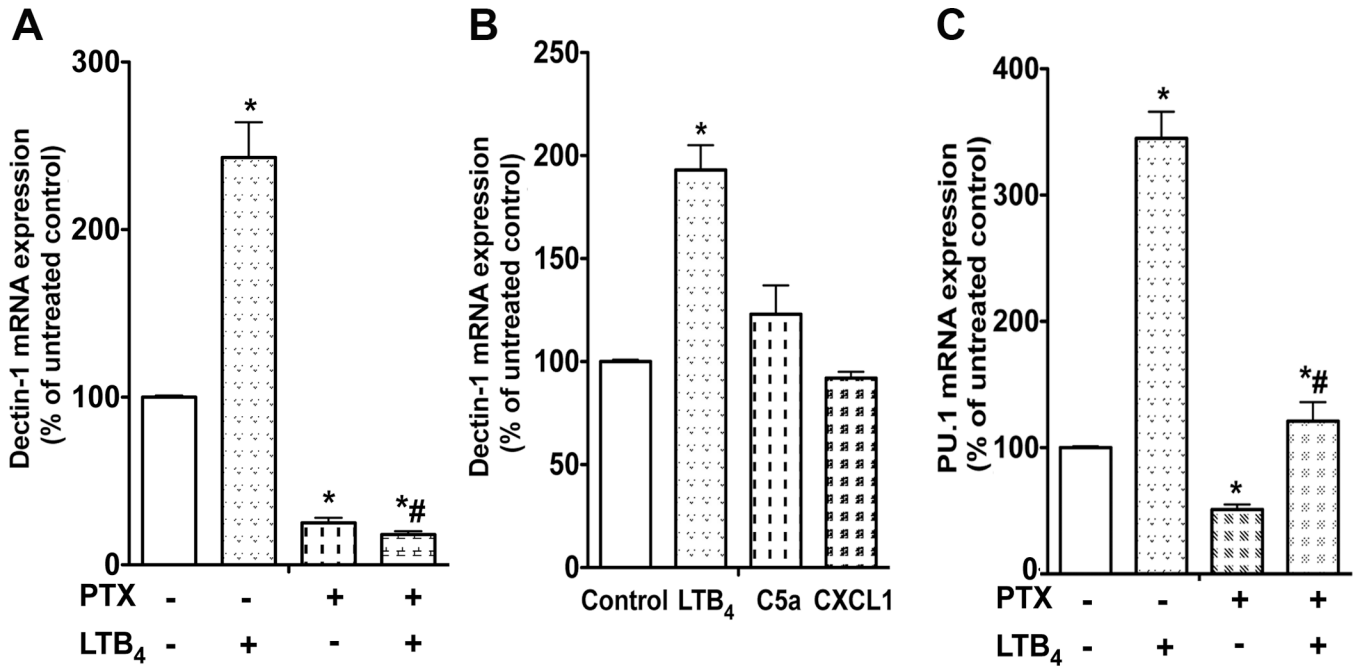

Fig. 5. Gαi signaling is required for LTB_4/BLT1-induced dectin-1 and PU.1 expression in macrophages

(**A**) WT elicited macrophages were pretreated with PTX (600 ng/ml) for 24 h and incubated with or without LTB_4 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 LTB_4 (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 LTB_4 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 3 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 LTB_4 only by ANOVA.

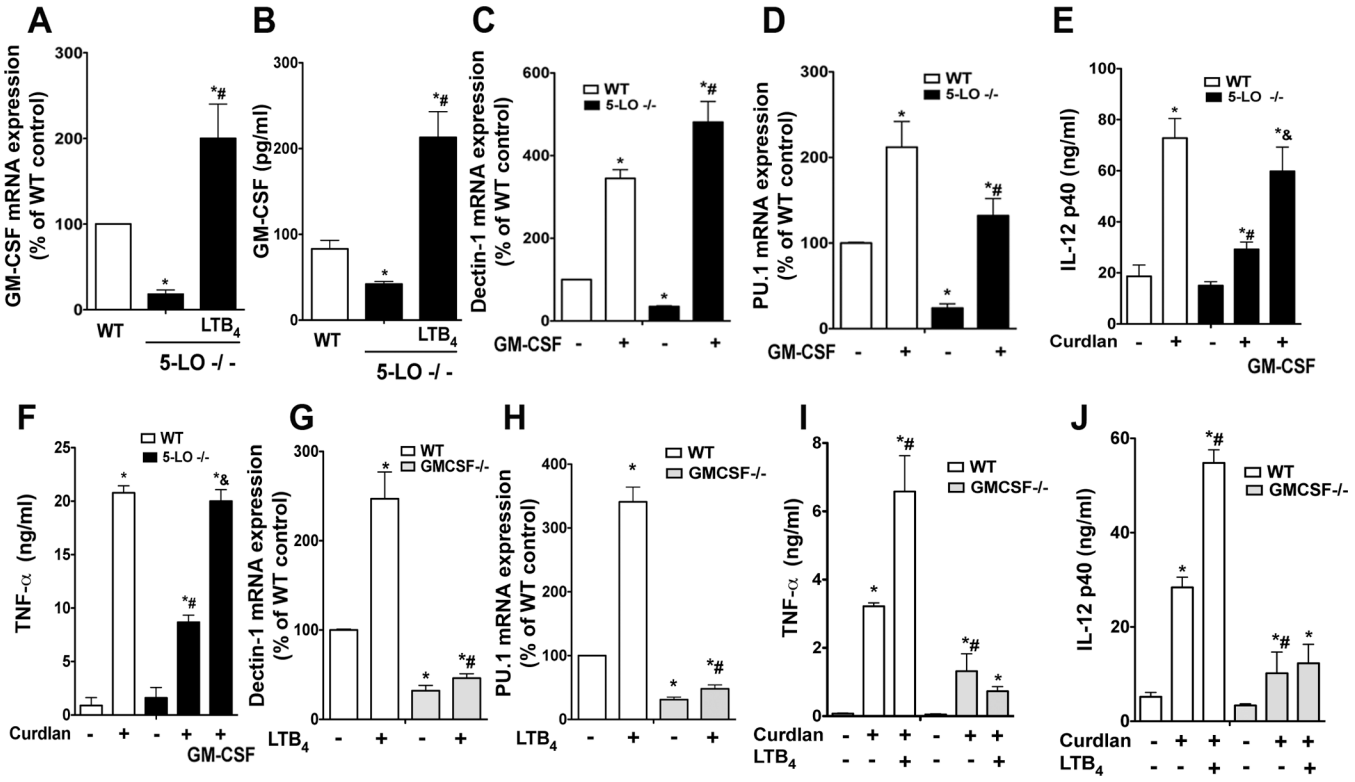

Fig. 6. GM-CSF mediates the enhancement by LTB_4 of PU.1 and dectin-1 in macrophages

(A) GM-CSF mRNA expression was determined by real-time RT-PCR in WT and 5-LO−/− macrophages incubated ± LTB_4 for 24 h. (**B**) GM-CSF protein was determined by ELISA in WT and 5-LO−/− macrophages incubated ± LTB_4 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 ± LTB_4 for 24 h and the expression of dectin-1 (**G**) and PU.1 (**H**) mRNA determined by real-time RT-PCR. (**I and J**) WT and GM-CSF−/− macrophages were pretreated ± LTB_4 for 24 hours followed by curdlan for another 24 h and the supernatant harvested to determine levels of TNF-α (**I**) and IL-12p40 (**J**) by ELISA. Data represent the mean ± SEM from 3 individual experiments, each performed in triplicate. *$p < 0.05$ versus WT Control; #$p < 0.05$ versus 5-LO−/− alone or LTB_4-stimulated macrophages; & $p < 0.05$ versus 5-LO−/− macrophages incubated with curdlan only by ANOVA.

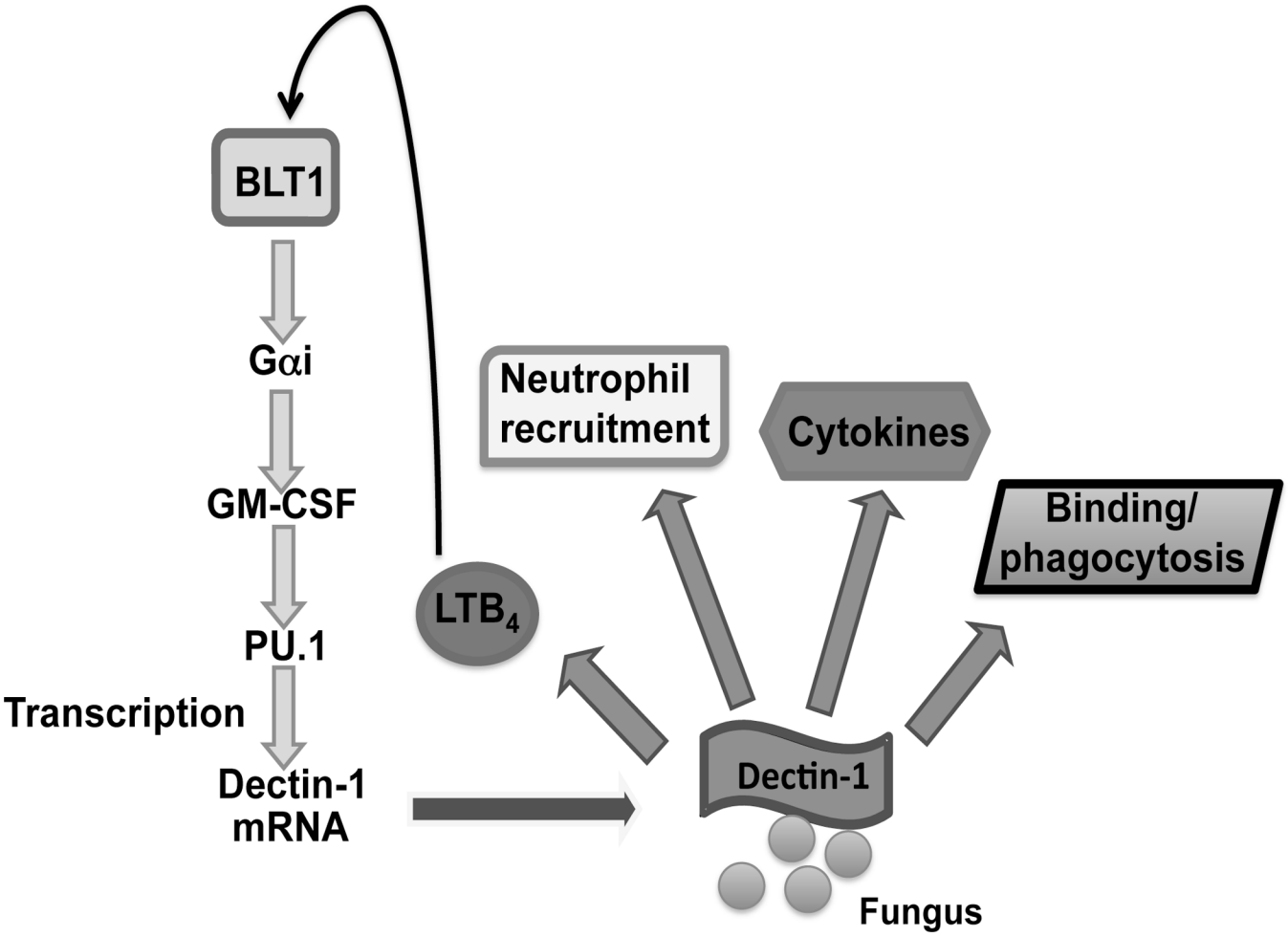

Fig. 7. Proposed model of LTB_4/BLT1 regulation of GM-CSF and PU.1 expression and enhancement of dectin-1 expression and responsiveness

Basal or dectin-1-activated LTB_4/BLT1 signaling elicits Gαi 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. LTB_4 generation in response to dectin-1 ligation represents an autocrine self-amplifying loop.