Leukotrienes Target F-actin/Cofilin-1 to Enhance Alveolar Macrophage Anti-fungal Activity*

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Candida albicans is the most common opportunistic fungal pathogen and causes local and systemic disease in immunocompromised patients. Alveolar macrophages (AMs) are pivotal for the clearance of C. albicans from the lung. Activated AMs secrete 5-lipoxygenase-derived leukotrienes (LTs), which in turn enhance phagocytosis and microbicidal activity against a diverse array of pathogens. Our aim was to investigate the role of LTB4 and LTD4 in AM antimicrobial functions against C. albicans and the signaling pathways involved.Pharmacologic and genetic inhibition of LT biosynthesis as well as receptor antagonism reduced phagocytosis of C. albicans when compared with untreated or WT controls. Conversely, exogenous LTs of both classes augmented base-line C. albicans phagocytosis by AMs. Although LTB4 enhanced mainly mannose receptor-dependent fungal ingestion, LTD4 enhanced mainly dextrin-1 receptor-mediated phagocytosis. LT enhancement of yeast ingestion was dependent on protein kinase C-δ (PKCδ) and PI3K but not PKCα and MAPK activation. Both LTs reduced activation of cofilin-1, whereas they enhanced total cellular F-actin; however, LTB4 accomplished this through the activation of LIM kinases (LIMKs) 1 and 2, whereas LTD₄ did so exclusively via LIMK-2. Finally, both exogenous LTB₄ and LTD₄ enhanced AM fungicidal activity in an NADPH oxidase-dependent manner. Our data identify LTB₄ and LTD₄ as key mediators of innate immunity against C. albicans, which act by both distinct and conserved signaling mechanisms to enhance multiple antimicrobial functions of AMs.

The importance of Candida albicans infection has grown as a result of the increased use of antimicrobial and immunosuppressive agents and of predisposing conditions such as cancer, diabetes, transplantation, HIV infection, and malnutrition (1–5). This pathogenic yeast can cause local infections at portals of entry, such as lung and genitourinary tract as well as disseminated infections. In the lung, alveolar macrophages (AMs)3 are important defenders against opportunistic fungal infections, preventing the hematogenous dissemination of C. albicans in immunocompromised hosts (6). AMs are able to recognize, ingest, and kill C. albicans through a range of pathogen recognition receptors (PRRs) including the C-type lectin-like receptor dectin-1 and the mannose receptor (CD206), representing the major macrophage receptors for β-glucan and mannan, respectively, involved in fungal recognition and ingestion (7). Binding of C. albicans to AMs causes the release of a myriad of proinflammatory mediators, including cytokines and bioactive lipids such as leukotrienes (LTs) (8, 9).

LTs are products of phospholipase A₂-derived arachidonic acid metabolism by the enzyme 5-lipoxygenase (5-LO) and the 5-LO activating protein (FLAP) and are synthesized by phagocytes in response to inflammatory or infectious stimuli (10). There are two main classes of LTs, namely LTB₄ and the cysteinyl-LTs (cysLTs), which include LTC₄, LTD₄, and LTE₄; these act by ligating the high affinity G protein-coupled receptors BLT1 and cysLT1, respectively (11, 12). LT receptor ligation enhances many aspects of AM activation, including leukocyte accumulation (11), microbial ingestion (13) and killing (14), and generation of proinflammatory mediators (10). We have previously characterized some of the signaling pathways by which LTs enhance AM antimicrobial functions against IgG-opsonized pathogens recognized by the Fcγ receptor (FcR) (15–17). Because of the diversity of signals derived from different phagocytic receptors, the importance of LTs in amplifying phagocytosis could be unique to IgG-coated target ingestion. In addition, during active acute infection, the importance of FcR signaling in the early events of host defense is controversial. Thus, it is of interest to investigate the importance of LTs in mediating AM phagocytosis by non-opsonic receptors.

There is increasing evidence that defects in LT synthesis contribute to impaired innate immunity in a variety of immunosuppressive states, such as malnutrition (18), bone marrow transplantation (19), and HIV infection (20, 21). In view of the importance of LTs in host defense along with the underprodu-

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3 The abbreviations used are: AM, alveolar macrophage; S-LO, 5-lipoxygenase; PRR, pathogen recognition receptor; FLAP, five-lipoxygenase activating protein; BLT1, leukotriene B4 receptor 1; LT, leukotriene; cysLT1, cysteinyl LT receptor 1; FcR, Fcγ receptor; ROI, reactive oxygen intermediate; CFU, colony forming unit; LIMK, LIM kinase.
tion of LTs observed in immunosuppressive states (22), the present study was undertaken to investigate the role of LTs and the signaling pathways involved in the anti-fungal activity of AMs against the opportunistic pathogen \textit{C. albicans}. Our data show that both endogenous as well as exogenous LTB$_4$ and LTD$_4$ enhance phagocytosis of \textit{C. albicans} by promoting F-actin polymerization and assembly and killing via NADPH oxidase activation and reactive oxygen intermediate (ROI) generation.

**EXPERIMENTAL PROCEDURES**

**Reagents**

RPMI 1640 was purchased from Invitrogen. LTB$_4$ and LTD$_4$ were purchased from Biomol. The inhibitors of protein kinase Ca (PKCa) (Ro-32-0432) and PKCd (rottlerin) were supplied by Calbiochem. PI3K inhibitors (LY290042 and wortmannin), 5-LO inhibitors (AA861 and Zileuton), the cysLT1 receptor antagonist MK571, and the NADPH oxidase inhibitor DPI were supplied by Enzo. CP105696 (BLT1 antagonist) was a generous gift of Pfizer. MK0591 (FLAP inhibitor) was from Merck. Alexa488-phalloidin and Alexa594-deoxyribonuclease I (DNase I) were from Molecular Probes. Laminarin (a soluble glucan prepared from \textit{Saccharomyces cerevisiae}) and mannan prepared from \textit{Laminaria digitata} and mannann prepared from \textit{Saccharomyces cerevisiae} were both from Sigma. 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**

Female pathogen-free 5-LO$^{-/-}$ (129-Alox5tm1Fun) mice (23), strain-matched wild-type (WT) sv129 mice, and Wistar rats were obtained from Central Laboratory Animal Medicine of University of São Paulo as well as Charles River Laboratories (Portage, MI). All experiments were in accord with ethical principles in animal research adopted by the Brazilian College of Animal Experimentation and the National Institutes of Health guidelines for the use of experimental animals, with the approval of the Animal Subject Committee of the Biomedical Sciences Institute, University of São Paulo, and the University of Michigan Committee for the Use and Care of Animals.

**Cell Isolation and Culture**

Rat or murine AMs were obtained by bronchoalveolar lavage as described (24). In general, rat cells were used for pharmacologic and signaling studies, and murine cells were used for comparisons between 5-LO$^{-/-}$ and WT genotypes. Murine resident peritoneal macrophages were obtained as described (25). 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**

\textit{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 (1% neopeptide, 2% dextrose (Difco)) with shaking. The cultures were washed in sterile nonpyrogenic PBS, counted with a hemocytometer, and diluted to 2 x 10$^3$ colony forming units (CFU)/ml in sterile nonpyrogenic PBS. \textit{C. albicans} was used either live or killed (through heating for 30 min at 56 °C) as indicated throughout the text and figure legends.

**Measurement of LTB$_4$ and CysLTs in the Supernatant of AM Cultures**

Levels of LTB$_4$ and cysLTs in the supernatants of AMs (5 x 10$^5$) stimulated with 10:1 live or heat-killed \textit{C. albicans} for different time points were determined using EIA kits (Cayman Chemical Co.) as described (26). In another experimental setting, AMs were pretreated with laminarin or mannan (100 μg/ml each) for 20 min and stimulated with live or heat-killed \textit{C. albicans} (10:1) for 15 min followed by LTB$_4$ determination.

**Phagocytosis Assays**

**Light Microscopic Assay**—2 x 10$^5$ WT and 5-LO$^{-/-}$ AMs were plated in 4-well chamber slides (Nunc). In another set of experiments, rat AMs were plated on 8-well glass coverslips in 24-well cell culture-treated dishes (BD Biosciences) and were pretreated or not with inhibitors of PKCd (6 μM rottlerin), PKCa (9 mM Ro-32-0432), PI3K (10 μM LY 290042 and 10 mM wortmannin), or ERK1/2 (by 2 μM PD98059) and p38 (by 10 μM SB202190) for 20 min before the addition of LTB$_4$ or LTD$_4$ (both at 10 or 100 nM as indicated in the legends). In another set of experiments, AMs were pretreated for 20 min with a 5-LO inhibitor (AA-861, 10 μM), a BLT1 antagonist (CP105696, 10 μM), or a cysLT1 antagonist (MK571, 10 μM) (24) followed by the addition of 30:1 live \textit{C. albicans}:AM for 90 min. The inhibitor doses used were based on previously published observations (24, 27–30). After 10 min of incubation with or without LTs, AMs were challenged with a multiplicity of infection of 30:1 live \textit{C. albicans} for 90 min at 37 °C. Wells were then washed three times with warmed PBS to remove noningested yeast. \textit{C. albicans} phagocytosis was assessed as described previously (31). Results were expressed as the phagocytic index, which was derived by multiplying the percent of macrophages containing at least one ingested target by the mean number of phagocytosed targets per positive macrophage.

**Fluorometric Assay**—The ability of AMs to phagocytose \textit{C. albicans} was also assessed using a previously published protocol for determining the ingestion of fluorescent, heat-killed FITC-labeled \textit{C. albicans} ($^{51}$FITC \textit{C. albicans}) (32). Briefly, heat-killed \textit{C. albicans} were labeled with FITC (33). 4 x 10$^5$ murine or rat AMs were seeded in replicates of 5- in 96-well tissue culture plates with opaque sides and optically clear bottoms (Costar, Corning Life Sciences). On the following day AMs were challenged with heat-killed \textit{C. albicans} using a multiplicity of infection of 10:1 for 90 min to allow phagocytosis to occur. In another set of experiments, AMs were incubated with laminarin and/or mannann (100 μg/ml each) or anti-decitin receptor (clone 2A11; 1 μg/ml) or anti-mannose receptor (clone 15–2; 10 μg/ml) for 30 min before the addition of LTs or \textit{C. albicans}. Trypan blue (250 μg/ml; Molecular Probes) was added for 10 min to quench the fluorescence of extracellular yeast, and fluo-
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rescence was determined using a Spectramax Gemini EM fluorometer at settings of 485 excitation/535 emission (Molecular Devices). The phagocytic index was calculated as previously described in relative fluorescence units (32).

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 described (34). Cofilin-1 mRNA was normalized to β-actin, and the respective untreated control was set to 100%.

RNA Interference
RNA interference experiments were performed according to a protocol provided by Dharmacon. AMs were transfected using DharmaFECT 1 reagent with 30 nm concentrations of nonspecific control or specific ON-TARGET SMARTpool cofilin-1. After 48 h of transfection, AMs were harvested for mRNA or fluorometric phagocytosis assay.

Confocal Microscopy
A total of $2 \times 10^7$ AMs were plated in 4-well chamber slides (Nunc), incubated with or without 10:1 heat-killed *C. albicans* for 15 min, and then washed with PBS. Slides were fixed in 4% paraformaldehyde in PBS for 30 min at room temperature and permeabilized with 0.5% Triton X-100 in PBS for 3 min. Cells were then blocked with 2% BSA in PBS and incubated with phospho-cofilin-1 (Ser-3) (1:1000, Cell Signaling), phospho-LIMK1 (Thr-505) (1:1000, from Biocytogen), and β-actin (1:10,000, Sigma). After incubation at 37°C for 24 h, CFU were counted, and the survival of ingested yeast was calculated by subtracting the background from each fluorophore (not shown). Cells were then stained with FITC-phalloidin (1 μm) to stain F-actin, according to the manufacturer’s protocol (Molecular Probes). Cells were washed three times in PBS, and fluorescence was detected using a Spectramax Gemini EM fluorometer at settings of 485 excitation/535 emission (Molecular Devices).

Western Blotting
2 × 10^5 AMs were plated in 6-well tissue culture dishes and were either stimulated or not with 100 nM LTB₄ or 100 nM LTD₄ for 5 min followed by the addition of 10:1 heat-killed *C. albicans* for 15 min. AMs were lysed in buffer (50 mM Tris–HCl (pH 7.4), 25 μM 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 Cl (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 (35) using primary antibodies against total and phospho-cofilin-1 (Ser-3) (1:1000, Cell Signaling), phospho-LIMK1 (Thr-508), total and phospho-LIMK-2 (Thr-505) (1:1000, from Abcam), and β-actin (1:10,000, Sigma). Densitometric analysis was as described (36); the intensity of phosphorylation was quantitated as the density of the phosphorylated protein band divided by that of the actin or the respective total protein band, 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.

Fungicidal Activity Assay
The ability of AMs to kill *C. albicans* was evaluated using an assay of CFU as described (37, 38). Briefly, WT and 5-LO⁻/⁻ mouse cultures or rat AMs (1 × 10^5) were plated in 12-well tissue culture dishes. The next day live *C. albicans* yeast cells were added to the macrophages at a multiplicity of infection of 5:1 (*C. albicans*:AMs) for 1 h to allow phagocytosis to occur. Then cells were stained with compounds of interest for 20 min or as indicated in the figure legends and were incubated at 37°C for 3 h. Subsequently, all wells were extensively washed to remove extracellular fungi, and the cells were collected and centrifuged at 4500 × g for 5 min. The pellet was resuspended with 1 ml of sterile water to lyse the cells. The contents were vortexed vigorously and then were serially diluted and plated on Sabouraud dextrose agar plates. Inspection of the initial lysate revealed only single colonies, 98% of which were still in the yeast phase. After incubation at 37°C for 24 h, CFU were counted, and the percentage of *C. albicans* cells that were killed was calculated by comparison to the CFU obtained from the original inoculum, which also was quantified by serial dilution and plating. Results were expressed as percent survival of ingested yeast, where the survival of ingested yeast = 100% × before inoculum CFU/CFU 3 h after AM incubation.

Measurement of ROI
AMs were added to 96-well plates at a concentration of 4 × 10^5 cells/well and cultured overnight in RPMI 1640 containing 10% FCS. The next day, medium was replaced with PBS con-
taining 10 μM H2DCF (a cell-permeable oxidant sensitive fluorophore), and the cells were cultured for 1 h. The medium was then replaced with warmed HBSS, and the cells were stimulated with heat-killed C. albicans using a multiplicity of infection of 10:1. ROI production was assessed after 90 min by measuring fluorescence using a Spectramax Gemini XS fluorometer (Molecular Devices) with excitation/emission setting at 493/522 nm. To assess the effect of LTs on ROI production by AMs, LTB₄ (100 nM) and LTD₄ (100 nM) and heat-killed C. albicans (10:1) were added to this solution before the addition to the AMs.

**Statistical Analysis**

Graphs represent the mean ± S.E. from three-six independent experiments. The means from different treatments were compared by analysis of variance. When significant differences were identified, individual comparisons were subsequently analyzed with the Bonferroni t test for unpaired values. When two groups were compared, we performed paired Student’s t tests. Statistical significance was set at a p value =0.05.

**RESULTS**

**Phagocytosis of C. albicans by AMs Requires the Generation of Both Classes of LTs**—First, we compared the ability of live and heat-killed C. albicans to induce LTB₄ and cysLT synthesis in rat AMs at time points relevant to the process of yeast ingestion. Both live and heat-killed C. albicans induced similar levels of LTB₄ (100 pg/ml, which corresponds to ~0.5 nM) after 5 min of fungal challenge. However, only live C. albicans further enhanced LTB₄ synthesis at the later time point tested (Fig. 1A). The synthesis of cysLTs was also induced by both live and heat-killed C. albicans but at levels lower than LTB₄ at all time points studied; this was expected, based on the known capacity for rat AMs to synthesize greater quantities of LTB₄ than cysLTs in response to a variety of stimuli (22, 39). Together, our data indicate that LT biosynthesis is a component of the normal macrophage response to this microbe. Next, we asked if LTs produced during C. albicans ingestion have a functional role in AM antimicrobial function. The phagocytic capacity over 90 min of AMs pretreated or not with the 5-LO inhibitor AA861 (10 μM) or the FLAP inhibitor MK886 (1 μM) was determined in C and D, fluorometric assay was performed, and phagocytic indices were calculated and expressed as a percentage of the control. Data are the mean ± S.E. from 3–5 separate experiments. *, p < 0.05 comparing treated to untreated groups or 5-LO⁻⁻⁻ to WT AMs.
Microscopically, LT inhibition by both the 5-LO and FLAP inhibitors reduced phagocytosis of live *C. albicans* by ~60% when compared with untreated cells (Fig. 1B). To confirm the effects of pharmacologic inhibition of LT synthesis on yeast ingestion determined microscopically, we utilized 5-LO−/− AMs (which cannot synthesize LTs) and control WT AMs in a fluorometric assay of heat-killed FITC-*C. albicans* phagocytosis. We observed a 60% decrease in yeast ingestion in 5-LO−/− cells when compared with WT AMs (Fig. 1C). We also saw a similar reduction in phagocytosis by LT-deficient peritoneal macrophages, suggesting that LTs are necessary for optimal phagocytosis in diverse macrophage populations (Fig. 1D). Thus, LT generation is required for optimal ingestion of both live and heat-killed *C. albicans*. The importance of individual LT classes in *C. albicans* ingestion was evaluated using BLT1 and cysLT1 antagonists. Pretreatment with BLT1 antagonist (CP105,696, 1 μM) and cysLT1 antagonist (MK571, 10 μM) resulted in a 45 and 60% reduction, respectively, in live *C. albicans* ingestion (Fig. 1E). These data suggest that LTs readily produced during *C. albicans* ingestion are required for optimal yeast phagocytosis.

Having established the importance of endogenously produced LTB₄ and LTD₄ for the optimal phagocytic capacity of AMs, we next investigated if the addition of LTs to AMs also amplified base-line yeast ingestion. Phagocytosis of live fungus was enhanced by both LTB₄ (Fig. 2A) and LTD₄ (Fig. 2B) in a dose-dependent fashion. A dose of 100 nM concentrations of each LT caused maximal ingestion of *C. albicans*, and thus, we chose this dose for subsequent experiments. Exogenous LTB₄ and LTD₄ each fully restored the deficient phagocytic capability of 5-LO−/− AMs, confirming the importance of LTs for macrophage phagocytosis (Fig. 2C). Interestingly, a combination of LTB₄ plus LTD₄, each used at a submaximal dose of 10 nM, amplified heat-killed FITC-*C. albicans* ingestion to a greater extent than did either LTB₄ or LTD₄ alone (Fig. 2C). The same pattern was also observed when testing the effects of exogenous LTs on fungal ingestion employing live *C. albicans* (Fig. 2D). Consistent with the greater level of LT synthesis in response to live versus heat-killed fungus, the dependence of phagocytosis on LTs as reflected by the magnitude of the phagocytic defect in 5-LO−/− AMs was likewise greater with live than with heat-killed *C. albicans* (Fig. 2, C and D). Together, these findings show that both classes of LTs are required for optimal *C. albicans* ingestion in AMs.

Identification of the Signaling Pathways Involved in LT Enhancement of Yeast Ingestion—Next, we sought to determine the role of dectin-1 and mannose receptor in yeast phagocytosis and the extent to which ingestion through each was influenced by both classes of LTs. Mannose receptor antagonism by both mannan and anti-mannose receptor antibody inhibited FITC-*C. albicans* phagocytosis by ~40%, and dectin-1 receptor blockade by both laminarin and anti-dectin-1 antibody inhibited fungal ingestion by ~60%. The combination of both mannan and laminarin treatment impaired yeast ingestion by ~80% (Fig. 3A). Although LTB₄ enhancement of yeast phagocytosis was abolished when AMs were pretreated with mannan and partially inhibited by laminarin, LTD₄ effects were shown to depend primarily on dectin-1 recognition. Conversely, inhibition of both mannose and dectin-1 receptors completely inhibited the stimulatory effect of both classes of LTs (Fig. 3B). We also investigated which PRR is responsible for LTB₄ production during *C. albicans* infection. We pretreated AMs with mannan and laminarin in block mannose receptor and dectin-1, respectively, followed by the addition of either live or heat-killed *C. albicans* for 15 min. Although LTB₄ production induced by live yeast was mainly dependent on dectin-1 and partially dependent on mannos receptor, that induced by heat-killed *C. albicans* was completely dependent on dectin-1 activation (Fig. 3, C and D).

Previously, we showed that BLT1 but not cysLT1 uses Gα₄ protein to enhance AM antimicrobial functions (17). However, the specific G proteins by which LTs enhance PRR-mediated phagocytosis of unopsonized pathogens is unknown. Here, we sought to investigate the role of Gα₄ in mediating LTB₄/LTD₄ effects on *C. albicans* ingestion. Gα₄ inhibition by pertussis toxin pretreatment, as described previously (17), abolished the effect of LTB₄, but not LTD₄, on *C. albicans* phagocytosis (Fig. 4A). These data show that Gα₄ mediates BLT1 effects on AM phagocytosis via PRRs.
Next, we investigated the downstream signaling pathways involved in the enhancement of yeast phagocytosis. We focused on the requirement of specific kinases previously shown to be important for LT enhancement of FcR-mediated phagocytosis, including PKCα and -δ, PI3K, and the MAPKs p38 and ERK1/2 (16). Inhibition of PKCδ, but not PKCα, abolished both LTB₄ and LTD₄ enhancement of C. albicans phagocytosis (Fig. 4B). As a confirmatory approach, we utilized intracellular delivery of a specific mouse monoclonal anti-PKCα or -δ IgG into rat AMs using a liposomal reagent (16). We have previously confirmed the specificity and efficacy of this technique (16, 40, 41). Anti-PKC-δ, but neither anti-PKCα nor nonspecific mouse IgG (not shown), blocked the ability of both LTs to augment phagocytosis (Fig. 4C). We did not observe any effect of the anti-PKCα or -δ Abs on basal phagocytosis (data not shown).

PI3K is pivotal for the engulfment of a variety of phagocytic targets (42). To investigate if PI3K activation is important for LT-enhanced phagocytosis, AMs were treated with two specific PI3K inhibitors, wortmannin (10 nm) and LY290042 (10 μM). We found that the potentiating effects of both LTB₄ and LTD₄ on yeast phagocytosis were abolished by both inhibitors (Fig. 4D).

Another class of kinases known to participate in C. albicans phagocytosis is the MAPKs, namely, p38 and ERK1/2 (43, 44). We investigated their role in LT amplification of C. albicans phagocytosis using well established pharmacologic inhibitors. The pharmacologic inhibition of ERK1/2 (by 2 μM PD98059) and p38 (by 10 μM SB203580) had no effect on LTB₄ or LTD₄ potentiation of phagocytosis (Fig. 4E). Moreover, neither ERK1/2 nor p38 influenced basal C. albicans phagocytosis (not shown). Collectively, our data demonstrate that both classes of LTs utilize similar kinase programs to enhance yeast phagocytosis.

**LTs Enhance F-actin Assembly during C. albicans Phagocytosis**—Actin polymerization is required for particle ingestion by phagocytes (45, 46). Both LTB₄ and LTD₄ are capable of
enhancing actin polymerization in leukocytes and epithelial cells (47). However, the role of LTs in assembly of polymerized F-actin during phagocytosis is unknown, and we interrogated this using WT and 5-LO/−/− AMs. By using confocal microscopy we observed that 5-LO/−/− AMs exhibited higher levels of G-actin and lower levels of F-actin when compared with WT cells (Fig. 5 A, i and ii). Interestingly, both exogenously provided LTB4 and LTD4 increased F-actin and reduced G-actin levels during yeast phagocytosis in 5-LO/−/− AMs (Fig. 5 A, iii and iv). We also determined fluorometrically the importance of LTs on F-actin levels present during live C. albicans ingestion in rat AMs treated with or without LTs; C. albicans enhanced F-actin levels in rat AMs, and both LTB4 and LTD4 potentiated yeast-induced F-actin polymerization. Moreover, the combination of both LTs elicited an additive effect (Fig. 5B). We next determined if kinases involved in potentiation of fungal ingestion by LTB4 and LTD4 also contribute to potentiation of F-actin levels. Indeed, both PI3K and PKCδ inhibition decreased F-actin levels in AMs challenged with both LTB4 and LTD4 during live C. albicans ingestion (Fig. 5C). These results indicate that potentiation of F-actin assembly in AMs is key to the enhancement of C. albicans phagocytosis by both LTB4 and LTD4 and is dependent on both PI3K and PKCδ.

Actin-binding proteins, including cofilin-1, regulate assembly and disassembly of actin filaments (48). Cofilin-1 causes depolymerization at the minus end of filaments, thereby preventing their reassembly (48), and inhibits FcR-mediated phagocytosis (49). However, the importance of this protein in C. albicans phagocytosis is unknown. Thus, we tested the possibility that LTs potentiate F-actin levels by inhibiting the activation of cofilin-1. Cofilin-1 mRNA was silenced with siRNA (Fig. 6A), and yeast ingestion was increased 2-fold when compared with control siRNA (Fig. 6A), identifying an effect for this actin-binding protein in limiting AM phagocytosis of C. albicans. Cofilin-1 is inactivated by phosphorylation on Ser-3, which results in inhibition of F-actin disassembly, thus allowing actin polymerization. To examine the effect of LTs on cofilin-1 phosphorylation in phagocytosing AMs, we utilized both confocal microscopy in WT and 5-LO−/− AMs (Fig. 6C) and immunoblotting (Fig. 6D). In control WT AMs, C. albicans challenge increased cofilin-1 phosphorylation and, therefore, actin polymerization. As shown in Fig. 6C, i and ii, 5-LO−/−
AMs displayed decreased cofilin-1 phosphorylation and concomitantly less actin polymerization upon exposure to *C. albicans* than did WT cells. That 5-LO−/− AMs were unable to phosphorylate cofilin-1 upon *C. albicans* challenge was confirmed by immunoblot (Fig. 6D). Next, we sought to investigate the role of specific LTs on cofilin-1 phosphorylation. Treatment of 5-LO−/− AMs with LTD4 and LTB4 restored both cofilin-1 phosphorylation and actin polymerization to levels observed in WT cells infected with *C. albicans* alone (Fig. 6C, iii and iv). Both classes of LTs also enhanced cofilin-1 phosphorylation in rat AMs, and again LTB4 was more effective than LTD4 in doing so (Fig. 6, E and F). LIM kinases (LIMKs) catalyze the phosphorylation of cofilin-1 on serine 3, and their overexpression reverses cofilin-induced actin depolymerization, leading to accumulation of actin filaments and aggregates (50). Thus, we asked if LTs enhanced activation of LIMK-1 and/or -2. *C. albicans* challenge itself increased phosphorylation of LIMK-1 but not LIMK-2 (Fig. 6, E and F). Interestingly, although LTB4 increased activation of both LIMK-1 and -2, LTD4 enhanced only the phosphorylation of LIMK-2. These results demonstrate that both LTB4 and LTD4 decreased the activation of cofilin-1, the cellular brake on actin polymerization, thereby enhancing F-actin assembly and optimizing *C. albicans* ingestion. However, LTB4 exerted stronger effects that correlated with an ability to act via both LIMK-1 and -2, as opposed to the effects of LTD4, which were limited to activation of LIMK-2.

**LTs Enhance AM Candidacidal Activity**—We previously reported that exogenous LTB4 and LTD4 enhanced intracellular killing of IgG-coated *Klebsiella pneumoniae* as well as *Leishmania amazonensis* (14, 25). We, therefore, hypothesized that LTs, in addition to enhancing *C. albicans* phagocytosis, also enhanced AM fungicidal activity. Because ingestion is a prerequisite for intracellular killing and because LTs enhance yeast ingestion, an assay that distinguishes effects on killing from those on phagocytosis was necessary. We accomplished this by adding LTB4 or LTD4 themselves after the ingestion of *C. albicans* was completed. With such a protocol, exogenous addition of both LTB4 and LTD4 enhanced basal fungicidal activity of LT-deficient AMs (Fig. 7A). Moreover, 5-LO−/− AMs exhibited reduced fungicidal activity (i.e. increased the survival of ingested yeast) than did WT AMs, suggesting that endogenously produced LTs are necessary for the optimal killing of *C. albicans* by AMs. *C. albicans* growth was not directly affected by the addition of LTs to macrophage-free cultures (data not shown). These data show that LTs are key mediators that promote two distinct and important steps in the control of yeast infection; that is, ingestion as well as killing.

**LT Enhancement of Candidacidal Activity Involves NADPH Oxidase Activation**—ROIs generated by NADPH oxidase are important in killing ingested yeast (51, 52), and LTs are known to activate this oxidase in AMs and other cell types (14, 26). Thus, we evaluated the role of NADPH oxidase-derived ROIs in LT enhancement of *C. albicans* killing. Cells were pretreated with the NADPH oxidase inhibitor DPI (53) followed by the addition of both classes of LTs and then *C. albicans*. DPI alone inhibited killing (i.e. increased survival of ingested *C. albicans*) by ~50% compared with untreated cells (Fig. 7B), indicating an important role for NADPH oxidase in the basal control of *C. albicans*, as previously reported (51). Exogenous LTB4 failed to overcome the killing defect in DPI-treated cells, whereas exogenous LTD4 did so significantly but...
incompletely. These results suggest that LTB4 augments candidacidal activity largely via activation of NADPH oxidase, whereas LTD4 accomplishes this partially via oxidase activation and partially via alternative microbicidal mechanisms. The impact of endogenously produced LTs on cellular ROI production in response to live \textit{C. albicans} infection was examined in WT and LT-deficient AMs. As shown in Fig. 7C, \textit{C. albicans} infection induced ROI production by WT AMs; this was inhibited in 5-LO/−/− AMs, suggesting that endogenous LTs are involved in NADPH oxidase activation and release of ROIs during \textit{C. albicans} infection. Accordingly, exogenous LTB4 and LTD4 further enhanced \textit{C. albicans}-induced ROI secretion in 5-LO/−/− cells (data not shown). As expected, DPI abolished ROI production in infected AMs (data not shown). Our results show that both classes of LTs enhance yeast killing in a manner-dependent to varying degrees on the activation of NADPH oxidase complex and ROI release.

DISCUSSION

\textit{C. albicans} is an opportunistic fungal pathogen that causes both local and disseminated infection (3, 54, 55). This pathogen can cause respiratory disease, especially in the setting of immunosuppressive therapy, bone marrow, organ transplants, and HIV infection. It is increasingly apparent that a variety of clinical circumstances are associated with an acquired defect in LT synthesis that itself confers increased susceptibility to infection; examples include HIV infection, bone marrow transplant, vitamin D3 deficiency, and cigarette smoking (10). Here we have explored the role of LTs in phagocytosis and killing of \textit{C. albicans} by AMs, the resident innate immune defender of the alveolar surface. Overall, our results show that 1) AMs quickly synthesize and release both LTB4 and cysLTs in response to \textit{C. albicans}, 2) both classes of LTs are required for optimal phagocytosis and killing of the fungi, 3) LTB4 enhances mannose and dectin-1 receptor phagocytosis, whereas LTD4 is
Leukotrienes Enhance Macrophage Anti-fungal Activity

**A**

**B**

**C**

FIGURE 7. LTs enhance AM fungicidal activity against *C. albicans*. A, AMs from 5-LO−/− and WT mice were incubated with live *C. albicans* at a ratio of 1:5 for 60 min to allow ingestion of the fungi. Cells were then treated with LTB₄ (100 nM) or LTD₄ (100 nM). The antifungal activity of AMs was measured after 3 h by comparing the CFU of each experimental group compared with control. B, rat AMs were pretreated with the NADPH oxidase inhibitor DPI (10 μM) for 20 min before the addition of live *C. albicans* (1:5). Thirty minutes later DPI was added back with or without LTB₄ (100 nM) or LTD₄ (100 nM). Fungal activity was determined as described under "Experimental Procedures." C, AMs were treated with or without LTB₄ (100 nM) or LTD₄ (100 nM) for 5 min, after which PBS containing 10 μM H2DCF was added, and the cells were cultured for 1 h. The medium was then replaced with warmed HBSS, and the cells were stimulated with heat-killed *C. albicans* (10:1). ROI production was assessed after 90 min by measuring fluorescence. Data are the mean ± S.E. from 4–8 separate experiments.

required for optimal dectin-1-mediated phagocytosis, 4) LT enhancement of phagocytosis involves the activation of PKCδ and PI3K, with subsequent activation of LIMKs, decreased cofilin-1 activation, and ultimately, F-actin assembly, and 5) LT enhancement of *C. albicans* killing involves NADPH oxidase activation and ROI generation.

Inhibition of LT synthesis or activity has likewise been reported to impair host defense against a myriad of pathogens, including bacteria (56, 57), viruses (58–60), fungi (20, 61), and parasites (25, 62). That endogenous generation of both classes of LTs is important in the macrophage phagocytic and microbicidal response during *C. albicans* infection was revealed through both pharmacologic and genetic means. *C. albicans* can be recognized by a variety of PRRs including dendin-1 and the mannose receptor, each of which can activate specific signaling pathways required for yeast ingestion (7). Here we show that LTB₄ production is required for optimal mannose receptor phagocytosis and partially necessary for dendin-1-mediated ingestion. In contrast, LTD₄ is essential for optimal dendin-1 phagocytosis. Such findings correlate with the differential role of dendin-1 and mannose receptor in generating LTB₄ during *C. albicans* ingestion. Although live and heat-killed yeast utilize dendin-1 to enhance LTB₄ production, mannose receptor participates only in live *C. albicans*-induced LT synthesis. The greater importance of dendin-1 recognition in mediating heat-killed *C. albicans*-induced LTB₄ production could reflect the exposure of β-glucan in the yeast exposed to high temperatures (63). In addition, our data are in agreement with previous reports showing that dendin-1 is the main receptor involved in both arachidonic acid release and LT secretion in macrophages (9, 64, 65). Little is known about the importance of arachidonic acid metabolites including LTs in enhancing fungal ingestion. Balestrieri et al. (67) identified no defect in phagocytosis of the yeast cell wall product zymosan in LTC₄ synthase-deficient peritoneal macrophages. In addition, Okamoto et al. (68) have shown that BLT₁−/− bone marrow-derived macrophages exhibit lower phagocytic capability of IgG-opsonized zymosan but not of non-opsonized zymosan. Although our work focused on AMs, we did observe a similar dependence on 5-LO products for phagocytosis by peritoneal macrophages. The discrepancy between these other reports and our own may, therefore, reflect differences in the PRRs recognizing *C. albicans* versus zymosan or strain of *C. albicans* and the possibility that LTC₄ synthase-deficient cells might overproduce LTB₄. Although Balestrieri et al. (38) showed that secretory PLA₂ activation was required for optimal *C. albicans* ingestion and killing, they did not address the role of specific arachidonic acid metabolites. In contrast, our work provides mechanistic insight into the anti-fungal actions of specific LTs.

Although Goq signaling was utilized exclusively by LTB₄/BLT₁, enhanced phagocytosis induced by both LTD₄ and LTB₄ depended on PKCδ and PI3K, as revealed by both kinase inhibitors and intracellular antibody blockade. We speculate that PI3K is upstream of PKCδ, based on previous work showing that PI3K-induced NADPH oxidase activation is dependent on PKCδ activation (69). Because both PI3K and PKCδ have been previously implicated in phagocytosis as well as in F-actin polymerization (70–72), we explored the effects of LTs on F-actin assembly and the possible signaling programs involved. Both classes of LTs enhanced F-actin assembly and decreased monomeric G-actin levels. Interestingly, LTD₄ has been reported to enhance F-actin polymerization in intestinal epithelial cells in a PKCδ- and PI3K-dependent manner (73). It also has been shown that LTB₄ enhances actin polymerization in neutrophils (74) and T cells (75). We show that both classes of kinases were required for enhanced F-actin polymerization during *C. albicans* challenge by LTB₄ and LTD₄. We also observed that LTB₄ enhanced the amount of F-actin surrounding the yeast, which co-localized with phosphorylated (deactivated) cofilin-1. Actin polymerization is regulated by phosphorylation/dephosphorylation of actin depolymerizing factors such as cofilin-1 (48). Cofilin-1 is a major actin-binding protein in leukocytes and plays an important role in restraining the respiratory burst and phagocytosis (49, 76), and its activity is decreased by phosphorylation at serine-3 by LIMKs. LIMKs can be phosphorylated by Rho-family GTPases via the activation of
Rho kinase (ROCK) or via Cdc42/Rac-mediated activation of PAK1, -2, or -4 (50, 77). Here, by employing two different approaches (Western blotting and confocal microscopy) we observed that 5-LO-deficient AMs exhibited increased cofilin-1 activation and that LTB₄ seems to be more effective than LTD₄ in decreasing cofilin-1-mediated F-actin depolymerization. Conversely, LTB₄ amplifies LIMK-1 and LIMK-2 phosphorylation, whereas LTD₄ only enhances LIMK-2 activity. The greater effect of LTB₄ than LTD₄ on cofilin-1 phosphorylation may reflect its action via both LIMK isoforms. The underlying reason for differences in actions between the two LTs remains unknown. However, LTB₄/BLT1 activates and employs a greater variety of signaling pathways than does LTD₄/cysLT1 to enhance AM FcR-mediated phagocytosis (78). Cross-talk between LT signaling and C. albicans signaling could conceivably involve specific cross-activation of dectin-1 and/or mannose receptors by LTD₄ and LTB₄, respectively, or translocation of those receptors to specific membrane microdomains such as lipid rafts, and association among BLT1 and/or cysLT1 and the fungal PRR(s). In this regard we have shown that BLT1 is more potent than cysLT1 in enhancing FcR-mediated phagocytosis (79). Furthermore, BLT1 associates with FcRI, forming a signaling platform composed of G proteins and kinases, and this platform is required for optimal phagocytosis (78). The observed divergence between BLT1 and cysLT1 in activating LIMKs could be due to activation of different upstream effectors. LIMK-1 is phosphorylated by p21 activated kinases (PAKs) 1–4, whereas LIMK-2 is phosphorylated by PKCθ. However, whether PKCθ is the kinase involved in LTB₄ or LTD₄-induced LIMK-2 phosphorylation remains to be determined.

Not only did LTs amplify phagocytosis, but they also increased AM candidacidal activity in a manner dependent on NADPH oxidase activation and ROI generation. We have previously confirmed the role of NADPH oxidase in LTB₄-mediated killing of K. pneumoniae as suggested by pharmacologic studies with AMs derived from gp91phox⁻/⁻ mice (14). In addition, LTB₄ can promote NADPH oxidase activation by various mechanisms, including enhancement of phosphorylation and membrane translocation of the NADPH oxidase subunit p47phox (14) and also by enhancing the expression of the gp91phox subunit (80). Interestingly, LTD₄-induced C. albicans killing was only partially dependent on NADPH oxidase activation. Thus, LTD₄ might induce the secretion of candidacidal molecules other than ROI, such as nitric oxide (81) and defensins (82). Recently, Flamand et al. (66) administered LTB₄ intravenously to monkeys and found increased α-defensin plasma levels and enhanced ex vivo antimicrobial activities of plasma.

Previous studies have detailed how signals emanating from G protein-coupled LT receptors enhance AM ingestion and killing of targets recognized by the opsonic Fcr (16, 78). The present work extends this concept of LT-mediated phagocytic signal amplification to ingestion via distinct PRRs recognizing C. albicans. We have recently reported that LTB₄/BLT1/Goα signaling also controls NFκB activation downstream of PRRs (36). Together, these studies highlight the emerging importance of cross-talk between LT receptors and PRR signaling.
