Leukotrienes (LTs) are lipid mediators that participate in inflammatory diseases and innate immune function. We sought to investigate the importance of LTs in regulating the microbialicidal activity of alveolar macrophages (AMs) and the molecular mechanisms by which this occurs. The role of LTs in enhancing AM microbialicidal activity was evaluated pharmacologically and genetically using in vitro challenge with *Klebsiella pneumoniae*. Exogenous LTs increased AM microbialicidal activity in a dose- and receptor-dependent manner, and endogenous production of LTs was necessary for optimal killing. Leukotriene B4 (LTB4) was more potent than cysteinyll LTs. An important role for nicotinamide adenine dinucleotide (NADPH) oxidase in LT-induced microbialicidal activity was indicated by the fact that bacterial killing was abrogated by the NADPH oxidase inhibitor diphenyleneiodonium (DPI; 10 μM) and in AMs derived from gp91phox-deficient mice. By contrast, LT-induced microbialicidal activity was independent of the generation of nitric oxide. LTs increased H2O2 production, and LTB4 was again the more potent agonist. Both classes of LTs elicited translocation of p47phox to the cell membrane, and LTB4 induced phosphorylation of p47phox in a manner dependent on protein kinase C-δ (PKC-δ) activity. In addition, the enhancement of microbicidal activity by LTs was also dependent on PKC-δ activity. Our results demonstrate that LTs, especially LTB4, enhance AM microbicidal activity through the PKC-δ-dependent activation of NADPH oxidase. (Blood. 2005;106:1067-1075) © 2005 by The American Society of Hematology

Introduction

Pneumonia is the leading cause of death from infection in the United States,1 and its global mortality is 4.3 million people per year.2 This problem is compounded by growing numbers of immunosuppressed patients and multidrug-resistant microorganisms. Developing more effective agents for the prevention and treatment of pneumonia requires a better understanding of how innate pulmonary defense mechanisms are regulated.

The alveolar macrophage (AM) patrols the epithelial surface of the distal lung and maintains sterility by phagocytosing and killing microorganisms.3,4 AMs are the first line of defense against invading microorganisms and, as such, are capable of secreting cytokines, lipid mediators, and microbialicidal molecules. Among the lipid mediators generated by AMs, the leukotrienes (LTs) play an important role in lung innate immunity, inducing neutrophil recruitment and enhancing macrophage antimicrobial functions.5 LTs are derived from the metabolism of the cell-membrane fatty acid arachidonic acid (AA) through the enzyme 5-lipoxygenase (5-LO), in concert with its helper protein, 5-LO–activating protein (FLAP).6 5-LO oxygenates AA to the intermediate 5-hydroperoxyeicosatetraenoic acid (5-HPETE), which is either enzymatically reduced by 5-LO to the unstable epoxide leukotriene A4 (LTA4) or alternatively is reduced to 5-hydroxyeicosatetraenoic acid (5-HETE). LTA4 can be hydrolyzed to form LTB4 or can be conjugated with glutathione to form the cysteinyll LTs (cysLTs), LTC4, LTD4, and LTE4.6

A role for LTs in host defense was first demonstrated by Wirth and Kierszenbaum7,8 in experiments showing that both LTB4 and LTC4 increased the phagocytosis and killing of *Trypanosoma cruzi* by peritoneal macrophages. Subsequently, numerous in vitro and in vivo models have revealed key roles for LTs in augmenting monocyte/macrophage phagocytosis9-12 and microbicidal activity.7,8,11-20 With respect to pulmonary innate immunity, 5-LO–deficient mice demonstrated impaired bacterial clearance and enhanced mortality during pneumonia caused by *Klebsiella pneumoniae*.11 In vitro, endogenous and exogenous LTs enhanced Fcγ-receptor–mediated phagocytosis by the AMs.10,21 However, effects of LTs on the microbialicidal activity of AMs and the relevant molecular mechanisms have not been carefully addressed.

Studies in non-AM cell types demonstrating LT-enhanced microbialicidal activity reveal potent effects on myriad antimicrobial molecules, including nitric oxide (NO).14,12,23 Lysozyme enzymes,24 defensins,25 and the activation of NADPH oxidase (NADPHox).26,27 NADPHox activation leads to the generation of reactive oxygen intermediates (ROIs) such as the superoxide anion (O2−) and H2O2, which are important weapons used by phagocytes (including AMs) to kill ingested bacteria.26-31 NADPHox functions as a large multisubunit complex assembled and activated through the phosphorylation and translocation of cytosolic subunits to the cytochrome b558 present in plasma membrane.32,33 Although free AA is implicated in activation,34 the
mechanisms whereby LTs enhance NADPHox activity remain obscure. In eosinophils, \(\text{LTB}_4\) was shown to enhance NADPHox activity, but the exact mechanisms responsible for this activation were not investigated.\(^{35,36}\) In neutrophils, \(\text{LTB}_4\) induced NADPHox activation through a Rac-dependent pathway; this effect was dependent on ERK and cytosolic phospholipase A\(_2\) activities but not on p38 kinase.\(^{37}\)

Despite the critical role of AMs in innate immunity, little is known about the activation of NADPHox in these cells. Moreover, although AMs are known to have a far greater capacity for LT synthesis than macrophages from other sites,\(^{38}\) virtually nothing is known about the influence of these lipid mediators on this process.

Here, we demonstrate that endogenous and exogenous LTs enhance bacterial killing in a manner dependent on NADPHox activation. We further demonstrate that LT-dependent activation of NADPHox is mediated by protein kinase C-\(\beta\) (PKC-\(\beta\)).

**Materials and methods**

**Reagents**

Dulbecco modified Eagle medium (DMEM) without phenol red and RPMI 1640 were purchased from Gibco-Invitrogen (Carlsbad, CA). Tryptic soy broth (TSB) was supplied by Difco (Detroit, MI). Saponin and MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) and type IV horseradish peroxidase (HRP) were purchased from Sigma (St Louis, MO). \(\text{LTB}_4\), \(\text{LTC}_4\), \(\text{LTD}_4\), 5(5)-HETE, phorbol myristate acetate (PMA), MK886 (FLAP inhibitor); A8361 (5-LO inhibitor); MK571 (CySLT receptor antagonist); the NADPHox-like flavoprotein inhibitor diphenyl-eneiodonium (DPI), and \(\text{N}^\text{a}\)-nitro-L-arginine methyl ester (L-NAME), a nitric oxide synthase inhibitor) were purchased from Biomol (Palo Alto, CA). CP105696 (B LT receptor \(1\) antagonist) was a generous gift from Dr Henry Showell (Pfizer, Groton, CT). Amplex Red (10-acetyl-3,7-dihydroxyphenoxazine) was purchased from Molecular Probes (Eugene, OR). PKC inhibitors rottlerin and Ro-32-0432 were supplied by Calbiochem (San Diego, CA). 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**

5-LO KO (129-Alox5\(^{5\text{mfn1/Pm}}\))\(^{39}\) and strain-matched wild-type (WT) sv129 mice were bred in the University of Michigan Unit for Laboratory Animal Medicine from breeders obtained from Jackson Laboratories (Bar Harbor, ME). All guP19-phox (B6.129S6-CyPLm1Jgr/J)\(^{40}\) strain- and strain-matched C57BL/6 mice were purchased from Jackson Laboratories, and female Wistar rats were obtained from Charles Rivers Laboratories (Portage, MI). The University Committee on Use and Care of Animals approved all animal protocols.

**Cell isolation and culture**

Resident AMs from mice and rats were obtained by ex vivo lung lavage, as previously described,\(^{41,38}\) and were resuspended in RPMI to a final concentration of 2 \(\times\) 10\(^6\) cells/mL. Cells were allowed to adhere to tissue culture–treated plates for 1 hour (37°C, 5% CO\(_2\)); this was followed by one wash with warm RPMI, resulting in more than 99% of adherent cells identified as AMs by use of a modified Wright-Giemsa stain (Diff-Quik; American Scientific Products, McGraw Park, IL). Cells were cultured overnight in RPMI containing 10% fetal bovine serum and were rested twice the next day with warm medium so that nonadherent cells could be removed.

**Tetrazolium dye reduction assay of bacterial killing**

The ability of bacteria to survive within the AM was quantified using a tetrazolium dye reduction assay, as described elsewhere.\(^{41,42}\) Briefly, 2 \(\times\) 10\(^6\) rat AMs, prepared as described previously, were seeded in duplicate 96-well tissue culture dishes. The next day, \(K\) pneumoniae were opsonized with 3% anti–\(K\) pneumoniae rat-derived immune serum, as previously described.\(^9\) Cells were then treated with compounds of interest for 20 minutes, or as indicated in the figure legends, and were infected with a 0.1-mL suspension of opsonized \(K\) pneumoniae (1 \(\times\) 10\(^6\) colony-forming units [CFU]/mL; multiplicity of infection [MOI], 50:1) for 30 minutes to allow phagocytosis to occur. The bacterial killing protocol was assessed as described elsewhere.\(^{42}\) The intensity of the absorbance at 595 nm was directly proportional to the number of intracellular bacteria associated with the macrophages.\(^{41,42}\) Results were expressed as percentage of survival of ingested bacteria, where the survival of ingested bacteria = 100% \(\times\) A\(_{595}\) control plate/A\(_{595}\) experimental plate. To determine the role of exogenous 5-LO metabolites in microbicidal activity, they were added 30 minutes after infection. The role of endogenous LT or PKC-\(\alpha\) and -\(\beta\) was assessed by including biosynthesis inhibitors, receptor antagonists, or specific PKC inhibitors 20 minutes before infection. Preliminary experiments compared this colorimetric assay with a conventional CFU-based (serial dilution) assay, and similar results were obtained (data not shown).

**Cell viability**

Neither experimental compounds nor vehicles showed adverse effects on AMs or on bacterial viability, as determined by a cell-based MTT assay (data not shown).

**H\(_2\)O\(_2\) detection**

AMs were plated in 96-well dishes, as described in “Tetrazolium dye reduction assay of bacterial killing.” H\(_2\)O\(_2\) secretion from AMs was determined colorimetrically using Amplex Red (Molecular Probes, Eugene, OR) reagent, according to the instructions of the manufacturer. A solution containing 50 \(\mu\)M Amplex Red reagent and 10 \(\mu\)M HRP was prepared in phosphate-buffered saline (PBS), and 0.1 mL of this was added to AMs (5 \(\times\) 10\(^6\) per well) at 37°C for 60 minutes to determine H\(_2\)O\(_2\) generation. After this time, A\(_{560}\) was assessed. The H\(_2\)O\(_2\) concentration was determined using a standard curve with known H\(_2\)O\(_2\) concentrations. The detection limit of this method was 0.625 nM. To assess the effect of exogenous 5-LO metabolites on H\(_2\)O\(_2\) production by AMs, compounds of interest were added to this solution before the addition to the AMs, as indicated in the legend of Figure 4. In separate experiments, AMs were pretreated with AA-861, MK886, or CP105696 for 20 minutes before the addition of the Amplex Red solution containing 50:1 opsonized \(K\) pneumoniae.

**Cell fractionation, immunoprecipitation, and Western blotting**

AMs (4 \(\times\) 10\(^6\)) were plated in 6-well tissue culture dishes and were stimulated for 5 minutes with 1 nM \(\text{LTB}_4\), 100 nM L\(\text{TD}_4\), 10 nM PMA, or vehicle control. After this time, AMs were lysed by sonication in ice-cold lysis buffer containing 150 mM Tris-HCl (pH 8.0), 100 mM NaCl, 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 50 mM NaF, and 1 \(\mu\)g/mL leupeptin, followed by ultracentrifugation at 100,000g for 20 minutes at 4°C. The cytosolic (soluble) fraction was harvested, and the membrane (insoluble) fraction was washed and subjected to another ultracentrifugation step as described above. The resultant pellet was resuspended in lysis buffer and sonicated. Protein concentrations were determined by a modified Coomassie dye-binding assay (Pierce Chemical, Rockford, IL). The cytosolic fraction was used for immunoprecipitation, as described previously,\(^{21}\) with some modifications. The fraction was incubated overnight at 4°C with anti-p47phox antibody (1:80; Upstate Biotechnology, Lake Placid, NY). Protein A-Sepharose was added to each sample and was incubated for 3 hours with rotation at 4°C. Beads were washed briefly 3 times with lysis buffer without Triton X-100, and samples containing 20 \(\mu\)g protein were separated on 8% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) gels transferred to nitrocellulose membranes. After blocking with 5% nonfat milk, membranes were probed with anti-p47phox antibody (1:500 dilution; Upstate Biotechnology); anti-FLAP (1:5000; provided by Dr. J. Evans, Merck Frosst) or antiposphoserine (1:900; Ki-191, clone IC8; Biomol) for 90 minutes,
followed by peroxidase-conjugated goat antirabbit (Amersham, Piscataway, NJ) or antimouse secondary (1:5000; Zymed, South San Francisco, CA), and were developed using enhanced chemiluminescence (ECL) detection (Amersham). Relative band densities were determined by densitometric analysis using National Institutes of Health Image software. In all instances, density values of bands were corrected by subtraction of the background values.

Statistical analysis

Data are represented as mean plus or minus SE and were analyzed with the Prism 3.0 statistical program (GraphPad Software, San Diego, CA). Comparisons between 2 experimental groups were performed with the Student t test. Comparisons among 3 experimental groups were performed with analysis of variance (ANOVA) followed by Bonferroni analysis. Differences were considered significant if P equaled .05. All experiments were performed on 3 separate occasions unless otherwise specified.

Results

5-LO products enhance the microbicidal activity of AMs infected with opsonized K pneumoniae

To determine the capacity of 5-LO products to modulate the microbicidal activity of AMs, we examined the effect of exogenously added LTB₄, LTC₄, LTD₄, and 5-HETE on the killing of immune serum–opsonized K pneumoniae. As demonstrated in Figure 1, all products caused a dose-dependent increase in microbicidal activity, reflected by a decrease in survival of bacteria that were ingested. The maximal effect (approximately 55% increase in killing) was similar for all metabolites, but potency differed—maximal effect was reached at 1 nM for LTB₄, 100 nM for cysLTs, and 1000 nM for 5-HETE.

Endogenous LTs augment the killing of ingested bacteria by AMs

Given that exogenously added LTs enhance bacterial killing by AMs and given that AMs have a high capacity to generate endogenous LTs when challenged with K pneumoniae, we next considered the role of endogenous LTs in AM killing by evaluating the microbicidal activity of AMs harvested from mice genetically deficient in 5-LO. In these animals, we observed an increase of approximately 55% in the survival of intracellular bacteria when compared with the AMs from WT mice. (Figure 2A).

As a complementary approach, we used pharmacologic means to inhibit the production of endogenous LTs by rat AMs or their ability to bind their respective receptors on the AM cell surface. As illustrated in Figure 2B, the inhibition of 5-LO by AA861 or FLAP by MK886 increased the survival of ingested bacteria by approximately 65% compared with untreated controls. To determine the specificity of these compounds, we performed “add-back” experiments, applying a mixture of optimal concentrations of exogenous LTs (1 nM LTB₄, 100 nM LTC₄, and 100 nM LTD₄) concomitantly with either AA861 or MK886 in rat AMs (Figure 2B) or in cells from 5-LO–deficient mice (Figure 2A). In all instances, exogenous LTs were able to overcome the effects of pharmacologic or genetic LT deficiency, restoring bacterial survival to the same degree observed in AMs from WT mice or untreated AMs from rat.

These data show a role for endogenous LTs but do not distinguish between LTB₄ and cysLTs. To determine the receptors through which the effects on bacterial killing of endogenously produced LTs were mediated, we used CP105696 (BLT1 antagonist) and MK571 (cysLT1 antagonist). Interestingly, AMs treated with the BLT1 antagonist exhibited suppressed intracellular killing, as evidenced by an increase of approximately 65% in the survival of ingested bacteria, whereas treatment of the AMs with MK571 had no effect. To exclude a possible role for cysLT2–mediating AM microbial activity, we examined the effect of a dual cysLT1/cysLT2 antagonist (BAY-u9773, 10 μM) and observed no effect on microbial activity compared with untreated controls (data not shown).
Leukotrienes do not augment nitric oxide–dependent killing of K pneumoniae

We assessed the effect of LTs on another important bactericidal molecule, NO. This reactive species has been shown to be crucial for the control of K pneumoniae infection in murine and in human AMs.46,47 For these experiments, AMs were pretreated with L-NAME (1 mM), a nonselective NO synthase inhibitor (or vehicle control), and then stimulated with LTB4, cysLTs, or the LT mixture. As evidenced in Figure 3C, inhibition of NO completely abolished the ability of AMs to control K pneumoniae infection, increasing the number of ingested bacteria by approximately 98%, but this defect was reversed by treatment with LTs. These data suggest that though NO has important bactericidal activities in AMs, it does not account for the enhanced killing induced by LTs.

5-LO products induce H2O2 secretion by rat AMs in a dose-dependent manner

The demonstration of a role for NADPHox activation in LT-mediated microbicidal activity suggested that LTs and 5-HETE could directly stimulate ROI production by AMs. Although LTs can induce ROI production in many cell types, including human and guinea pig eosinophils,48 murine and human neutrophils,49,50 and astroglia cell,51 only LTC4 has been shown to stimulate its production by peritoneal macrophages.52 As illustrated in Figure 4A, the stimulation of AMs with LTs or 5-HETE induced the secretion of H2O2 in a dose-dependent manner. Similar to the effects on bacterial killing, LTB4 was the most effective NADPHox activator in terms of magnitude (LTB4 = 5-HETE > LTD4 and LTC4) and potency (LTB4 > LTD4 > 5-HETE).

The impact of endogenously produced LTs on cellular H2O2 production in response to K pneumoniae infection was explored using the LT biosynthesis inhibitors AA-861 and MK886 and the BLT1 antagonist CP105696. As shown in Figure 4B, K pneumoniae infection induced H2O2 production by rat AMs; this was inhibited by LT synthesis inhibitors and the BLT1 receptor antagonist, suggesting that endogenous LTB4 is involved in NADPHox activation and release of H2O2 during K pneumoniae infection.
inhibitor did not alter the effect of LTB₄, whereas the PKC-δ inhibitor suppressed the phosphorylation of p47phox when compared with LTB₄ alone. The total amount of cytosolic p47phox did not change in any condition tested (Figure 5B). In addition, we observed the same role of PKC isoforms in p47phox translocation that we observed for its phosphorylation (Figure 5B), supporting a contribution of PKC-δ but not -α. Inhibition of PKC activity in AMs by Ro-32-0432 or rottlerin was isoform specific, as determined by immunoblot analysis of the cytosolic protein fraction against phospho-PKC-α and -δ (data not shown). In addition, to ensure equal protein loading, blots were stripped and reprobed against FLAP. Thus, our data show that LTB₄-induced phosphorylation and subsequent translocation of p47phox are mediated by PKC-δ.

**LTB₄ enhances AM microbialic activity by increasing PKC-δ activity**

Once it was determined that LTB₄ was able to activate NADPHox through p47phox phosphorylation and membrane translocation in a manner dependent on PKC-δ, we sought to evaluate whether this phenomenon was also important for LTB₄-induced microbialic activity in AMs. To do this, AMs were pretreated with selective inhibitors of PKC-α (10 nM Ro-32-0432) or PKC-δ (6 μM rottlerin) and then by LTB₄ (1 nM) after 30 minutes of infection. As observed in Figure 6, LTB₄ increased AM microbialic activity by approximately 34%; however, this effect was abolished when the cells were pretreated with PKC-δ inhibitor. Interestingly, treatment with the PKC-α inhibitor did not alter the effect of LTB₄. Thus, this result establishes that PKC-δ is an upstream target for the effects of LTB₄ on enhancing microbialic activity, which results in NADPHox activation and bacterial killing.

**Discussion**

This study establishes the importance of LTs in AM bactericidal activity and characterizes some of the molecular mechanisms involved in this phenomenon. Our results show that (1) LTs greatly enhance the microbialic activity of AMs infected by the relevant Gram-negative pathogen *K pneumoniae*; (2) LTB₄ is the major

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**Figure 5. LTB₄ elicits translocation and phosphorylation of p47phox in a PKC-δ-dependent manner.** (A) Rat AMs (4 x 10⁵/well) were stimulated with 1 nM LTB₄, 100 nM LTC₄, 100 nM LTD₄, and 1000 nM 5(S)-HETE or 10 nM PMA for 5 minutes and then were harvested and fractionated as described in “Materials and methods.” Immunoblotting of membrane fractions was performed using anti-p47phox (1:500). Results are representative of those from 3 experiments. (B) Rat AMs (4 x 10⁵/well) were pretreated with 10 nM Ro32-0432 (PKC-α inhibitor), 6 μM rottlerin (PKC-δ inhibitor), or vehicle control for 20 minutes, stimulated with 1 nM LTB₄ for 5 minutes, and then harvested and fractionated as described in “Materials and methods.” The cytosolic fraction was immunoprecipitated, and immunoprecipitated protein was immunoblotted using antiphosphoserine (1:900). The total cytosolic fraction and the membrane fraction were probed for total p47phox (1:500). Results are representative of those from 2 experiments. Densitometry analysis is from numbers under lanes indicate relative density of phosphorylated or membrane-translocated p47phox. To ensure equal protein loading, blots were stripped and reprobed against FLAP.

**Figure 6. Role of PKC-δ and -α in LTB₄ enhancing AM killing of opsonized K pneumoniae.** Rat AMs were pretreated with vehicle control, the PKC-δ inhibitor rottlerin (6 μM), or the PKC-α inhibitor RO32-0432 (1 nM) for 20 minutes before the addition of opsonized *K pneumoniae*. Thirty minutes after infection, drugs were added back with or without 1 nM LTB₄ as described above. Microbicidal activity was assessed; data are expressed as mean ± SE. Percentage of survival of ingested bacteria of quadruplicate values from 1 of 4 representative experiments. *P < .05 compared with untreated control; ANOVA.
contributing endogenous LT, and its effects are mediated by the
BLT1 receptor; (3) the microbicidal activity induced by LTs occurs
because of the activation of NADPHox, not because of NO
generation; (4) LTs increase the phosphorylation and translocation
of p47phox in a manner dependent on PKC-δ activation; (5) PKC-δ
activation is also a determinant of the AM microbicidal activity
enhanced by LTB4.

A role for LTs in amplifying leukocyte microbicidal activity has
been suggested by studies documenting enhanced leukocyte func-
tions, such as phagocytosis, pathogen killing, release of neutrophil
granules, defensin production, and generation of antimicrobial
oxidants.7,9,11,14,16,19,25,58,59 However, information is lacking regard-
ing the relative importance of the various LTs in immunoregulation
and which molecular mechanisms evoked by LTs are involved in
these processes. In particular, there is a paucity of information in
the AM, a cell type critical to the antimicrobial defense of the lung.

To examine these issues, our first approach was to stimulate the
AM with exogenous LTB4, cysLTs, and 5-HETE and to assess their
individual effects on the killing of ingested K. pneumoniae. Because
LTs are known to enhance Fc-γ receptor–mediated phagocytosis by
AMs,10 it was important to use a bacteria-based assay that could
quantify the effects on bacterial killing independently of effects on
phagocytosis. To achieve this, we used a well-characterized
bactericidal assay91 that allowed the addition of LTs after phagocy-
tosis had occurred and that included a (4°C) control to account for
differences in bacterial ingestion.

Our results indicate that all the 5-LO products tested enhanced
bacterial killing but that LTB4 was the most potent. This finding is
of interest because cysLTs were slightly more potent than LTB4 in
promoting bacterial phagocytosis by AMs.9 Wirth and Kierszen-
baum7,8 showed that LTB4 and LTC4 increased phagocytosis and T
cruzi killing; however, they did not evaluate the relative potency
of both LTs. We confirmed that the effects of LTB4 and the cysLTs
were specifically mediated by the BLT1 and cysLT1 receptors,
respectively. Comparison between these data also indicates that a
lower concentration of exogenous cysLTs is required to enhance
phagocytosis (1 nM)9 than bacterial killing (100 nM). It is notable
that blocking the BLT1 receptor itself suppressed the ability of
AMs to kill ingested bacteria, illustrating the importance of
endogenously produced LTB4 in AM function. However, pharma-
ilogic blockade of the cysLT receptors did not affect basal rat AM
microbicidal activity. These results may best be explained by the
fact that the rat (and human) AM capacity to produce cysLTs is
approximately 10 times lower than that of LTB4.9 These results are
in contrast to studies of phagocytosis of K. pneumoniae, in which
blocking cysLT1 suppressed rat AM phagocytosis.9

We can contrast these findings with our examination of the link
between the NO cascade and LT signaling (Figure 3C). Although
NO production is required for the AM to kill K. pneumoniae,
blocking NO production did not abrogate the microbicidal enhance-
ment induced by LTs. Other investigators have found a relationship
between LT-induced antimicrobial activity and the NO cascade.14,38
For example, Talvani et al14 showed that LTs enhanced the ability
of peritoneal macrophages to kill T. cruzi organisms in a manner
dependent on NO production. The reasons for this discrepancy are
unclear but may reflect differences in the pathogen studied or the
experimental design. Unlike our model, in which infection with K
pneumoniae was of a short duration (90 minutes), the T. cruzi
infection model was more prolonged (7 days).

Because LTs enhance Fc-γ receptor–mediated phagocytosis by
the AM10 and immunoglobulin G (IgG)–opsonized targets induce
the activation of NADPHox,86 we wanted to examine the ability of
LTs to stimulate NADPHox. To accomplish this, we used genetic
and pharmacologic approaches to characterize relationships be-
tween LT signaling and NADPHox activity. Using the specific
NADPHox inhibitor DPI and AMs derived from gp91phox-
deficient mice, we confirmed the important role of this enzyme
complex in generating ROIs lethal to invading bacteria. Import-
antly, when the activation of NADPHox was abrogated, the ability
of LTs to enhance bacterial killing was lost, strongly suggesting
that NADPHox activity mediates the effects of LTs. Our study also
documented that LTs can stimulate AM H2O2 production in the
absence or presence of infection. This was shown using exogenous
LTs, inhibitors of LT synthesis, and LT receptor antagonists.

The constitutive membrane component of NADPHox is the
flavocytochrome b558, which is formed by gp91phox and p22phox
and which represents the catalytic core of the holoenzyme.
Activation of flavocytochrome b558 requires translocation of the
cytosolic components p47phox, p40phox, p67phox, and rac-1 or
rac-2. NADPHox activation often follows the stimulation of
cell-membrane G-protein–coupled receptors and involves mul-
tiple downstream signaling pathways that culminate in the phosphor-
ylation of enzyme complex components by specific kinases such as
PKC.61,62 mitogen-activated protein (MAP) kinases,63,64 and pro-
tein kinase B/Akt.55,66

To study NADPHox activation in vitro, we used immunoblot
analysis to quantify the phosphorylation and translocation of
p47phox to the cell membrane. We found that all 5-LO metabolites
enhanced the translocation of p47phox. In most instances, the
translocation of p47phox requires its phosphorylation, though
to its absence does exist for lipids such as AA, linoleic acid, and
diacylglycerol eliciting translocation without phosphorylation.57,58
We verified that LTB4 did indeed cause the phosphorylation of
p47phox. We similarly observed that both classes of LTs induced
the phosphorylation and translocation of p40phox to the plasma
membrane (data not shown). To our knowledge, this is the first
report showing that LTB4 can stimulate the phosphorylation and
translocation of NADPHox components.

The NADPHox complex of monocytes/macrophages has in
general been less studied than that of neutrophils. Few reports show
the molecular mechanisms involved in the phosphorylation, trans-
location, and assembly of the phox proteins in macrophages,
especially the AM. Yamamoto et al59 showed that PI3-kinase
mediates the phosphorylation of p47phox in HL-60 cells stimulated
by N-formyl-methionyl-leucyl-phenylalanine (fMLP) through acti-
vation of the classical PKC and PKC-δ. Zhou et al70 reported that

t-butyl hydroperoxide increased p47phox translocation to the AM
membrane. However, our results are the first to demonstrate that
LTs can regulate the phosphorylation and translocation of cytosolic
components of NADPHox.

We investigated the effect of Ca++-dependent PKC-α and
Ca++-independent PKC-δ in the phosphorylation of p47phox. We
studied these isoforms of PKC because they are implicated in
NADPHox activation and are present in AMs.55,71 Using the
cell-permeable PKC inhibitors rottlerin (PKC-δ) and Ro-32-0432
(PKC-α), we found that PKC-δ mediates the LTB4-simulated
phosphorylation and subsequent translocation of p47phox (Figure
6A-C). These results are supported by the findings of O’Flaherty et
al72 that LTB4 can induce unconventional PKC activation in
human neutrophils. In addition, Beyer et al73 demonstrated that in
human monocytes stimulated by opsonized zymosan, p47phox
phosphorylation and membrane translocation is dependent on
PKC-δ but not PKC-α.
Once we determined the molecular mechanism of NADPHox activation by LTB₄, we sought to evaluate whether this mechanism is also involved in AM microbicidal activity. Our results indicated that PKC-δ is an upstream target of LTs required for the amplification of AM microbicidal activity. That PKC is involved in macrophage microbicidal activity has been demonstrated. However, the role of PKCs in AM effector function is not well understood. Considering phagocytosis and microbicidal activity in macrophages, Heale and Speert 14 showed that PKC agonists increase phagocytosis of Pseudomonas aeruginosa by murine AMs. St-Denis et al 23 demonstrated that PKC-α is involved in the killing of Leishmania donovani and Legionella pneumophila in RAW 264.7. In addition, PKC-ε-deficient mice experience a decreased period of survival during Gram-negative bacterial infection. 76 However, the role of PKC-δ and microbicidal activity has never been assessed in macrophages.

We noted that optimal concentrations of LTs and cysLTs exhibited no additive effects on bacterial killing. This can likely be explained by the convergence in signaling pathways downstream from BLT1 and cysLT1 because both are coupled to Gq and to Gi and are resistant to Gβγ. LTs may also act indirectly to increase cPLA₂ activity (dashed line and question mark).

Figure 7. Proposed model of NADPHox activation by LTB₄.

Either binding of IgG-coated targets with the FcγR or ligation of BLT1 by LTB₄ results in increased levels of intracellular Ca²⁺ and diacylglycerol (DAG). Ca²⁺ activates cPLA₂ activity, which results in enhanced AA release and subsequent generation of LTs. LTB₄ stimulates PKC-δ through an unknown mechanism, whereas DAG directly activates this enzyme. Once activated, PKC-δ induces phosphorylation and translocation of p47phox to the plasma membrane, generating ROIs. LTs may also act indirectly by increasing cPLA₂ activity (dashed line and question mark).

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Leukotrienes enhance the bactericidal activity of alveolar macrophages against *Klebsiella pneumoniae* through the activation of NADPH oxidase

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