Leukotriene B$_4$ Augments Neutrophil Phagocytosis of *Klebsiella pneumoniae*

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Neutrophils play a critical role in the clearance of bacteria from the lung and other organs by their capacity for phagocytosis and killing. Previously, we identified an important role for the leukotrienes in rat alveolar macrophage phagocytosis of *Klebsiella pneumoniae*. In this report, we explored the possibility that the leukotrienes play an important role in phagocytosis by neutrophils as well. Inhibition of endogenous leukotriene synthesis by 5-lipoxygenase knockout in mice or by pharmacologic means in human peripheral blood neutrophils attenuated phagocytosis of opsonized *K. pneumoniae*. Reduced phagocytosis was also observed in human neutrophils pretreated with a leukotriene B$_4$ receptor but not a cysteinyl-leukotriene receptor antagonist. While leukotriene B$_4$ reconstituted defective phagocytosis in leukotriene-deficient neutrophils and enhanced phagocytosis in neutrophils capable of leukotriene synthesis, leukotriene C$_4$, leukotriene D$_4$, 5-hydroperoxyeicosatetraenoic acid, and 5-oxo-eicosatetraenoic acid were ineffective. To determine the opsonin dependence of the leukotriene B$_4$ augmentation of phagocytosis, we assessed the ability of leukotriene B$_4$ to modulate neutrophil phagocytosis and the adherence of sheep erythrocytes opsonized with immunoglobulin G or the complement fragment C3bi. While leukotriene B$_4$ augmented both Fc receptor- and complement receptor-mediated phagocytosis, increased adherence to leukotriene B$_4$-treated neutrophils was limited to complement opsonized targets. In conclusion, we have identified a novel role for leukotriene B$_4$ in the augmentation of neutrophil phagocytosis mediated by either the Fc or complement receptor.

The alveolar epithelial surface represents the body's largest interface with the external environment, and it is continually challenged with bacteria aspirated from the oropharynx that evade the mechanical clearance mechanisms of the upper respiratory tract. The alveolar macrophage (AM) is the primary means of innate host defense against bacterial pathogens that attempt to reach the alveolar space. When the bacterial burden in the alveolar space is great enough to activate a sufficient number of AMs to secrete chemotactic substances such as chemokines, leukotriene B$_4$ (LTB$_4$), and complement fragments, polymorphonuclear leukocytes (PMNs) are recruited from a marginated pool in the pulmonary vasculature to the alveolar epithelial surface (19). PMNs play a critical role in clearing encapsulated bacteria, such as *Klebsiella pneumoniae*, from the lung and in preventing bacterial dissemination to the systemic circulation (26). The ability of these cells to perform this function can be enhanced by inflammatory mediators that activate the PMN for increased phagocytosis and killing of ingested bacteria.

The LTs are potent lipid mediators of inflammation derived from the 5-lipoxygenase (5-LO)-catalyzed oxygenation of arachidonic acid (AA). The enzyme 5-LO in concert with the 5-LO activating protein can oxygenate AA to form the intermediate metabolite, 5-hydroperoxyeicosatetraenoic acid. This 5-LO product can either be dehydrated to LTA$_4$ or reduced to 5-hydroxyeicosatetraenoic acid (5-HETE). LTA$_4$ can be hydrolyzed to form the potent PMN chemoattractant LTB$_4$ or conjugated with glutathione to form the cysteinyl-LTs (LTC$_4$, LTD$_4$, and LTE$_4$), well known to elicit smooth muscle contraction and microvascular permeability (15).

LTs are readily acknowledged as important mediators of pathologic states of inflammation such as asthma. To ascertain whether LTs participate in the homeostatic form of inflammation, i.e., antimicrobial host defense, we subjected 5-LO knockout (KO) mice to an intrapulmonary challenge with *K. pneumoniae* (2). Compared to their wild-type (WT) counterparts, the LT-deficient mice exhibited enhanced lethality and reduced bacterial clearance from the lung in this model. This defect in 5-LO KO mice in vivo was associated with reduced AM phagocytosis and killing of *K. pneumoniae* in vitro. Further in vitro studies indicated that the inhibition of LT synthesis by pharmacologic means also reduced AM phagocytosis of bacteria. Moreover, exogenous LTs restored phagocytosis when added to LT-deficient AMs and also augmented phagocytosis above baseline capacity when added to LT-competent AMs (17) (2). Finally, mechanistic studies revealed that the LT augmentation of phagocytosis in AMs was largely Fc receptor (FcR) mediated and protein kinase C dependent in AMs (16).

Since PMNs are professional phagocytes known to synthesize LTs and express plasma membrane receptors for LTs, we explored the possibility that these mediators modulate PMN phagocytosis of *K. pneumoniae* as they do in AMs. In this study, we demonstrate that PMN phagocytosis of *K. pneumoniae* is indeed augmented by LTB$_4$ and that the enhancement in PMN phagocytosis can occur through FcR- or complement receptor (CR)-mediated phagocytosis.

MATERIALS AND METHODS

Cell isolation and culture. Human PMNs, isolated from venous blood from healthy volunteers, were purified by centrifugation through Polymorphprep (Nycomed Pharma, Oslo, Norway), followed by hypotonic lysis of erythrocytes (3).

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Elicited PMNs were obtained from 129/SvEv WT and 5-LO KO mice by peritoneal lavage 4 h after an intraperitoneal injection of 1% glycogen solution in saline. Ninety percent of the cells obtained by peritoneal lavage were identified as PMNs by a modified Wright-Giemsa stain (Diff-Quik; American Scientific Products, McGaw Park, Ill.). Following PMN isolation, the cells were enumerated using a hemocytometer and suspended in RPMI 1640 (Gibco, Grand Island, N.Y.) to a final concentration of 5×10^5 cells/ml. For phagocytosis experiments, 10^5 PMNs were adhered to glass eight-well Falcon culture slides (Becton Dickinson, Franklin Lakes, N.J.) for 1 h in RPMI 1640.

K. pneumoniae **preparation.** *K. pneumoniae* strain 43816 (serotype 2; American Type Culture Collection) was grown in tryptic soy broth (Difco, Detroit, Mich.) for 18 h at 37°C. The concentration of bacteria in culture was determined spectrophotometrically (A_{600}) (10).

Pharmacologic modulation of PMNs. In some experiments, PMNs were pretreated for 15 min with the 5-LO enzyme inhibitor zileuton (10 μM; Abbott Laboratories, Chicago, Ill.), the 5-LO activating protein inhibitor MK-886 (1 μM; Merck-Frosst, Montreal, Quebec, Canada), the cysteinyl-LT receptor antagonist LY171883 (1 μM), or the LTB$_4$ receptor antagonist LY292476 (1 μM) (the latter two from Eli Lilly, Indianapolis, Ind.), each diluted in Hanks balanced salt solution. The use of these pharmacologic agents at these doses was previously shown to inhibit LT synthesis and block the LT receptors (4, 7) and reduce phagocytosis of *K. pneumoniae* in AMs (17).

Phagocytosis of *K. pneumoniae*. Following the addition of 10^6 *K. pneumoniae* opsonized with 1% immune serum (17), PMN cultures were incubated for 30 min at 37°C. Following the incubation period, the extracellular bacteria were removed by three washes with Hanks balanced salt solution. The monolayers containing bacteria were stained with Diff-Quik and enumerated. For each slide, a standard pattern of high-powered fields was examined by light microscopy (1,000 × objective) to enumerate 100 cells. By comparing the phagocytic index in the presence and absence of cytochalasin D (20), we determined that 90% of the cell-associated bacteria using this method were actually internalized.

Preparation of E opsonized with IgG or C3bi. Sheep erythrocytes (E; ICN Biomedicals, Costa Mesa, Calif.) were washed and opsonized with either rabbit anti-E immunoglobulin G (IgG) (ICN Biomedicals, Inc.) or IgM (ICN Biomedicals) and C3bi (Sigma) as described previously (1, 29). Opsonized E were enumerated using a hemocytometer and diluted to a final concentration of 20×10^7 E/ml.

Phagocytosis of opsonized E. E-IgG or E-C3bi (10^7) was added to the PMN monolayers following pretreatment with medium alone (control), LTs, or LT modulators for 5 to 10 min; 15 min later, internalization of E-C3bi was initiated by the addition of 15 nM phorbol myristate acetate (PMA). After a 30-min incubation at 37°C, water was added to each well for 10 s followed by 3× saline to lyse the surface-bound E and to restore isotonic conditions. A phagocytic index was calculated as described previously (17).

Adherence of opsonized E. To assess adherence of E-IgG or E-C3bi to the surface of PMNs, monolayers were incubated with cytochalasin D (5 μg/ml) at 37°C in 5% CO$_2$, which would allow surface binding of E's, but not phagocytosis (13). Thirty minutes after the addition of opsonin-coated E's, the total number of adherent targets was enumerated for 100 cells, using the microscopic procedure described above for phagocytosis. The adherence index was calculated by determining the total number of adhered E-IgG or E-C3bi per 100 PMNs.

Statistical analysis. A minimum of three replicate wells per condition was studied in each experiment, and the number of individual experiments is indicated in the figure legends. Data are expressed as the mean ± standard error (SE). Where appropriate, mean values were compared using a paired *t* test, a one-way analysis of variance, or a Kruskal-Wallis test on ranks for nonparametric data. The Dunnett's test or the Student-Newman-Keuls tests were used for mean separation. In all cases, a *P* value of <0.05 was considered significant.

RESULTS

Inhibition of endogenous LT synthesis reduces PMN phagocytosis of *K. pneumoniae*. To determine if endogenously produced LTs have a functional role in PMN phagocytosis, human PMN monolayers were pretreated with either zileuton (10 μM) or MK-886 (1 μM) at concentrations known to inhibit LT synthesis. They were then incubated with *K. pneumoniae* opsonized with immune serum. Both agents, which block LT synthesis by distinctly different mechanisms of action, reduced phagocytosis of opsonized *K. pneumoniae* by approximately 35% (*P* < 0.05) (Fig. 1). The results of this experiment indicate

that endogenously produced LTs are necessary for maximal bacterial phagocytosis in human PMNs.

Decreased phagocytosis of opsonized *K. pneumoniae* by elicited PMNs from 5-LO KO mice. During bacterial infection of the lung and other organs, PMNs are recruited to tissue from the peripheral circulation in response to a chemoattractant gradient. During the recruitment process, the PMN encounters a number of mediators which prime the cells for enhanced antimicrobial defense. These include tumor necrosis factor alpha, gamma interferon, interleukin-12, and LTs. To assess the importance of endogenously generated LTs in phagocytosis by PMNs recruited to a site of inflammation, we assessed phagocytosis of opsonized *K. pneumoniae* in PMNs recruited in response to glycogen instillation into the peritoneal cavity of 5-LO KO and WT mice. Phagocytosis by elicited peritoneal PMNs obtained from 5-LO KO mice was reduced by approximately 50% compared with cells from WT animals (*P* < 0.05) (Fig. 2). This reduction in phagocytosis underscores the importance of LTs in the antimicrobial function of PMNs at a site of inflammation.

LTB$_4$ receptor antagonist attenuates phagocytosis. In a previous study (17), we determined that receptor antagonists to both LTB$_4$ and cysteinyl-LTs in rat AMs reduced phagocytosis. This indicated that endogenous production of both classes of LTs augmented phagocytosis. We used this same approach to determine the contribution of endogenously produced LTs of each class to phagocytosis by human PMNs. The LTB$_4$ receptor antagonist LY292476 reduced bacterial phagocytosis by approximately 40% (*P* < 0.05) (Fig. 3), indicating that endogenously produced LTB$_4$ was produced during phagocytosis, exited the cell, and activated the plasma membrane receptor for LTB$_4$. In contrast, the cysteinyl-LT receptor antagonist LY171886 had a minimal effect on PMN phagocytosis.

Exogenous LTB$_4$ restores phagocytosis in LT-deficient human and murine PMNs. To determine the specificity of the relationship between various LTs and phagocytosis, we examined whether exogenous LTs could restore the impairment of phagocytosis observed in human PMNs pretreated with zileuton and elicited PMNs from 5-LO KO mice. Prior to the addition of opsonized *K. pneumoniae*, exogenous LTB$_4$ or LTC$_4$ (1 nM) was added to human PMNs pretreated for 15 min with zileuton (10 μM) or to murine elicited PMNs. The addition of exogenous LTB$_4$ completely restored phagocytosis in human PMNs (Fig. 4A) and nearly restored phagocytosis in murine elicited PMNs (Fig. 4B). In contrast, the addition of exogenous LTC$_4$ did not enhance phagocytosis in either instance (data not shown).

Exogenous LTB$_4$ dose dependently augments phagocytosis of opsonized *K. pneumoniae* in PMNs capable of LT synthesis. Having established the importance of endogenously produced LTB$_4$ for full phagocytic capacity of PMNs, we considered the possibility that exogenously added LTs may augment PMN phagocytosis even in cells competent for LT synthesis. Human PMNs were incubated with various doses (0.01 to 100 nM) of exogenous LTB$_4$, LTD$_4$, LTC$_4$, 5-oxo-eicosatetraenoic acid (5-oxo-ETE), or 5-HETE for 5 min prior to the addition of opsonized *K. pneumoniae*. As shown in Fig. 5, phagocytosis was enhanced above the basal level with LTB$_4$ in a dose-dependent fashion, reaching statistical significance at 1 to 100 nM. In contrast, none of the other lipids were able to significantly
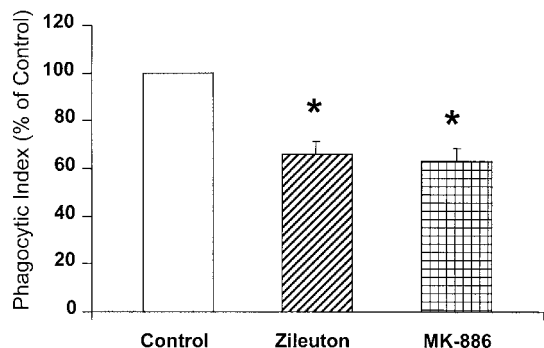
FIG. 1. Effect of endogenous LT synthesis inhibition on PMN phagocytosis of opsonized *K. pneumoniae*. Human PMNs were pretreated in the absence (control) or presence of zileuton (10 μM) or MK-886 (1 μM) for 15 min prior to the addition of *K. pneumoniae* opsonized with 1% immune serum. The phagocytic index of the control was 93 ± 23. Data are presented as the mean ± SE ($n = 5$). *, $P < 0.05$ with respect to the control, using a paired t test.
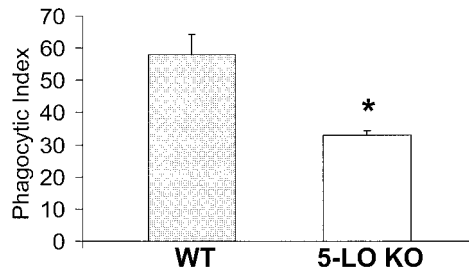

FIG. 2. Effect of 5-LO gene knockout on phagocytosis of opsonized *K. pneumonia* by elicited peritoneal PMNs. Data are presented as the mean ± SE ($n = 3$). *, $P < 0.05$ with respect to the control, using a paired t test.

enhance phagocytosis at any of the doses tested (data not shown).

LTB$_4$ increases human PMN phagocytosis of E-IgG. The phagocytic target used in all of the previously described experiments was *K. pneumoniae* opsonized with 1% immune serum. Immune serum would be expected to contain two distinct opsonins, IgG and complement. To determine whether FcR-dependent phagocytosis in human PMNs is modulated by LTB$_4$, cells were pretreated with increasing doses of LTB$_4$ for 10 min prior to a 30-min incubation with E-IgG. After incubation, extracellular Es were removed by hypotonic lysis and a phagocytic index was calculated to determine the number of ingested E-IgG per 100 PMNs. In a dose-dependent fashion, LTB$_4$ enhanced phagocytosis of E-IgG, with the maximum effect (~3.5-fold the control level) observed at 10 to 100 nM (Fig. 6). We next explored the possibility that LTB$_4$ enhanced phagocytosis of E-IgG by increasing target adherence. Following pretreatment with LTB$_4$, PMN monolayers were cultured at 37°C and cooled to 15°C prior to the addition of E-IgG, in order to allow adherence but not phagocytosis (9). In contrast to the effects on phagocytosis demonstrated in Fig. 6, E-IgG adherence to human PMNs was not significantly affected by LTB$_4$ pretreatment at doses up to 100 nM (control phagocytic index of 100 versus phagocytic index with 100 nM LTB$_4$ of 99 ± 11). These results indicate that LTB$_4$ augments the ability of the PMN to internalize, rather than to bind, E-IgG.

LTB$_4$ enhances human PMN adherence and PMA-stimulated phagocytosis of E-C3bi. Since LTB$_4$ is known to cause mobilization of CR to the cell surface (18), we next evaluated whether LTB$_4$ increased the ability of the PMN to bind complement-opsonized targets. PMNs were pretreated with increasing doses of LTB$_4$ (0.01 to 100 nM) prior to the addition of E-C3bi. LTB$_4$ at 0.1 to 100 nM significantly increased E-C3bi adherence to human PMNs (Fig. 7). To determine if LTB$_4$-mediated enhancement of E-C3bi adherence is associated with increased phagocytosis, human PMNs were pretreated with various concentrations of LTB$_4$ prior to the addition of E-C3bi. Phagocytosis was initiated 15 min later with the addition of 15 nM PMA, since phagocytic cells must be acti-

vated by an additional stimulus in order to phagocytose complement-opsonized targets (9). LTB$_4$ dose dependently augmented phagocytosis of E-C3bi following treatment with PMA (Fig. 8). These results indicate that LTB$_4$ enhances CR-dependent phagocytosis, and that this may occur, at least in part, via an effect on increasing target adherence.

DISCUSSION

In this study, we used both genetic and pharmacologic approaches to demonstrate that endogenously produced LTs promote PMN phagocytosis of bacteria opsonized with immune serum. This conclusion was based on the observations that PMNs elicited from the peritoneum of 5-LO KO mice and human PMNs pretreated with zileuton and MK-886, two drugs that block LT synthesis by distinctly different mechanisms (5, 23), exhibited reduced phagocytosis. It is very likely that LTB$_4$ is the endogenous 5-LO product responsible for this effect on phagocytosis, since (i) it is the principal 5-LO product in PMNs and (ii) LTB$_4$ receptor antagonist, but not cysteinyl-LT receptor antagonist, also reduced phagocytosis. Although they do not synthesize LTC$_4$, some PMN effector functions have been shown to respond to cysteinyl-LTs (11, 14). Taken together, the results of these experiments suggest that endogenously produced LTB$_4$ acts as an autocrine stimulus for enhanced PMN phagocytosis of opsonized *K. pneumonia*.

Experiments demonstrating the ability of exogenous LTB$_4$ to restore phagocytosis in elicited PMNs from 5-LO KO mice and human PMNs pretreated with the LT synthesis inhibitor zileuton supported the conclusion that reduced phagocytosis of opsonized bacteria was indeed due to the lack of LT synthesis rather than other potential phenotypic abnormalities. Based on our previous studies with AMs (17), we considered the possibility that LTs might have pharmacologic effects on PMN phagocytosis of bacteria. While LTB$_4$, LTC$_4$, 5-HETE, and 5-oxo-ETE all enhanced phagocytosis of opsonized *K. pneumonia* by normal AMs in our previous study (17), only LTB$_4$ stimulated increased PMN phagocytosis in the present study. It is also noteworthy that the concentration (1 to 100 nM) of exogenously added LTB$_4$ that restored phagocytosis in LT-deficient PMNs and enhanced phagocytosis in LT-competent cells was within the physiologic range. Nanomolar quantities of LTB$_4$ have been recovered from the bronchoalveolar lavage fluid of pneumonia patients (12). Moreover, concentrations of LTB$_4$ at which binding to the two types of PMN LTB$_4$ recep-
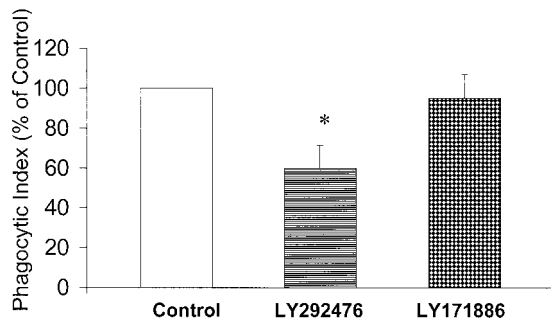
FIG. 3. Effect of LT receptor antagonists on phagocytosis of opsonized *K. pneumoniae* by human PMNs. The phagocytic index of the control was 93 ± 23. Data are presented as the mean $\pm$ SE ($n = 4$). *, $P < 0.05$ with respect to the control, using a paired t test.

tors (BLT) are half-maximal are 1.1 nM (BLT1) and 20 nM (BLT2) (30). These results indicate that only LTB_4, the principal 5-LO product of PMNs (8), is capable of enhancing phagocytosis in these cells at physiologic levels.

The positive effect of LTB_4 on PMN phagocytosis is not surprising since there is ample evidence demonstrating that LTB_4 activates many PMN functions, including upregulation of the CR (18), superoxide generation (28), and increased calcium mobilization (22). PMNs recruited to a site of inflammation have been exposed to various inflammatory mediators and are representative of the activated cells called upon for antimicrobial effector functions (24). The fact that the phago-
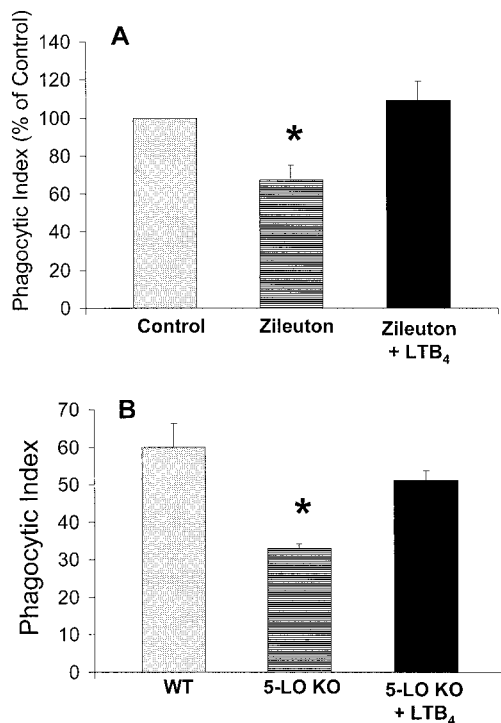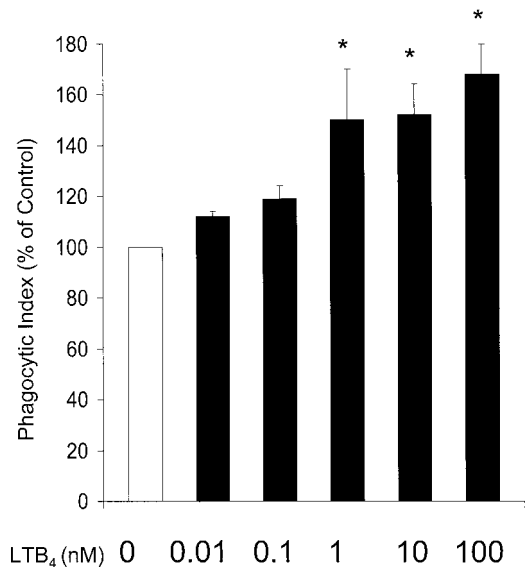

FIG. 5. Effect of exogenous LTB_4 dose on human PMN phagocytosis of *K. pneumoniae* opsonized with 1% immune serum. Human PMNs were incubated with LTB_4 in the dose indicated for 5 to 10 min prior to the addition of opsonized *K. pneumoniae*. The phagocytic index of the control was 40 ± 3. Data are expressed as the mean $\pm$ SE ($n = 3$). *, $P < 0.05$ with respect to the control by analysis of variance.

cytic capacity of elicited PMNs from 5-LO KO mice was compromised suggests that LTs play an important role in PMN activation for enhanced bacterial phagocytosis.

It is well known that optimal recognition of phagocytic targets is mediated by surface receptors for specific opsonins (e.g., complement and IgG) (13). Initial experiments showing LTB_4 enhancement of phagocytosis utilized bacteria opsonized with complete immune serum that would contain complement and IgG. Whether both or either of these opsonins was required for the LTB_4 augmentation of phagocytosis was addressed by coating inert targets (E) with only a single opsonin, either IgG or
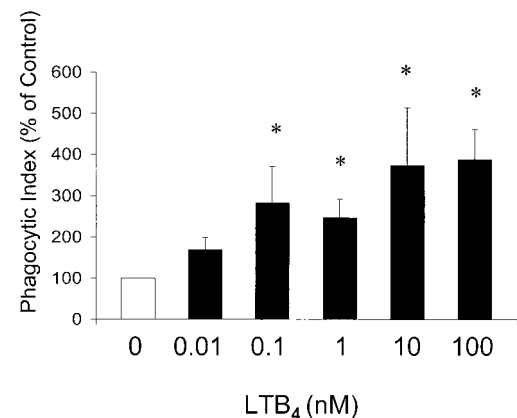

FIG. 4. Ability of exogenous LTB_4 to restore phagocytosis in human PMNs pretreated with zileuton (10 μM) for 15 min (A) or in elicited murine PMNs from 5-LO KO mice (B). The phagocytic index of the control (A) was 50 ± 2. Data are expressed as the mean $\pm$ SE ($n = 3$). *, $P < 0.05$ with respect to the control, using a paired t test.

FIG. 6. Effect of exogenous LTB_4 dose on human PMN phagocytosis of E-IgG. Human PMNs were incubated with LTB_4 for 5 to 10 min prior to the addition of E-IgG. The phagocytic index of the control was 20 ± 5. Data are expressed as the mean $\pm$ SE ($n = 3$). *, $P < 0.05$ with respect to the control by Kruskal-Wallis test on ranks, using Dunnett's test for means separation.
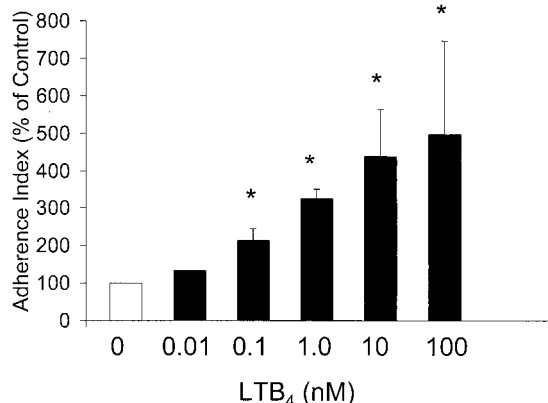
FIG. 7. Effect of exogenous LTB$_4$ dose on human PMN adherence of E-C3bi. Human PMNs were incubated with LTB$_4$ for 5 to 10 min prior to the addition of E-C3bi. The adherence index of the control was 96.7 ± 51. Data are expressed as the mean $\pm$ SE ($n = 3$). *, $P < 0.05$ with respect to the control by Kruskal-Wallis test on ranks, using Dunnett's test for means separation.

C3bi. These experiments also served to exclude the possibilities that other opsonins that may be present in the rat serum or nonopsonic recognition of a moiety that may be present on the surface of *K. pneumoniae* were responsible for the LTB$_4$ modulation of phagocytosis. They revealed that the effects of LTB$_4$ on phagocytosis could be mediated through either the FcR or the CR. This result was in contrast to our previous study that concluded that the effects of exogenous LTs on rat AM phagocytosis were limited to IgG-opsonized targets. The fact that rat AMs express low levels of the CR would explain this disparity (21, 27).

Subsequent experiments addressed the possibility that LTs modulate FcR- and CR-dependent phagocytosis by increasing adherence of targets to PMNs. This could result from increases in receptor number or enhanced affinity of the receptor for its
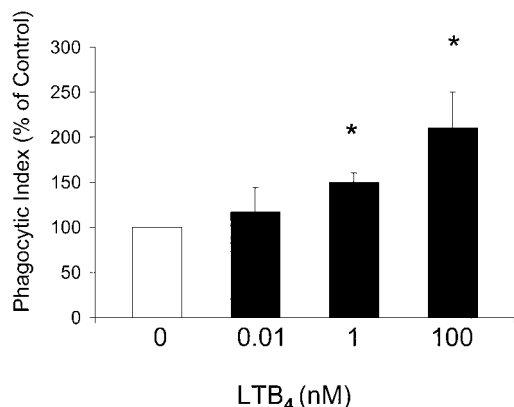

FIG. 8. Effect of exogenous LTB$_4$ dose on human PMN phagocytosis of E-C3bi. Human PMNs were incubated with LTB$_4$ for 5 to 10 min prior to the addition of E-C3bi. E-C3bi were allowed to adhere to the PMNs for 15 min, and phagocytosis was stimulated by a 15-min incubation with PMA (15 nM). The phagocytic index of the control was 22 ± 5. Data are expressed as the mean $\pm$ SE ($n = 4$). *, $P < 0.05$ with respect to the control by Kruskal-Wallis test on ranks, using Dunnett's test for means separation.

ligand. Indeed, LTB$_4$ is recognized to enhance CR expression in PMNs (18). While exogenous LTB$_4$ had no effect on the adherence of E-IgG, it induced an increase in the internalization of this target. In addition, LTB$_4$ enhanced E-C3bi adherence in a dose-dependent fashion, indicating that its ability to augment PMN phagocytosis may involve enhanced adherence of complement-opsonized targets.

In summary, we have revealed an important role for endogenously produced LTB$_4$ in the augmentation of PMN phagocytosis of opsonized bacteria. This enhancement of phagocytosis can be mediated through either the FcR or CR. In view of the role of endogenous LTB$_4$ in promoting phagocytosis by PMNs, increased susceptibility to infection observed in conditions such as human immunodeficiciency virus infection (6) and malnutrition (25) may relate to their recognized LT deficiency.

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