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Intrapulmonary Administration of Leukotriene B\(_4\) Augments Neutrophil Accumulation and Responses in the Lung to Klebsiella Infection in CXCL1 Knockout Mice

Sanjay Batra,* Shanshan Cai,∗ Gayathriy Balamayooran,* and Samithamby Jeyaseelan*†

In prior studies, we demonstrated that 1) CXCL1/KC is essential for NF-κB and MAPK activation and expression of CXCL2/MIP-2 and CXCL5/LPS-induced CXC chemokine in Klebsiella-infected lungs, and 2) CXCL1 derived from hematopoietic and resident cells contributes to host immunity against Klebsiella. However, the role of CXCL1 in mediating neutrophil leukotriene B\(_4\) (LTB\(_4\)), reactive oxygen species (ROS), and reactive nitrogen species (RNS) production is unclear, as is the contribution of these factors to host immunity. In this study, we investigated 1) the role of CXCL1 in LTB\(_4\), NADPH oxidase, and inducible NO synthase (iNOS) expression in lungs and neutrophils, and 2) whether LTB\(_4\) postinfection reverses innate immune defects in CXCL1\(^{-/-}\) mice via regulation of NADPH oxidase and iNOS. Our results demonstrate reduced neutrophil influx, attenuated LTB\(_4\) levels, and decreased ROS and iNOS production in the lungs of CXCL1\(^{-/-}\) mice after Klebsiella pneumoniae infection. Using neutrophil depletion and repletion, we found that neutrophils are the predominant source of pulmonary LTB\(_4\) after infection. To treat immune defects in CXCL1\(^{-/-}\) mice, we intrapulmonarily administered LTB\(_4\). Postinfection, LTB\(_4\) treatment reversed immune defects in CXCL1\(^{-/-}\) mice and improved survival, neutrophil recruitment, cytokine/chemokine expression, NF-κB/MAPK activation, and ROS/RNS production. LTB\(_4\) also enhanced myeloperoxidase, H\(_2\)O\(_2\), RNS production, and bacterial killing in K. pneumoniae-infected CXCL1\(^{-/-}\) neutrophils. These novel results uncover important roles for CXCL1 in generating ROS and RNS in neutrophils and in regulating host immunity against K. pneumoniae infection. Our findings suggest that LTB\(_4\) could be used to correct defects in neutrophil recruitment and function in individuals lacking or expressing dysfunctional CXCL1. *The Journal of Immunology, 2012, 188: 3458–3468.

Gram-negative bacterial pneumonia continues to be a major cause of morbidity, mortality, and health care costs (1–3). Neutrophils are the first responders to migrate toward the site of infection to clear causative bacteria; however, their excessive accumulation is associated with devastating pathological consequences including acute lung injury and acute respiratory distress syndrome (4–6). ELR\(^{+}\) CXC chemokines, including CXCL1/KC, CXCL2/MIP-2, and CXCL5/LIX, are potent chemotactic mediators for neutrophils (7–12). To determine the impact of CXCL1 on host immunity in the lung, we previously used a mouse model of Klebsiella pneumoniae infection and found CXCL1 to be important for neutrophil-dependent bacterial clearance in the lung (13). We also demonstrated that CXCL1 regulates the activation of NF-κB and MAPKs and the expression of other neutrophil chemokines, including CXCL2 and CXCL5 (13).

Bacterial clearance by neutrophils depends on the generation of reactive oxygen species (ROS) and reactive nitrogen species (RNS) (14, 15). Formation of ROS is catalyzed by NADPH oxidase and myeloperoxidase (MPO), whereas NO synthases catalyze the reaction to form RNS (16, 17). Upon activation, oxygen consumption in neutrophils increases, and the oxygen molecule is univalently reduced to superoxide by the membrane-bound NADPH oxidase complex (18, 19). Although the core enzyme consists of five subunits including p67\(_{phox}\), p47\(_{phox}\), p40\(_{phox}\), p22\(_{phox}\), and gp91\(_{phox}\), only p67\(_{phox}\), p47\(_{phox}\), and p40\(_{phox}\) exist in the cytoplasm in an unactivated state (18, 19). Upon cell activation, p67\(_{phox}\), p47\(_{phox}\), and p40\(_{phox}\) translocate onto the membrane. This complex is an electron transport chain that produces H\(_2\)O\(_2\) in combination with superoxide dismutate (18, 19). Superoxide is further converted to reactive hypochlorite by MPO (18, 19). Furthermore, NO is produced from guanidino nitrogen during the conversion of l-arginine to l-citrulline by NO synthases (20).

Leukotriene B\(_4\) (LTB\(_4\)) has been shown to be a neutrophil chemotactant derived from membrane phospholipids (21, 22). The role of LTB\(_4\) in the context of ROS and RNS production and bacterial killing has largely been explored in macrophages. LTB\(_4\) induces NADPH oxidase activation in alveolar macrophages (AMs) in response to Klebsiella infection. LTB\(_{4}\)-deficient human AMs exhibit impaired phagocytosis and killing of pneumococci, and these defects can be restored by addition of exogenous LTB\(_4\) (23). Genetic deletion of 5-lipoxygenase or pharmacological inhibition of LTB\(_4\) biosynthesis in mice results in enhanced mortality and attenuated microbial clearance after pneumococcal infection; this occurs via recruitment of macrophages but not neutrophils (24, 25). One of these reports also demonstrated that LTB\(_4\) augmented p47\(_{phox}\) expression and bacterial clearance in primary lung macrophages (24). In this regard, LTB\(_4\) has been
shown to augment killing of K. pneumoniae by murine AMs via ROS but not RNS (26). In human AMs, NO has been shown to be important in Klebsiella clearance (27). However, more detailed mechanisms underlying LTβr restoration in the lung or in macrophages have yet to be explored.

Despite the critical role of neutrophil recruitment and responses during pulmonary K. pneumoniae clearance, little is known about the role of CXCL1, LTβr, NADPH oxidase, or inducible NO synthase (iNOS) in neutrophils during K. pneumoniae infection. We illustrate that CXCL1 controls neutrophil immunity by regulating LTβr ROS, and RNS production after Klebsiella infection. Compared with wild-type (WT) controls, exogenous LTβr corrected host immunity in CXCL1−/− mice by restoring neutrophil influx, bacterial clearance, cytokine/chemokine production, as well as expression of ROS and RNS. Moreover, LTβr restored ROS and RNS generation and bacterial killing capacity in K. pneumoniae-stimulated CXCL1−/− neutrophils. These findings identify novel molecular and cellular mechanisms underlying the enhancement of neutrophil immunity in CXCL1−/− mice by a single dose of intrapulmonary LTβr administration.

Materials and Methods

Animals

Eight- to ten-week-old CXCL1 gene-deficient (CXCL1−/−) female mice were back-crossed 10 times with C57BL/6 mice for this study; therefore, C57BL/6 (WT) mice were used as age- and gender-matched controls (28). Eight- to ten-week-old CXCL1 gene-deficient (CXCL1−/−) female mice were used 6–8 wk after bone marrow transfer. We found that greater than 84% of blood leukocytes were derived from donor mice at the time of infection. To obtain cytosolic and membrane fractions, harvested lungs were homogenized for 30 s in PBS containing 0.2% antibiotic (neomycin sulfate) for the first 3 wk. Transplanted mice were cannulated with a 20-gauge catheter, then a total of 0.9 ml BALF or lungs was collected for LTB4 determination as described in our previous publications (24).

LTβr administration

LTβr (Cayman Chemicals, Ann Arbor, MI) was prepared in PBS containing 0.1% BSA to a final concentration of 2 μg/ml, and 50 μl/mouse (100 ng/mouse) was administered intratracheally (i.t.) at 1 h after Klebsiella challenge as described (23). After 48 h postinfection, bronchoalveolar lavage fluid (BALF) or lungs was collected for LTβr determination as described in our previous publications (24).

BALF isolation and lung harvesting

BALF was collected as described previously (13, 29–31). In brief, tracheae were cannulated with a 20-gauge catheter, then a total of 0.9 ml BAL buffer was introduced, flushed four times, and retrieved. A total of 3.0 ml BALF was retrieved from each mouse. Cytospin slides prepared with 0.5 ml BALF were stained with Diff-Quick reagents (Fisher, Chicago, IL) to enumerate leukocyte subtypes based on their cellular and nuclear morphological properties. Lungs were excised at designated time points after K. pneumoniae challenge and were immediately snap frozen followed by storage at −80°C for later use.

Total WBC, polymorphonuclear leukocyte, cytokine/chemokine, and LTβr determination

We used BALF and lungs that were obtained from animals after K. pneumoniae infection, K. pneumoniae plus LTβr administration, or K. pneumoniae plus BSA (vehicle) instillation. ELISA kits for TNF-α and IL-6 were obtained from eBioscience (San Diego, CA), and kits for CXCL1, MIP-2, LIX, and LTβr were obtained from R&D Systems (Minneapolis, MN). The minimum detection limit was 8 pg/ml cytokine/chemokine protein, whereas the detection limit for LTβr was 13.7 pg/ml (13, 29–32). For cellular recruitment, cytospin samples were subsequently prepared from BALF cells and stained with Diff-Quick. Total leukocytes in BALF were determined using a hemocytometer, whereas leukocyte subsets were examined by direct counting of stained slides based on cell and nuclear morphology (13, 29–32).

Subcellular fractionation

To obtain cytosolic and membrane fractions, harvested lung tissues were fractionated using a cell fractionation kit (Biovision) following the manufacturer’s recommendations (33). Membrane and cytoplasm fractions were obtained, and equal amounts of protein for each fraction were subjected to SDS-PAGE. Western blots were probed with anti-p67phox and p47phox Abs. The same membranes were immunoblotted with GADPH and pan-cadherin to indicate the cytosol and membrane fractions and to demonstrate equal loading on gels.

Neutrophil purification

Neutrophils were purified from bone marrow-derived cells by negative magnetic selection (StemCell Technologies, Vancouver, BC, Canada). Bone marrow cells were flushed out from tibiae and femurs using PBS. The cell suspension was passed through a 0.7-μm filter then resuspended in RoboSep buffer (PBS without Ca2+/Mg2+, 2% FBS, 1 mM EDTA); Purified neutrophils were obtained from bone marrow cells by using a custom antibody mixture containing Abs to CD5, CD4, CD45RB/B220, TER119, F4/80, CD11c, and c-Kit (cat. no. 19709). Briefly, bone marrow cells were incubated at 4°C in RoboSep buffer containing 5% normal rat serum along with the custom Ab mixture, biotin selection mixture, and magnetic colloid according to the manufacturer’s instructions. Samples were then placed in the EasySep magnet. After 3 min of incubation, samples highly enriched with neutrophils were pooled off into the new tube (34). Purified cells were washed and resuspended in RPMI 1640 containing 10% FBS. Cells were then counted using a hemocytometer and used for neutrophil-mediated bacterial killing, MPO activity, and release of H2O2 and NO and to determine the expression levels of the components of the NADPH oxidase system after K. pneumoniae infection. Neutrophil purity near 91%, as assessed by cytospin slides and flow cytometry (data not shown).

Neutrophil depletion

The neutrophil depletion protocol (GR1/Ly6G) used in this study has been described earlier (13, 31). A total of 50 μg anti-mouse Ly6G mAb (clone IIA8; BD Pharmingen) per mouse was administered i.p. in 50 μl at 12 and 2 h prior to bacterial infection. As a control, 50 μg isotype-matched control mAb in an equal volume was administered prior to infection. To validate the efficiency of anti-Gr1/Ly6G mAb in neutrophil depletion, we determined blood neutrophil counts every 12 h up to 3 d and observed that after depletion, only 2–3% of neutrophils remained in circulation during this period (data not shown).

Neutrophil repletion

To examine the role of neutrophils in contributing to LTβr polymorphonuclear leukocyte (PMN) repletion performed i.t. in neutropenic mice with PMNs (107 cells/mouse) isolated from WT or CXCL1−/− mice 30 min prior to K. pneumoniae infection.

Bone marrow transplantation

Generation of CXCL1 chimeras has been described in our earlier publications (13, 35). Briefly, donor and recipient animals (between 6 and 8 wk old) were used to make chimeras. Recipients were gamma-irradiated in two 525-rad doses delivered over 3 h. Isolated bone marrow cells (8 × 107) mouse) were injected into the tail vein of recipients, and animals were kept on 0.2% antibiotic (neomycin sulfate) for the first 3 wk. Transplanted mice were used 6–8 wk after bone marrow transfer. We found that greater than 84% of blood leukocytes were derived from donor mice at the time of experiments.

Immunoblotting

At the designated times, harvested lungs were homogenized for 30 s in 1 ml buffer containing 0.1% Triton X-100 in PBS with complete protease and phosphatase inhibitor mixture (Roche, Indianapolis, IN) and then centrifuged at maximum speed in a microcentrifuge at 4°C. The resulting supernatant fluids were used for immunoblotting. To ensure that equal amounts of protein were loaded onto the gel, a Bradford protein assay was used to measure protein levels in the sample (Bio-Rad, Hercules, CA). The whole-lung homogenates were resolved on 10% SDS-PAGE, and the resolved proteins were transferred to polyvinylidene fluoride membrane using standard protocols (13, 29). Abs to p67phox, p47phox, and GADPH were obtained from Santa Cruz Biotechnology (Santa Cruz, CA), and Abs...
animal survival was examined over 15 d (13, 29).

Survival studies

WT and CXCL1−/− mice were inoculated i.t. with 10^5 CFU K. pneumoniae in 50 µl 0.9% saline and subsequently divided into two groups. One group received LTB₄ in PBS containing 0.1% BSA to a final concentration of 2 µg/ml; 50 µl/mouse was administered i.t. at 1 h after Klebsiella challenge. The control animals received 50 µl/mouse PBS containing 0.1% BSA, and animal survival was examined over 15 d (13, 29).

Lung bacterial CFUs

The lungs of mice were weighed and homogenized in 1 ml 0.9% saline using a tissue homogenizer. Supernatants were serially diluted, and 20-µl aliquots of each sample were plated on MacConkey agar and tryptic soy agar plates. The number of colonies was enumerated after incubation at 37°C overnight (13, 29).

Phagocytosis assay

Klebsiella expressing GFP were opsonized in RPMI 1640 containing 10% FBS for 30 min at 37°C with constant agitation. Purified neutrophils and bacteria at a 1:1 ratio in polystyrene tubes (1 MOI) with agitation at 37°C in 100 µl RPMI 1640 containing 10% FBS. After incubation at designated time-points, tubes were placed on ice to stop phagocytosis, and gentamicin was added to tubes at a final concentration of 100 µg/ml. The rationale for using 100 µg/ml gentamicin is that this dose was sufficient to kill 100% of extracellular bacteria, as confirmed by others (39, 40). After 15 min of incubation on ice, the suspension was washed twice and resuspended in PBS for flow cytometric analysis. As a control, one tube containing neutrophils only (no bacteria) was carried through the procedure to evaluate the background fluorescence of PMNs alone (41, 42). Antibacterial activity of gentamicin was determined by plating the samples at each time point on MacConkey agar. At the 100 µg/ml concentration, gentamicin was able to kill 100% of extracellular bacteria (data not shown). Similar findings have been reported in earlier studies (39, 40).

Bacterial killing activity of neutrophils

A neutrophil killing assay was performed as described earlier with slight modifications (43). Briefly, 1 × 10^6 neutrophils were suspended in RPMI 1640 with 10% v/v FBS, and 1 × 10^6 opsonized bacteria were added to polypropylene tubes (1 MOI). The tubes were incubated in a shaking water bath at 37°C for 180 min with continuous agitation, then samples were harvested at 30, 60, or 180 min, and a portion of the sample was spun at 100 × g for 10 min to collect the viable bacteria in media. Gentamicin (100 µg/ml) was added to the neutrophil pellet for 15 min to kill extracellular bacteria (39, 40). The neutrophil pellet was resuspended in 1 ml PBS with 0.05% (w/v) saponin, and the debris was broken up using a ground-glass homogenizer to evaluate engulfed bacteria. Colony counting of viable bacteria was conducted by plating 20-µl aliquots of each sample on MacConkey agar and tryptic soy agar plates. The number of colonies was enumerated after incubation at 37°C overnight.

Statistical analysis

Data were analyzed by Student t test for two-way comparisons; for comparisons among three or more groups, analysis was carried out by two-way ANOVA followed by Bonferroni post hoc test. Survival curves were compared by Wilcoxon signed-rank test. All statistical calculations were performed using InStat software and GraphPad Prism 4.0 (GraphPad, San Diego, CA). Significance was defined as p < 0.05.

Results

CXCL1 controls neutrophil recruitment and LTB₄ production in the lungs after K. pneumoniae infection

Leukocyte recruitment to the lungs is a critical step in host immunity to clear bacteria. In our previous report, we showed that pulmonary Klebsiella challenge leads to substantially reduced total WBC and neutrophil counts in the BALF of CXCL1−/− mice compared with those of their littermate controls (13). In the current study, we extended these observations by specifically examining neutrophil-specific responses that might be regulated by CXCL1 deficiency. We began by examining MPO and found it to be significantly decreased in the lungs of CXCL1−/− mice at 24 and 48 h postinfection (Fig. 1A). Because LTB₄ is a neutrophil chemoattractant lipid, we also examined LTB₄ levels in CXCL1−/− mice after K. pneumoniae administration. We found significantly reduced levels of LTB₄ in CXCL1−/− mice at 24 and 48 h postinfection compared with those of littermate controls (13). These findings indicate that CXCL1 is an essential regulator of neutrophil influx and LTB₄ production in the lungs during K. pneumoniae infection.

CXCL1 regulates the expression of NADPH oxidase and iNOS in the lung after K. pneumoniae infection

The NADPH oxidase complex includes p67phox and p47phox, which regulate the production of H₂O₂, whereas iNOS controls the production of RNS; both mechanisms are critical for bacterial degradation (14–17). Therefore, we next examined whether CXCL1 is important for the expression of NADPH oxidase and iNOS in the lung after K. pneumoniae infection. We found that the expression of p47phox, a key component of NADPH oxidase, was decreased in CXCL1−/− mice at 24 and 48 h postinfection, whereas the expression of iNOS was decreased at 48 h postinfection, the time point at which it was first detected in WT (Fig. 1C, 1D). Furthermore, the expression of p67phox was not reduced in CXCL1−/− mice compared with WT at either 24 or 48 h postinfection (Fig. 1C, 1D). Nevertheless, the expression of both p67phox and p47phox was higher at 24 h, whereas the expression of iNOS was higher at 48 h (Fig. 1C, 1D). To determine the level of...
activation of NADPH oxidase, we explored the translocation of p67phox and p47phox from the cytoplasm to the plasma membrane in lung cells 48 h after K. pneumoniae challenge. Our results show attenuated p67phox and p47phox translocation in the lungs of CXCL1−/− mice compared with that of their littermate controls (Fig. 1E, 1F). These data suggest that CXCL1 is essential for the regulation of ROS and RNS generation in the lungs.

**Neutrophils are the major source of LTB₄, NADPH oxidase, and iNOS expression in the lung after Klebsiella infection.**

Neutrophils are known to be critical in controlling K. pneumoniae infection in the lung (13, 31). To determine whether neutrophils are important for NADPH oxidase and iNOS expression in the lung during K. pneumoniae infection, we depleted granulocytes in WT and CXCL1−/− mice using anti–Gr-1/Ly6G Ab, then determined the expression of LTB₄, p67phox, p47phox, and iNOS in lungs postinfection. To our surprise, we found substantial reduction in LTB₄, p67phox, p47phox, and iNOS in the lungs of granulocyte-depleted mice after K. pneumoniae challenge (Fig. 2A–C). Furthermore, intratracheal neutrophil repletion in neutropenic mice supported the predominant role of neutrophils in LTB₄ production (Fig. 2D), as bone marrow chimera experiments demonstrated that bone marrow cells were a major source of LTB₄ (Fig. 2E). These observations suggest that neutrophils are the predominant contributors of NADPH oxidase and iNOS expression in the lungs of K. pneumoniae-infected mice.

**CXCL1 is essential for expression of NADPH oxidase and formation of oxygen free radicals and NO in neutrophils after K. pneumoniae infection in vitro.**

Because neutrophil depletion reduced the expression/activation of NADPH oxidase components and iNOS, we specifically examined whether CXCL1 plays an essential role in the expression of NADPH oxidase components and iNOS in purified neutrophils. We found reduced expression of p67phox and p47phox in neutrophils isolated from CXCL1−/− mice at 180 min after K. pneumoniae stimulation (Fig. 3A, 3B). We also observed attenuated MPO, NO, and H₂O₂ production from neutrophils of CXCL1−/− mice at this time point, whereas NO production was reduced in neutrophils even at an earlier time point (Fig. 3C). Furthermore, bacterial killing and phagocytosis by neutrophils were impaired in CXCL1−/− neutrophils (Fig. 3D, 3E).

**LTB₄ administration augments survival, bacterial clearance, cellular recruitment, and expression of cytokines/chemokines in the lungs of CXCL1−/− mice after K. pneumoniae infection.**

Because CXCL1 regulates LTB₄ as well as ROS and RNS during bacterial infections, we wanted to determine whether LTB₄ reg-

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**FIGURE 1.** MPO activity and LTB₄ production in the lungs is dependent on CXCL1 during K. pneumoniae infection. (A and B) MPO activity (A) and LTB₄ levels (B) in homogenized (unlavaged) whole lungs of WT (C57BL/6) and CXCL1−/− mice infected with K. pneumoniae (10³ CFU/mouse) for 24 and 48 h. Data are presented as means ± SEM; n = 6–8 mice/group. *p < 0.05 (compared with CXCL1−/− mice). (C) The levels of p67phox, p47phox, and iNOS in K. pneumoniae-infected homogenized whole lungs of WT and CXCL1−/− mice at 24 and 48 h postinfection. The blot is representative of three individual blots with identical results. (D) Densitometric analysis of p67phox, p47phox, and iNOS expression in homogenized lungs. Densitometry was performed from three separate blots. Data are expressed as means ± SEM; n = 6–8 mice/group. *p < 0.05 (compared with CXCL1−/− mice). (E) Levels of p67phox and p47phox in cytosolic and membrane fractions of the lung upon K. pneumoniae infection. This is a representative blot of three independent experiments. GAPDH is a cytosolic marker; pan-cadherin is a plasma membrane marker. (F) Densitometric analysis of p67phox and p47phox levels in cytosol and membrane fractions of the lung after K. pneumoniae infection. Protein expression was quantitated from three separate blots. Data are shown as means ± SEM. For experiments (E) and (F), a total of 6–8 mice were used in each group. *p < 0.05 (compared with CXCL1−/− mice).
ulates ROS- and RNS-dependent host immune mechanisms in K. pneumoniae-infected CXCL1−/− mice. We administered LTB4 i.t. to CXCL1−/− mice at 1 h after K. pneumoniae infection. WT and CXCL1−/− mice were i.p. injected with anti-Gr-1/Ly6G or control Ab at 12 and 2 h before i.t. infection with K. pneumoniae. Data are presented as means ± SEM. *p < 0.05 (compared with control Ab-administered mice). (B) Protein levels of p67phox, p47phox, and iNOS in the lungs of neutrophil-depleted WT and CXCL1−/− mice after K. pneumoniae infection (10^3 CFU/mouse). This blot is representative of three separate blots. (C) Densitometric analysis of p67phox, p47phox, and iNOS levels in the lungs of neutrophil-depleted WT and CXCL1−/− mice after K. pneumoniae (10^3 CFU/mouse) infection. Western blots from three independent experiments were used to quantify protein levels compared with GAPDH. Data are expressed as mean ± SE. *p < 0.05 (compared with control Ab-administered mice). For experiments (A)–(C), n = 6–9 mice/group. Role of hematopoietic and resident cells in LTB4 production. (D) Levels of LTB4 in the lungs of i.t. neutrophil repleted (10^7 cells/mouse) neutropenic WT and CXCL1−/− mice at 48 h after K. pneumoniae infection. Data are presented as means ± SEM. *p < 0.05 [compared with depleted (non-repleted) mice]. (E) Bone marrow chimeras were generated, and LTB4 levels in lungs were measured at 48 h after K. pneumoniae challenge. A total of 5–7 mice/group were used. *p < 0.05 (compared with CXCL1−/−→CXCL1−/− mice).

LTB4 administration improves activation of NF-κB, MAPKs, and expression of cellular adhesion molecules in the lungs of CXCL1−/− mice after K. pneumoniae infection. To explore LTB4-mediated host immune mechanisms in CXCL1−/− mice in more detail, we measured the activation of NF-κB, MAPKs, and expression of ICAM-1 and VCAM-1 in the lungs after K. pneumoniae infection. Subsequent to LTB4 administration in K. pneumonia-infected CXCL1−/− mice, we observed augmented activation of NF-κB and MAPKs along with increased expression of ICAM-1 in CXCL1−/− mice (Fig. 5A, 5B). These findings demonstrate that augmentation of host immune mecha-
nisms in K. pneumoniae-infected CXCL1−/− mice after LTB₄ administration involve activation of NF-κB and MAPKs (JNK and ERK) and upregulation of cell adhesion molecules. LTB₄ augments expression of NADPH oxidase components in the lungs of CXCL1−/− mice after K. pneumoniae infection.

We next examined whether LTB₄ administration in K. pneumoniae-challenged CXCL1−/− mice leads to increased expression of NADPH oxidase and iNOS. Our results show that LTB₄ augments expression of p67phox, p47phox, and iNOS in CXCL1−/− mice (Fig. 5C, 5D). In contrast, LTB₄ administration in K. pneumoniae-infected WT mice did not alter the expression levels of either NADPH oxidase components or iNOS (Fig. 5C, 5D).

LTB₄ treatment enhances phagocytosis, bacterial killing, and ROS/RNS production in neutrophils of CXCL1−/− mice after K. pneumoniae stimulation.

The local restoration of components important for neutrophil bactericidal activity after i.t. LTB₄ administration suggested that the deficit in bacterial killing in CXCL1−/− neutrophils was dependent on LTB₄. To determine whether LTB₄ treatment augments bacterial killing, we purified neutrophils from bone marrow by negative selection and infected them with K. pneumoniae. Our findings show that LTB₄ treatment improved bacterial killing by CXCL1−/− neutrophils (Fig. 6A). The mechanism for this appeared to be multifactorial, as LTB₄ administration led to enhanced phagocytosis of K. pneumoniae-infected WT and CXCL1−/− neutrophils at 30 min posttreatment (MOI 1). For experiments (A)–(E), a total of 5-8 mice/group were used. *p < 0.05 (compared with CXCL1−/− neutrophils).

Discussion

Previous studies had shown that CXCL1 enhanced neutrophil recruitment to the lungs during infectious inflammation, as inhibition of CXCL1 by a blocking Ab resulted in attenuation of neutrophil migration to the airspaces after Escherichia coli LPS challenge in a rat model (7, 8). Furthermore, transgenic CXCL1 mice, which constitutively express CXCL1 within the lungs, have more neu-
trophic influx and less bacterial burden in their organs after challenge with *K. pneumoniae* (44). In subsequent studies, the signaling cascades associated with neutrophil-dependent bacterial clearance have been explored using CXCL1 gene-deleted mice (13, 28). Using CXCL1−/− mice, we reported previously that CXCL1 derived from both hematopoietic and resident cells is essential for expression of CXCL2/MIP-2 and CXCL5/LIX and activation of NF-κB and MAPKs in the lungs during *Klebsiella* infection (13).

Our current study is the first, to our knowledge, to determine the mechanisms by which CXCL1 regulates innate immunity to *Klebsiella*-induced pneumonia. Our results demonstrate that 1) CXCL1 mediates neutrophil accumulation in the lungs via LTB4 production during *K. pneumoniae* infection; 2) neutrophil-depleted animals exhibit reduced LTB4, NF-κB activation, and iNOS expression whereas neutrophil repletion in neutropenic mice enhances production of LTB4; 3) i.t. administration of LTB4 restores survival, neutrophil recruitment, cytokine/chemokine production, expression of NADPH oxidase components and iNOS, as well as activation of NF-κB and MAPKs in *K. pneumoniae*-infected CXCL1−/− mice; and 4) LTB4 treatment improves the expression of NADPH oxidase components and iNOS as well as bacterial killing capacity of *K. pneumoniae*-infected CXCL1−/− neutrophils. The model depicting the mechanisms underlying CXCL1-mediated neutrophil immunity in *K. pneumoniae* infection in the lung is shown in Fig. 7. During *K. pneumoniae* infection, CXCL1 is produced by local bone marrow-derived and resident cells and causes neutrophil recruitment into the lungs. These recruited neutrophils produce LTB4, which activates NF-κB and MAPKs essential for cytokine (TNF-α and IL-6), chemokine (MIP-2 and LIX), and ROS/RNS production. In turn, these events are important for neutrophil-mediated bacterial clearance in the lungs. Cytokines/chemokines and ROS/RNS can also induce more CXCL1 via a positive feedback loop involving autocrine and paracrine mechanisms. Although some of the cascades are not validated by the current investigation, future studies are required to explore these cascades.

A lipid mediator produced via the 5-lipoxygenase pathway of arachidonic acid metabolism (45–47), LTB4 is the major player in the formation of oxygen and nitrogen free radicals in myeloid cells, including macrophages and neutrophils (48–51). In addition, LTB4 is an important neutrophil chemoattractant that has been shown to regulate chemotaxis, degranulation, release of lysosomal enzymes, and formation of oxygen free radicals in neutrophils (52, 53). Furthermore, Ab-mediated MIP-2 blocking negatively regulates LTB4 production, an example of cross-talk that can occur between chemokines and LTB4 (54). Our studies illustrate roles for CXCL1 in mediating both neutrophil recruitment and bacterial killing by ROS and RNS via regulation of endogenous LTB4 production after infection. Because of the presence of the LTB4 receptors, BLT-1 and BLT-2, in both myeloid and lung-resident cells, the effects of LTB4 could be mediated through both autocrine and paracrine mechanisms (21, 26, 55–57).

The critical role of NADPH oxidase in host immunity is clearly shown by the immune deficiency syndrome chronic granulomatous disease, which is caused by an autosomal deficiency in NADPH oxidase that renders individuals vulnerable to life-threatening bacterial infections (58). Bacterial factors are known to induce oxidative stress in tissues, as observed in gastric epithelium during *H. pylori* infection as well as in the lungs after bacterial infection (59–62). In CXCL1−/− mice, we saw reduced expression and activation of NADPH oxidase components p67phox and p47phox after *K. pneumoniae* infection. Thus, as we observed reduced LTB4 in CXCL1−/− mice upon *K. pneumoniae* infection, we favor the interpretation that LTB4 is responsible for the production of ROS and RNS via the activation of NADPH oxidase.

Our studies suggest that the reduction in NADPH oxidase and iNOS expression observed in the lungs of CXCL1−/− mice after *K. pneumoniae* infection is due to either attenuated neutrophil...
accumulation and/or activation of bone marrow/resident cells in the lung. Notably, these findings demonstrate a predominant role for neutrophils in producing LTB4 and different components of NADPH oxidase and iNOS in the lungs. The bone marrow chimeras and neutrophil repletion experiments additionally support the conclusion that neutrophils are the major contributor of LTB4 in the lung. Although our studies clearly established roles for neutrophils in mediating LTB4 production, LTB4 can be produced by other bone marrow cells including AMs or lung-resident cells (23, 26). Our findings confirm this as neutrophil depletion did not completely abolish LTB4 levels in the lung after *K. pneumoniae* infection (Fig. 2). As LTB4 concentrations were decreased after Gr1 depletion (Fig. 2A), the majority of lung LTB4 did appear to be either directly or indirectly produced by neutrophils.

We focused on neutrophils in CXCL1−/− mice because of the critical importance of this unique cell type in *K. pneumoniae* clearance (44). In prior studies, we demonstrated that depletion of neutrophils resulted in modest changes in the production of CXCL1 and no change in the levels of CXCL2 and CXCL5 in the lungs after *K. pneumoniae* infection. These results are consistent with the fact that myeloid cells other than neutrophils and/or resident cells are involved in CXCL1, CXCL2, and CXCL5 production during intrapulmonary *K. pneumoniae* challenge (13). Conversely, we observed a substantial reduction in TNF-α levels in the lungs of...
neutrophil-depleted WT and CXCL1\(^{-/-}\) mice (13). Although it has been shown previously that TNF-\(\alpha\) can induce the synthesis of LTB4, it is likely that neutrophil-derived TNF-\(\alpha\) can induce LTB\(_4\) synthesis in the lungs via autocrine and/or paracrine mechanisms due to the presence of TNF receptors, such as TNFR1 and TNFR2, on both myeloid and resident cells in the lung (63, 64).

Notably, a single intrapulmonary administration of LTB4 corrected neutrophil recruitment and bacterial clearance in K. pneumoniae-infected CXCL1\(^{-/-}\) mice (Fig. 4). In line with this observation, earlier studies have shown that LTB4 is important for macrophage-mediated, but not neutrophil-mediated, host immunity via recruitment (65). This conclusion is supported by the fact that unlike macrophage influx, neutrophil recruitment to the lungs was unaffected after pneumococcal infection in mice (66). Our results show that neutrophils depleted by LTB4 treatment of CXCL1-deficient mice were used. *p < 0.05 (compared with BSA-treated neutrophils).

**FIGURE 6.** Impaired bacterial killing, ROS and RNS generation, and phagocytosis in K. pneumoniae-stimulated CXCL1\(^{-/-}\) neutrophils is restored by LTB4. (A) Bacterial killing capacity of K. pneumoniae-infected, LTB4-treated neutrophils from WT and CXCL1\(^{-/-}\) deficient mice was determined at 30, 60, and 180 min by assessing intracellular and extracellular CFUs. (B) Relative phagocytosis of K. pneumoniae-infected WT and CXCL1\(^{-/-}\) neutrophils at 30 min posttreatment (MOI 1). Relative phagocytosis index was calculated as described in Materials and Methods. (C) MPO release, intracellular MPO, NO, and H\(_2\)O\(_2\) production in K. pneumoniae-infected WT and CXCL1\(^{-/-}\) neutrophils at 30 and 180 min post-LTB4 treatment. For experiments (A)-(C), a total of 7–9 mice/group were used. *p < 0.05 (compared with BSA-treated neutrophils).

**FIGURE 7.** Scheme depicting CXCL1-dependent bacterial clearance in the lung after K. pneumoniae infection. K. pneumoniae induces CXCL1 production by hematopoietic and resident cells, causing neutrophil accumulation in the lungs. Neutrophil influx into the lungs is important for LTB4 production by bone marrow-derived cells. LTB4 subsequently activates NF-\(\kappa\)B and MAPKs, resulting in the production of cytokines/chemokines and the generation of ROS/RNS. Cytokines/chemokines and ROS/RNS regulate each other through signaling cascades. Cytokines/chemokines and ROS/RNS can regulate the production of LTB4 via a positive feedback loop. ROS/RNS ultimately lead to bacterial clearance from the lungs.

The production of cytokines and chemokines is mediated by transcription factors, including NF-\(\kappa\)B and MAPKs including ERK, p38, and JNK kinases (13, 29, 32, 65, 66). In this regard, investigations have unequivocally demonstrated that NF-\(\kappa\)B and MAPK activation is pivotal to inflammation during bacterial pneumonia (13, 29, 32, 65, 66).

An earlier study with K. pneumoniae infection in macrophages showed that LTB4 induced phosphorylation of p47\(\text{phox}\) and enhanced bacterial killing (24). In the current investigation, we demonstrated that isolated neutrophils produce substantial LTB4 and express both NADPH oxidase and iNOS. We also found that LTB4 treatment improved bacterial killing, phagocytosis, and the production of oxidants in K. pneumoniae-infected CXCL1\(^{-/-}\) neutrophils. Our results are in agreement with reported findings that LTB4 can augment neutrophil phagocytosis of K. pneumoniae via Fc- and complement-mediated mechanisms (41).

Observations from our current investigation have translational importance in identifying new avenues to augment host immunity in patients with nonfunctional CXCL1 who have bacterial pneumonia. In this regard, three single-nucleotide polymorphisms in human CXCL1 have been reported (67), although their association with host immune defects has not yet been examined. Although MIP-2 and TNF-\(\alpha\) were augmented in WT mice by exogenous LTB4 at a concentration of 100 ng/mouse, LTB4 did not affect neutrophil immunity in WT mice (Fig. 4). To augment neutrophil immunity, numerous cytokines, including IFN-\(\gamma\) and G-CSF, could be administered locally rather than systemically. The chemotactic lipid LTB4 has advantages over cytokine proteins be-
cause it is less immunogenic and more cost-effective than the cytokines and can be successfully administered locally.

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Disclosures
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