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Intrapulmonary Administration of Leukotriene B4 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 B4 (LTB4), 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 LTB4, NADPH oxidase, and inducible NO synthase (iNOS) expression in lungs and neutrophils, and 2) whether LTB4 postinfection reverses innate immune defects in CXCL1−/− mice via regulation of NADPH oxidase and iNOS. Our results demonstrate reduced neutrophil influx, attenuated LTB4 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 LTB4 after infection. To treat immune defects in CXCL1−/− mice, we intrapulmonarily administered LTB4. Postinfection, LTB4 treatment reversed immune defects in CXCL1−/− mice and improved survival, neutrophil recruitment, cytokine/chemokine expression, NF-κB inhibition, and these defects can be restored by addition of exogenous LTB4 in neutrophils and in regulating host immunity against K. pneumoniae infection. Our findings suggest that LTB4 could be used to correct defects in neutrophil recruitment and function in individuals lacking or expressing malfunction CXCL1.

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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 p67phox, p47phox, p40phox, p22phox, and gp91phox, only p67phox, p47phox, and p40phox exist in the cytoplasm in an unactivated state (18, 19). Upon cell activation, p67phox, p47phox, and p40phox translocate onto the membrane. This complex is an electron transport chain that produces H2O2 in combination with superoxide dismutase (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 B4 (LTB4) has been shown to be a neutrophil chemoattractant derived from membrane phospholipids (21, 22). The role of LTB4 in the context of ROS and RNS production and bacterial killing has largely been explored in macrophages. LTB4 induces NADPH oxidase activation in alveolar macrophages (AMs) in response to Klebsiella infection. LTB4-deficient human AMs exhibit impaired phagocytosis and killing of pneumococci, and these defects can be restored by addition of exogenous LTB4 (23). Genetic deletion of 5-lipoxygenase or pharmacological inhibition of LTB4 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 LTB4 augmented p47phox expression and bacterial clearance in primary lung macrophages (24). In this regard, LTB4 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 LTβ4 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, LTβ4, NADPH oxidase, or inducible NO synthase (iNOS) in neutrophils during *K. pneumoniae* infection. We illustrate that CXCL1 controls neutrophil immunity by regulating LTβ4, ROS, and RNS production after *Klebsiella* infection. Compared with wild-type (WT) controls, exogenous LTβ4 corrected host immunity in CXCL1−/− mice by restoring neutrophil influx, bacterial clearance, cytokine/chemokine production, activation of NF-κB and MAPKs, as well as expression of ROS and RNS. Moreover, LTβ4 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 LTβ4 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). Animal experiments were conducted as per the Louisiana State University Animal Welfare Committee’s approved protocol.

**Bacterial preparation and infection**

The *K. pneumoniae* strain (ATCC 43816) was grown in tryptic soy broth overnight to mid-logarithmic phase at 37°C while shaking at 200 rpm. After PBS washings, bacteria were resuspended in isotonic saline at a concentration of 10^7 CFU/μl per mouse. For infection, a ketamine/xylazine mixture was used to anesthetize mice, and the trachea was exposed for inoculation with 10^7 CFU/mouse (13, 29). A 10-fold serially diluted suspension of initial inoculum was plated onto tryptic soy agar plates and MacConkey plates for validation of the inoculum.

**LTβ4 administration**

LTβ4 (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 LTβ4 determination as described in our previous publications (24).

**BALF isolation and lung harvesting**

BALF was collected as described previously (13, 29–31). In brief, trachea 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. Cytosin slides prepared with 0.5 ml BALF were stained by 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 infection, and were immediately snap frozen followed by storage at −80°C for later use.

**Total WBC, polymorphonuclear leukocyte, cytokine/chemokine, and LTβ4 determination**

We used BALF and lungs that were obtained from animals after *K. pneumoniae* infection, *K. pneumoniae* plus LTβ4 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 LTβ4 were obtained from R&D Systems (Minneapolis, MN). The minimum detection limit was 8 pg/ml cytokine/chemokine/protein, whereas the detection limit for LTβ4 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 GAPDH and pan-cadherin to indicate the cytoplasm 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 from tibiae and femurs using PBS. The cell suspension was passed through a 0.7-μm filter and then resuspended in RoboSep buffer (PBS without Ca^2+ and Mg^2+; 2% FBS, 1 mM EDTA). Purified neutrophils were obtained from bone marrow cells using a by using a custom mixture containing Abs to CD5, CD4, CD45R/B220, TER119, F4/80, CD11c, and e-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 poured 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 H_2O_2 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 IA8; 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 LTβ4, polymorphonuclear leukocyte (PMN) repletion was performed i.t. in neutropenic mice with PMNs (10^7 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 × 10^7 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 homogenate samples 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 GAPDH were obtained from Santa Cruz Biotechnology (Santa Cruz, CA), and Abs...
Hydrogen peroxide release by neutrophils

Hydrogen peroxide levels were measured to evaluate ROS production by using the Fluorescent H₂O₂/Peroxidase Detection Kit (Cell Technology, Mountain View, CA). The Fluoro H₂O₂ detection kit uses a nonfluorescent detection reagent to detect H₂O₂. H₂O₂ oxidizes the detection reagent in a 1:1 stoichiometry to produce a fluorescent product, resorufin, which is measured using a colorimetric assay kit (Cayman Chemical Company). A standard curve was plotted by diluting standards with incubation media (36, 37).

MPO activity in neutrophils and lungs

MPO release by the neutrophils was measured as previously described with minor modifications (13, 29). Briefly, culture media obtained at different time intervals were stored at −80°C until evaluation. The reaction mixture consisted of 100 μl culture media and 50 μl 1.0% hexadecyltrimethylammonium bromide (HTAB) (Sigma Chemical, St. Louis, MO) in 50 μl 1 M potassium phosphate buffer (pH 6) containing 1 mM dianisidine hydrochloride (0.167 mg/ml; Sigma Chemical) and 0.0005% hydrogen peroxide. Absorbance change at 460 nm was recorded with a spectrophotometer (U-2001; Hitachi, Tokyo, Japan) every 5 min at room temperature. For MPO activity in the lung and neutrophils, tissues were homogenized or cells were lysed in HTAB-phosphate buffer containing 0.1% dianisidine hydrochloride and hydrogen peroxide, and MPO activity was measured in cells as described in our earlier publication (13, 29–31).

Survival studies

WT and CXCL1−/− mice were inoculated i.t. with 10³ 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 were mixed at a 1:1 ratio in polystyrene tubes (1 ML) 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 × 10⁶ neutrophils were suspended in RPMI 1640 with 10% v/v FBS, and 1 × 10⁶ 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 with 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 immunidadaptability 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 chemotactic lipid, we also examined LTB₄ levels in the lungs of 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 (Fig. 1B). 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 determined 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, IF). These data suggest that CXCL1 is essential for the regulation of ROS and RNS generation in the lungs.

**Neutrophils are the major source of LTB$_4$, 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 LTB$_4$, p67phox, p47phox, and iNOS in lungs postinfection. To our surprise, we found substantial reduction in LTB$_4$, 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 LTB$_4$ production (Fig. 2D), as bone marrow chimera experiments demonstrated that bone marrow cells were a major source of LTB$_4$ (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 H$_2$O$_2$ 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).

**LTB$_4$ 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 LTB$_4$ as well as ROS and RNS during bacterial infections, we wanted to determine whether LTB$_4$ reg-

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**FIGURE 1.** MPO activity and LTB$_4$ production in the lungs is dependent on CXCL1 during K. pneumoniae infection. (A and B) MPO activity (A) and LTB$_4$ levels (B) in homogenized (unlavaged) whole lungs of WT (C57BL/6) and CXCL1−/− mice infected with K. pneumoniae (10$^3$ 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 LTB\(_4\) i.t. to CXCL1\(^{-/-}\) mice at 1 h after *K. pneumoniae* infection (10\(^3\) CFU/mouse). Intriguingly, we observed that LTB4 treatment dramatically improved survival and bacterial clearance of the lungs and bacterial dissemination in CXCL1\(^{-/-}\) mice, but not in WT mice, after *K. pneumoniae* infection (Fig. 4A–C). We next examined whether LTB\(_4\) treatment affects pathologic consequences in the lungs of CXCL1\(^{-/-}\) mice during Gram-negative bacterial pneumonia. Total leukocyte count (Fig. 4D), neutrophil count (Fig. 4E), and expression of cytokines (TNF-\(\alpha\), IL-6) and chemokines (MIP-2 and LIX) were enhanced in CXCL1\(^{-/-}\) mice upon LTB\(_4\) administration (Fig. 4F). These findings show that LTB\(_4\) can correct impaired host immune defects in CXCL1\(^{-/-}\) mice after *K. pneumoniae* infection.

**LTB\(_4\) administration improves activation of NF-\(\kappa\)B, MAPKs, and expression of cellular adhesion molecules in the lungs of CXCL1\(^{-/-}\) mice after *K. pneumoniae* infection.** To explore LTB\(_4\)-mediated host immune mechanisms in CXCL1\(^{-/-}\) mice in more detail, we measured the activation of NF-\(\kappa\)B, MAPKs, and expression of ICAM-1 and VCAM-1 in the lungs after *K. pneumoniae* infection. Subsequent to LTB\(_4\) administration in *K. pneumoniae*-infected CXCL1\(^{-/-}\) mice, we observed augmented activation of NF-\(\kappa\)B 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* stimulation

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 p67^phox^, p47^phox^, 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, 60, and 180 min postinfection. Experiments were performed in triplicate. (D) Bacterial killing capacity of *K. pneumoniae*-infected neutrophils from WT and CXCL1^−/−^ deficient mice was determined by assessing extracellular and intracellular CFUs at 30, 60, and 180 min after infection with *K. pneumoniae* (MOI 1). (E) Relative 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 neutrophils.
FIGURE 4. Impaired survival, bacterial clearance, neutrophil influx, and cytokine/chemokine production in the lungs of CXCL1−/− mice are restored by exogenous LTB4. (A) Mortality in WT or CXCL1−/− mice infected with 10^3 CFU K. pneumoniae and administered LTB4 (100 ng/mouse) or vehicle (BSA) 1 h after infection; survival was assessed up to 15 d. Data are presented as percent survival (n = 20 mice/group) and analyzed using Wilcoxon signed-rank test. *p < 0.05 (difference between LTB4- or vehicle (BSA) control-treated CXCL1−/− mice); †p < 0.05 (difference between WT plus LTB4 and CXCL1−/− plus LTB4 mice). (B and C) Bacterial clearance in the lungs and dissemination were examined in lung homogenates of LTB4- or BSA-administered WT and CXCL1−/− mice at 48 and 96 h after K. pneumoniae challenge (10^3 CFU/mouse). Data are presented as mean ± SE (n = 5 to 6 mice/group). *p < 0.05 [compared with BSA (vehicle)-administered mice]. (D and E) Cellular infiltration in airspaces at 48 h after i.t. treatment with LTB4 (100 ng/mouse) or vehicle (BSA) control. (F) Concentrations of CXCL2/2, MIP-2, IL-6, TNF-α, and CXCL5/LIX in BALF from CXCL1−/− or WT mice infected with 10^3 CFU K. pneumoniae and administered LTB4 or vehicle (BSA). For experiments (D)–(F), n = 6–8 mice/group. *p < 0.05 (compared with BSA-administered mice).

trophil 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 lung 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, NADPH oxidase, 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 during 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 p67(phox) and p47(phox) 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). We focused on neutrophils in CXCL12/−/− 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 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\textsuperscript{−/−} mice (13). Although it has been shown previously that TNF-\textalpha can induce the synthesis of LTB\textsubscript{4}, it is likely that neutrophil-derived TNF-\textalpha can induce LTB\textsubscript{4} synthesis in the lungs via autocrine and/or paracrine mechanisms due to the presence of TNF receptors, such as TNFR\textsubscript{1} and TNFR\textsubscript{2}, on both myeloid and resident cells in the lung (63, 64).

Notably, a single intrapulmonary administration of LTB\textsubscript{4} corrected neutrophil recruitment and bacterial clearance in the lungs via autocrine and/or paracrine mechanisms due to the presence of TNF receptors, such as TNFR\textsubscript{1} and TNFR\textsubscript{2}, on both myeloid and resident cells in the lung (63, 64).

An earlier study with \textit{K. pneumoniae} infection in macrophages showed that LTB\textsubscript{4} induced phosphorylation of p47\textsubscript{phox} and enhanced bacterial killing (24). In the current investigation, we demonstrated that isolated neutrophils produce substantial LTB\textsubscript{4} and express both NADPH oxidase and iNOS. We also found that LTB\textsubscript{4} treatment improved bacterial killing, phagocytosis, and the production of oxidants in \textit{K. pneumoniae}-infected CXCL1\textsuperscript{−/−} neutrophils. Our results are in agreement with reported findings that LTB\textsubscript{4} can augment neutrophil phagocytosis of \textit{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, investigations have unequivocally demonstrated that NF-\kappaB and MAPK activation is pivotal to inflammation during bacterial pneumonia (13, 29, 32, 65, 66). In this regard, investigations have unequivocally demonstrated that NF-\kappaB and MAPK activation is pivotal to inflammation during bacterial pneumonia (13, 29, 32, 65, 66).
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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