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Intrapulmonary Administration of Leukotriene B₄ Augments Neutrophil Accumulation and Responses in the Lung to *Klebsiella* Infection in CXCL1 Knockout Mice

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Abstract

In prior studies we demonstrated that: 1) CXCL1/KC is essential for NF- κ B 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. Here, we investigated: 1) the role of CXCL1 in LTB₄, NADPH oxidase, and iNOS expression in lungs and neutrophils; and 2) whether LTB₄ after infection 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 following *K. pneumoniae* infection. Using neutrophil depletion and repletion, we found that neutrophils are the predominant source of pulmonary LTB₄ following infection. To treat immune defects in CXCL1^{-/-} mice, we intrapulmonary administered LTB₄. Post-infection LTB₄ treatment reversed immune defects in CXCL1^{-/-} mice and improved survival, neutrophil recruitment, cytokine/chemokine expression, NF- κ B/MAPK activation, and ROS/RNS production. LTB₄ also enhanced MPO, H₂O₂, 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 LTB₄ could be used to correct defects in neutrophil recruitment and function in individuals lacking or expressing malfunctioning CXCL1.

INTRODUCTION

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 towards the site of infection in order 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- κ B 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 nitric oxide synthases (NOSs) 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 H₂O₂ in combination with superoxide dismutase (18, 19). Superoxide is further converted to reactive hypochlorite by myeloperoxidase (18, 19). Furthermore, nitric oxide is produced from guanidino nitrogen during the conversion of L-arginine to L-citrulline by NOSs (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 (5-LO) or pharmacological inhibition of LTB₄ biosynthesis in mice results in enhanced mortality and attenuated microbial clearance following pneumococcal infection; this occurs via recruitment of macrophages but not neutrophils (24, 25). One of these reports also demonstrated that LTB₄ augmented p47^{phox} 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, nitric oxide has been shown to be important in *Klebsiella* clearance (27). However, more detailed mechanisms underlying LTB₄ 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, LTB₄, NADPH oxidase, or iNOS in neutrophils during *K. pneumoniae* infection. We illustrate that CXCL1 controls neutrophil immunity by regulating LTB₄, ROS, and RNS production following *Klebsiella* infection. Compared to WT controls, exogenous LTB₄ corrected host immunity in CXCL1^{-/-} mice by restoring neutrophil influx, bacterial clearance, cytokine/chemokine production, activation of NF- κ B and MAPKs, as well as expression of ROS and RNS. Moreover, LTB₄ 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 LTB₄ administration.

MATERIALS AND METHODS

Animals

Eight to ten-week old CXCL1 gene-deficient (CXCL1^{-/-}) female mice were backcrossed 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 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. Following PBS washings, bacteria were resuspended in isotonic saline at a concentration of 10³ CFU/50 µl/mouse. For infection, a ketamine/xylazine mixture was used to anesthetize mice and the trachea was exposed for inoculation with 10³ CFU/mouse (13, 29). A 10-fold serially diluted suspension of initial inoculum was plated onto Tryptic Soy Agar (TSA) plates and MacConkey plates for validation of the inoculum.

LTB₄ administration

LTB₄ (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 i.t. at 1 h post-*Klebsiella* challenge as described (23). Following 48 h post-infection, bronchoalveolar lavage fluid (BALF) or lungs were collected for LTB₄ 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 4 times, and retrieved. A total of 3.0 ml BALF was retrieved from each mouse. Cytospin slides prepared with 0.5 ml of 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 post-*K. pneumoniae* challenge and were immediately snap frozen followed by storage at -80°C for later use.

Total WBC, PMN, cytokine/chemokine, and LTB₄ determination

We used BALF and lungs that were obtained from animals following *K. pneumoniae* infection, *K. pneumoniae*+LTB₄ administration or *K. pneumoniae* +BSA (vehicle) instillation. ELISA kits for TNF-α and IL-6 were obtained from eBioscience San Diego, CA) whereas kits for CXCL1, MIP-2, LIX and LTB₄ were obtained from R&D Systems (Minneapolis, MN). The minimum detection limit was 8 pg/ml of cytokine/chemokine protein, whereas the detection limit for LTB₄ 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, City, State) 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-p67^{phox} and p47^{phox} antibodies. 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, Canada). Bone marrow cells were flushed from tibias and femurs using PBS. The cell suspension was passed through a 0.70 μ filter then resuspended in RoboSep buffer (PBS without $\text{Ca}^{2+}/\text{Mg}^{2+}$, 2% FBS, 1 mM EDTA). Purified neutrophils were obtained from bone marrow cells by using a custom mixture containing Abs to CD5, CD4, CD45R/B220, TER119, F4/80, CD11c, 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 antibody cocktail, biotin selection cocktail, and magnetic colloid according to manufacturer's instructions. Samples were then placed in the EasySep magnet. Following three minutes 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 , nitric oxide (NO) and to determine the expression levels of the components of NADPH oxidase system after *K. pneumoniae* infection. Neutrophil purity neared 91%, as assessed by cytospin slides and flow cytometry (data not shown).

Neutrophil depletion

The neutrophil depletion protocol (GR1/Ly6G) used here has been described earlier (13, 31). A total of 50 μg of anti-mouse Ly6G mAb (clone IA8; BD Pharmingen, City, State) per mouse was administrated i.p. in a 50 μl at 12 and 2 h prior to bacterial infection. As a control, 50 μg of isotype-matched control mAb in an equal volume was administrated 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 days 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 LTB_4 , 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 6–8 wk old) were used to make chimeras. Recipients were γ -irradiated in two 525-rad doses delivered over 3 h. Isolated bone marrow cells (8×10^6 /mouse) were injected into the tail vein of recipients and animals were kept on 0.2% antibiotic (neomycin sulfate) for the first 3 wks. 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 in 1 ml of buffer containing 0.1% Triton X-100 in PBS, with complete protease and phosphatase inhibitor cocktail (Roche Co, Indianapolis, IN) for 30 s and 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 PVDF membrane using standard protocols (13, 29). Antibodies to p67^{phox}, p47^{phox}, and GAPDH were obtained from Santa Cruz Biotechnology (Santa Cruz, CA); whereas

antibodies to iNOS, ICAM-1, VCAM-1, phospho-NF- κ B (Ser536), NF- κ B, phospho-I κ -B α (Ser 32/36), I κ -B α , phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204), phospho-p38 MAPK (Thr180/Tyr182), phospho-JNK (Thr183/Tyr185), total p38 MAPK, and pan-cadherin were obtained from Cell Signaling Technology (Danvers, MA). Primary antibodies were added at a 1:1000 dilution whereas monoclonal Ab to mouse GAPDH was added at 1:5000 dilution. Immunostaining was performed using appropriate secondary Ab at a dilution of 1: 5000, and developed with ECL plus Western blot detection system (ThermoFisher, Piscataway, NJ). The intensity of immunoreactive bands was determined using gel Digitizing Software (UN-SCAN-IT gel™ from Silk Scientific, Inc., City, Utah) for densitometric analysis.

Nitric oxide release by neutrophils

The NO assay was performed as described in previous reports (36, 37). Neutrophils isolated from bone marrow were infected with 1 MOI of *K. pneumoniae* for different time intervals. Media were collected at designated time-points for the detection of NO₂ and NO₃ anions using a colorimetric assay kit (Cayman Chemical Company, MI). A standard curve was plotted by diluting standards with incubation media (36, 37)

Hydrogen peroxide release by neutrophils

Hydrogen peroxide levels were measured to evaluate reactive oxygen species production by using the Fluorescent H₂O₂/Peroxidase Detection Kit (Cell Technology, Inc, Mountain View, CA). The Fluoro H₂O₂ detection kit utilizes a non-fluorescent detection reagent to detect H₂O₂. H₂O₂ oxidizes the detection reagent in a 1:1 stoichiometry to produce a fluorescent product, resorufin, which is catalyzed by peroxidase in a homogeneous no wash assay system. After incubation, fluorescence was measured at an excitation of 540 nm and an emission of 595 nm (38).

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 was stored at -80 °C until evaluation. The reaction mixture consisted of 100 μ l of culture media and 50 μ l of 1.0% hexadecyltrimethylammoniumbromide (HTAB) (Sigma Chemical, St. Louis, MO) in 100 mmol/l phosphate buffer (pH 6.0) and 50 μ l of 50 mmol/l potassium phosphate buffer (pH 6.0) containing o-dianisidine hydrochloride (0.167 mg/ml; Sigma Chemical, St. Louis, MO) 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 o-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 of *K. pneumoniae* in 50 μ l of 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 μ g/ml; 50 μ l/mouse was administered i.t. at 1 h post- *Klebsiella* challenge. The control animals received 50 μ l/mouse PBS containing 0.1% BSA and animal survival was examined over 15 days (13, 29).

Lung bacterial CFUs

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

Phagocytosis assay

Klebsiella expressing green fluorescent protein (GFP) were opsonized in RPMI containing 10% FBS for 30 mins at 37°C with constant agitation. Purified neutrophils and bacteria were mixed at a 1:1 ratio in polystyrene tubes (1 MOI) with agitation at 37°C in 100 µl of RPMI 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). Antibactericidal 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 $100 \times g$ 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 of 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 TSA plates. The number of colonies was enumerated after incubation at 37°C overnight. .

Statistical analysis

Data were analyzed by Student's *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 (San Diego, CA). Significance was defined as $p < 0.05$.

Results

CXCL1 controls neutrophil recruitment and LTB₄ production in the lungs following *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 as compared to 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 post-infection (Fig. 1A). Since LTB₄ is a neutrophil chemotactic lipid, we also examined LTB₄ levels in the lungs of CXCL1^{-/-} mice following *K. pneumoniae* administration. We found significantly reduced levels of LTB₄ in CXCL1^{-/-} mice at 24 and 48 h post-infection as compared to 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 following *K. pneumoniae* infection

The NADPH oxidase complex includes p67^{phox} and p47^{phox}, which regulate the production of H₂O₂, whereas inducible nitric oxide synthase (iNOS) controls the production of reactive nitrogen species; 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 following *K. pneumoniae* infection. We found that the expression of p47^{phox}, a key component of the NADPH oxidase, was decreased in CXCL1^{-/-} mice at 24 and 48 h post-infection, whereas the expression of iNOS was decreased at 48 h post-infection, the time point at which it was first detected in WT (Figs. 1C-D). Furthermore, the expression of p67^{phox} was not reduced in CXCL1^{-/-} mice compared to WT at either 24 or 48 h post-infection (Figs. 1C-D). Nevertheless, the expression of both p67^{phox} and p47^{phox} was higher at 24 h, whereas the expression of iNOS was higher at 48 h (Figs. 1C-D). To determine the level of activation of NADPH oxidase, we explored the translocation of p67^{phox} and p47^{phox} from the cytoplasm to the plasma membrane in lung cells 48 h post-*K. pneumoniae* challenge. Our results show attenuated p67^{phox} and p47^{phox} translocation in the lungs of CXCL1^{-/-} mice compared to their littermate controls (Fig. 1E-F). 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 following *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 antibody, then determined the expression of LTB₄, p67^{phox}, p47^{phox}, and iNOS in lungs after infection. To our surprise, we found substantial reduction in LTB₄, p67^{phox}, p47^{phox} and iNOS in the lungs of granulocyte-depleted mice following *K. pneumoniae* challenge (Figs 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 nitric oxide in neutrophils following *K. pneumoniae* infection *in vitro*

Since 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 p67^{phox} and p47^{phox} in neutrophils isolated from CXCL1^{-/-} mice at 180 min after *K. pneumoniae* stimulation (Fig 3A-B). 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-E).

LTB₄ administration augments survival, bacterial clearance, cellular recruitment, and expression of cytokines/chemokines in the lungs of CXCL1^{-/-} mice following *K. pneumoniae* infection

Because CXCL1 regulates LTB₄ as well as ROS and RNS during bacterial infections, we wanted to determine whether LTB₄ regulates ROS- and RNS-dependent host immune mechanisms in *K. pneumoniae*-infected CXCL1^{-/-} mice. We administered LTB₄ i.t. to CXCL1^{-/-} mice at 1 h post-*K. pneumoniae* challenge, based on an earlier report showing that this route was the most efficient for administering LTB₄ in mice (24). Intriguingly, we observed that LTB₄ treatment dramatically improved survival and bacterial clearance the lungs and bacterial dissemination in CXCL1^{-/-} mice, but not WT mice, following *K. pneumoniae* infection (Fig.4 A-C). We next examined whether LTB₄ administration affects pathologic consequences in the lungs of CXCL1^{-/-} mice during Gram-negative bacterial pneumonia. Total leukocyte count (Fig. 4D), neutrophil count (Fig. 4E), expression of cytokines (TNF- α , IL-6) and chemokines (MIP-2 and LIX) were enhanced in CXCL1^{-/-} mice upon LTB₄ administration (Fig. 4F). These findings show that LTB₄ can correct impaired host immune defects in CXCL1^{-/-} mice following *K. pneumoniae* infection.

LTB₄ administration improves activation of NF- κ B, MAPKs, and expression of cellular adhesion molecules in the lungs of CXCL1^{-/-} mice following *K. pneumoniae* infection

To explore LTB₄-mediated host immune mechanisms in CXCL1^{-/-} mice in more detail, we measured the activation of NF- κ B, MAPKs, and expression of ICAM-1 and VCAM-1 in the lungs following *K. pneumoniae* infection. Subsequent to LTB₄ administration in *K. pneumoniae*-infected CXCL1^{-/-} mice, we observed augmented activation of NF- κ B and MAPKs along with increased expression of ICAM-1 in CXCL1^{-/-} mice (Fig 5A-B). These findings demonstrate that augmentation of host immune mechanisms in *K. pneumoniae*-infected CXCL1^{-/-} mice after LTB₄ administration involve activation of NF- κ B and MAPKs (JNK and ERK), and upregulation of cell adhesion molecules.

LTB₄ augments expression of NADPH oxidase components in the lungs of CXCL1^{-/-} mice following *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 p67^{phox}, p47^{phox}, and iNOS in CXCL1^{-/-} mice (Fig 5C-D). On the other hand, LTB₄ administration in *K. pneumoniae*-infected WT mice did not alter the expression levels of either NADPH oxidase components or iNOS (Fig. 5C-D).

LTB₄ treatment enhances phagocytosis, bacterial killing, and ROS/RNS production in neutrophils of CXCL1^{-/-} mice following *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. In order 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 multi-factorial, as LTB₄ administration led to enhanced phagocytosis of *K. pneumoniae* in CXCL1^{-/-} neutrophils (Fig 6B), and increased neutrophil MPO activity and H₂O₂ production (Fig 6C). These findings suggest that LTB₄ is critical for phagocytosis and bacterial clearance in CXCL1^{-/-} neutrophils via oxidative stress.

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 neutrophil 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- κ B and MAPKs in the lung during *Klebsiella* infection (13).

Our current study is the first to determine the mechanisms by which CXCL1 regulates innate immunity to *Klebsiella*-induced pneumonia. Our results demonstrate that: (i) CXCL1 mediates neutrophil accumulation in the lungs via LTB₄ production during *K. pneumoniae* infection; (ii) neutrophil-depleted animals exhibit reduced LTB₄, NADPH oxidase, and iNOS expression while neutrophil repletion in neutropenic mice enhances production of LTB₄; (iii) 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- κ B and MAPKs in *K. pneumoniae*-infected CXCL1^{-/-} mice; and (iv) 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- κ B 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 (5-LO) 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, antibody-mediated MIP-2 blocking negatively regulates LTB₄ production, an example of crosstalk 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 following 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 following

bacterial infection (59-62). In CXCL1^{-/-} mice we saw reduced expression and activation of NADPH oxidase components p67^{phox} and p47^{phox} following *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 following *K. pneumoniae* infection is due to either attenuated neutrophil accumulation and/or activation of bone marrow/resident cells in the lung. Interestingly, 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 following 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 following *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). While it has been shown previously that TNF- α can induce the synthesis of LTB₄, it is likely that neutrophil-derived TNF- α can induce LTB₄ 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).

Interestingly, 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, but not neutrophil, mediated host immunity via recruitment (24). This conclusion is supported by the fact that, unlike macrophage influx, neutrophil recruitment to the lungs was unaffected following pneumococcal infection in mice (24). Our results show LTB₄ restored CXCL1-dependent cytokine and chemokine production in the lungs, suggesting that LTB₄ interaction with its G-protein coupled receptors (BLT-1 and BLT-2) induces downstream signaling leading to cytokine/chemokine production. The production of cytokines and chemokines is mediated by transcription factors, including NF- κ B and MAPKs including ERK, p38 and JNK kinases (13, 29, 32, 65, 66). In this regard, investigations have unequivocally demonstrated that NF- κ 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^{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. While MIP-2 and TNF- α 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- γ and G-CSF could be administered locally rather than systemically. The chemotactic lipid, LTB₄ has advantages over cytokine proteins because it is less immunogenic and more cost effective than the cytokines, and can be successfully administered locally.

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List of Abbreviations

ICAM-1	Intracellular cell-adhesion molecule-1
LIX	Lipopolysaccharide-induced CXC chemokines
KC	Keratinocyte cell derived chemokines
MIP-2	macrophage inflammatory protein-2
VCAM-1	Vascular cell-adhesion molecule-1
ROS	Reactive oxygen species
RNS	Reactive nitrogen species
BALF	Bronchoalveolar lavage fluid
MPO	Myeloperoxidase

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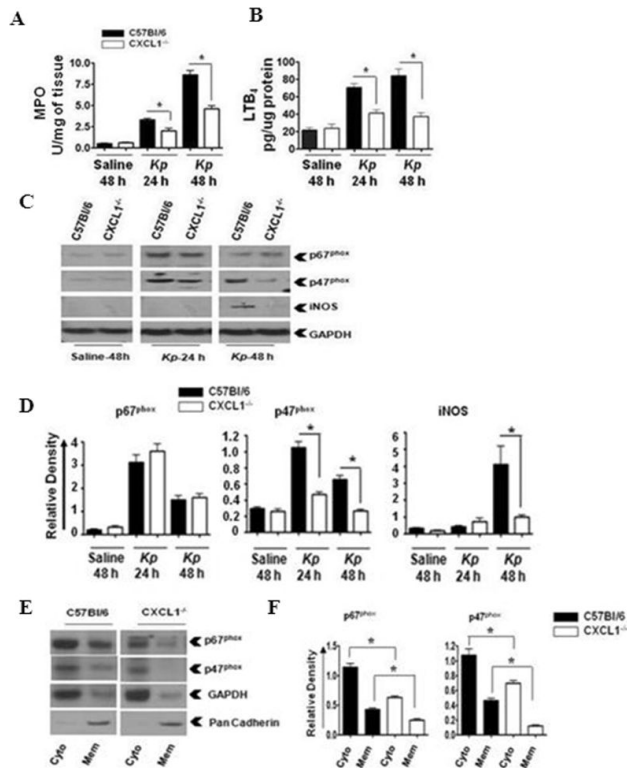


Figure 1. MPO activity and LTB₄ production in the lungs is dependent on CXCL1 during *K. pneumoniae* infection

MPO activity (A) and LTB₄ levels (B) in homogenized (unlabeled) 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. (* indicates p<0.05 compared with CXCL1^{-/-} mice). The levels of p67^{phox}, p47^{phox}, and iNOS (C) in *K. pneumoniae*-infected homogenized whole lungs of WT and CXCL1^{-/-} mice at 24 and 48 h post-infection. The blot is representative of three individual blots with identical results. Densitometric analysis of p67^{phox}, p47^{phox} and iNOS expression (D) in homogenized lungs. Densitometry was performed from three separate blots. Data are expressed as means ± SEM. n=6-8 mice/group. (* indicates p<0.05 compared to CXCL1^{-/-} mice). Levels of p67^{phox} and p47^{phox} in cytosolic and membrane fractions of the lung (E) 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. Densitometric analysis of p67^{phox} and p47^{phox} levels (F) in cytosol and membrane fractions of the lung following *K. pneumoniae* infection. Protein expression was quantitated from three separate blots. Data are shown as means ± SEM. For experiments E-F, a total of 6-8 mice were used in each group. (* indicates p<0.05 as compared to CXCL1^{-/-} mice.)

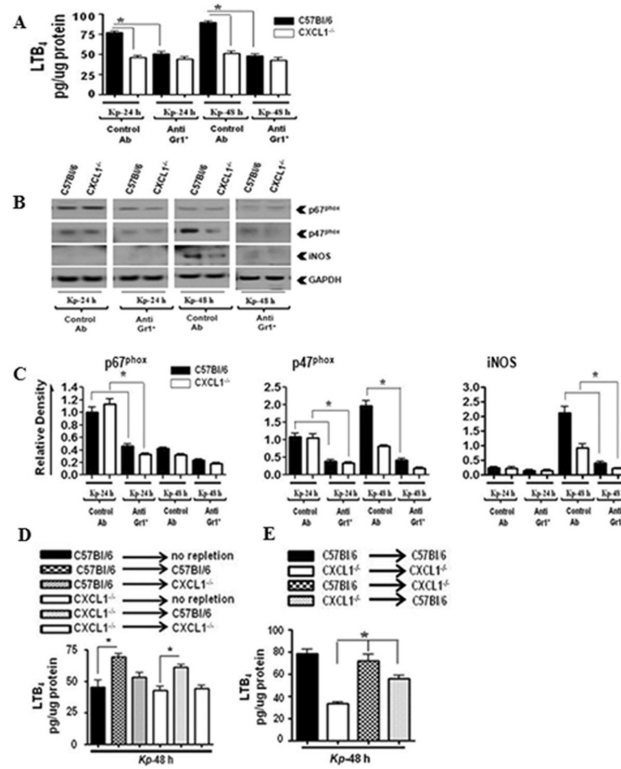


Figure 2. Neutrophil depletion impairs LTB₄, iNOS, and NADPH oxidase component expression in lung tissues during *K. pneumoniae* infection

LTB₄ levels (A) in the lungs of neutrophil-depleted WT and CXCL1^{-/-} mice at 24 and 48 h following *K. pneumoniae* infection (10³ CFUs/mouse). 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. (* indicates *p* < 0.05 compared with control Ab-administered mice.) Protein levels of p67^{phox}, p47^{phox}, and iNOS in whole lung homogenates (B) of neutrophil-depleted WT and CXCL1^{-/-} mice after *K. pneumoniae* infection (10³ CFUs/mouse). This blot is representative of three separate blots. Densitometric analysis (C) of p67^{phox}, p47^{phox}, and iNOS levels in the lungs of neutrophil-depleted WT and CXCL1^{-/-} mice following *K. pneumoniae* (10³ CFUs/mouse) infection. Western blots from three independent blots experiments were used to quantify protein levels compared to GAPDH. Data are expressed as mean ± SE. For experiments A-C, n=6-9 mice/group. (* indicates *p*<0.05 as compared to control Ab-administered mice.) Role of hematopoietic and resident cells in LTB₄ production. Levels of LTB₄ (D) in the lungs of i.t. neutrophil repleted (10⁷ cells/mouse) neutropenic WT and CXCL1^{-/-} mice at 48 h following *K. pneumoniae* infection. Data are presented as means ± SEM. (* indicates *p*<0.05 compared to depleted (non-repleted) mice.) (E) Bone marrow chimeras were generated and LTB₄ levels in lungs were measured at 48 h post *K. pneumoniae* challenge. A total of 5-7 mice/group were used. (* indicates *p* < 0.05 compared with CXCL1^{-/-}→CXCL1^{-/-} mice.)

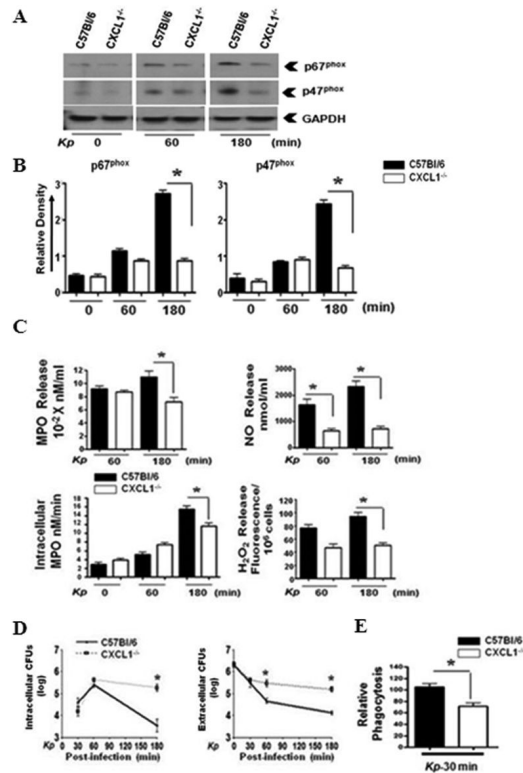


Figure 3. CXCL1 is essential for *Klebsiella*-induced expression of p67^{phox}, p47^{phox}, and ROS/RNS production by neutrophils
 Levels of p67^{phox} and p47^{phox} (A) in bone marrow-derived neutrophils from WT and CXCL1^{-/-} mice after infection with *K. pneumoniae* (MOI of 1). This is a representative blot from three separate experiments. Densitometric analysis from three separate blots (B) shows the expression of p67^{phox} and p47^{phox} in *K. pneumoniae* stimulated-neutrophils that were normalized against GAPDH. (* indicates p<0.05 as compared to CXCL1^{-/-} neutrophils). MPO activity and nitrite and H₂O₂ release (C) in WT and CXCL1^{-/-} neutrophils stimulated with *K. pneumoniae*. The levels of MPO, nitrite, and H₂O₂ were measured in infected neutrophils at 30, 60, and 180 min post-infection. Experiments were performed in triplicates. Bacterial killing capacity (D) of *K. pneumoniae*-infected neutrophils from WT and CXCL1^{-/-} deficient mice was determined by assessing extracellular and intracellular CFUs at 30, 60, and 180 mins post-infection with *K. pneumoniae* (MOI of 1). Relative phagocytosis (E) of *K. pneumoniae*-infected WT and CXCL1^{-/-} neutrophils at 30 min post-treatment (MOI of 1). For experiments A-E, a total of 5-8 mice/group were used. (* indicates p<0.05 compared with CXCL1^{-/-} neutrophils).

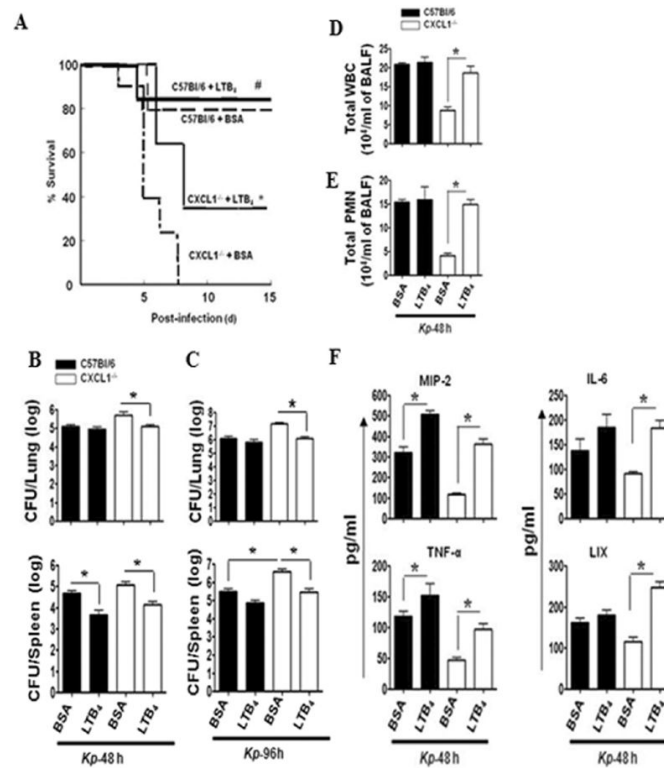


Figure 4. Impaired survival, bacterial clearance, neutrophil influx, and cytokine/chemokine production in the lungs of CXCL1^{-/-} mice are restored by exogenous LTB₄
Mortality (A) in WT or CXCL1^{-/-} mice infected with 10³ CFUs of *K. pneumoniae* and administered with LTB₄ (100 ng/mouse) or vehicle (BSA) 1 h later, and survival was assessed up to 15 days. Data are presented as % survival ($n=20$ mice/group) and analyzed using Wilcoxon signed-rank test. * indicates the difference between LTB₄ or vehicle (BSA) control treated CXCL1^{-/-} mice ($p<0.05$) and # indicates the difference between WT+LTB₄ and CXCL1^{-/-}+LTB₄ mice.) Bacterial clearance (B, C) in the lungs and dissemination were examined in lung homogenates of LTB₄ or BSA administered WT and CXCL1^{-/-} mice at 48 and 96 h post-*K. pneumoniae* challenge (10³ CFUs/mouse). Data are presented as Mean \pm SE ($n=5-6$ mice/group). (* indicates $p<0.05$ compared with BSA (vehicle) administered mice.) Cellular infiltration (D,E) in airspaces at 48 h after i.t. treatment with LTB₄ (100 ng/mouse) or vehicle (BSA) control. Concentrations of CXCL2/MIP-2, IL-6, TNF- α , and CXCL5/LIX (F) in BALF from CXCL1^{-/-} or WT mice infected with 10³ CFUs of *K. pneumoniae* and administered with LTB₄ or vehicle (BSA). For experiments D-F, $n=6-8$ mice/group. (* indicates $p<0.05$ as compared to BSA administered mice).

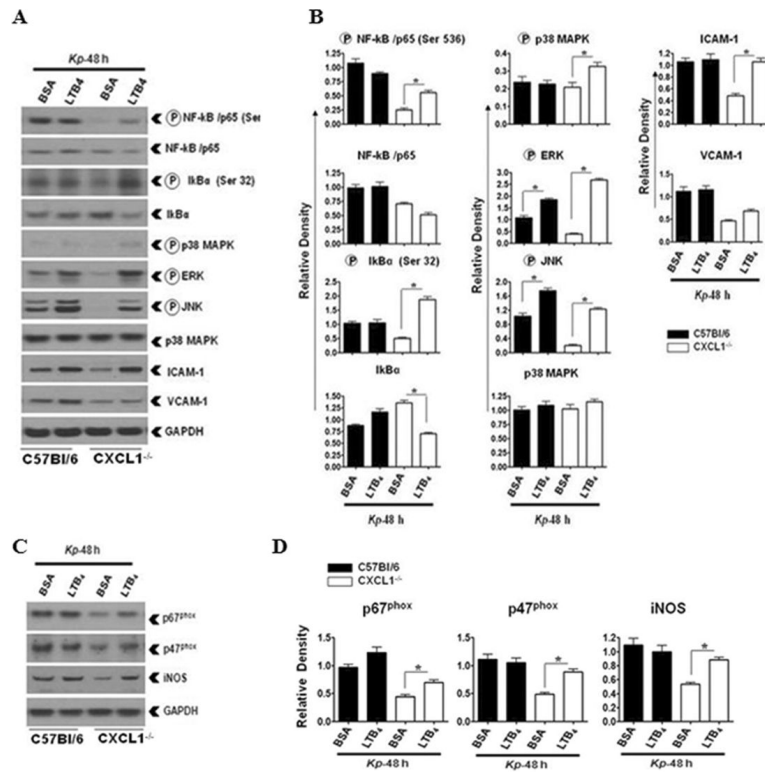


Figure 5. Defective *K. pneumoniae*-induced activation of NF- κ B, MAPKs, and expression of ICAM-1 in the lungs of CXCL1^{-/-} mice is corrected by LTB₄
 Activation of NF- κ B and MAPKs and expression of ICAM-1 and VCAM-1 (A) in *K. pneumoniae*-infected and LTB₄ administered homogenized whole lungs at 48 h post-infection. WT and CXCL1^{-/-} mice were infected with 10³ CFU of *K. pneumoniae* cells via the i.t. route and treated with LTB₄ (100 ng/mouse) or vehicle administration 1 h later. Data are presented as a representative of three independent blots/experiments. Densitometric analysis (B) of activation of NF- κ B and MAPKs and expression of ICAM-1 and VCAM-1 in the lung homogenates after LTB₄ or vehicle treatment. Data represent the means \pm SEM of arbitrary densitometric units for each band from three independent blots/experiments. (* indicates $p < 0.05$ as compared to BSA treated C57Bl/6 or CXCL1^{-/-} mice). Expression levels of p67^{phox}, p47^{phox}, and iNOS (C) in homogenized lungs of *K. pneumoniae*-infected and i.t.-treated with vehicle or LTB₄ (100 ng) 1 h post-infection. This blot is representative of three independent blots. Densitometric analysis of iNOS, p67^{phox}, and p47^{phox} levels in lung (D) homogenates from three independent experiments ($p < 0.05$). (* indicates $p < 0.05$ compared with C57Bl/6 or CXCL1^{-/-} vehicle (BSA) administered mice ($n = 5-6$ /group)). For experiments A-D, a total of 6-9 mice/group was used.

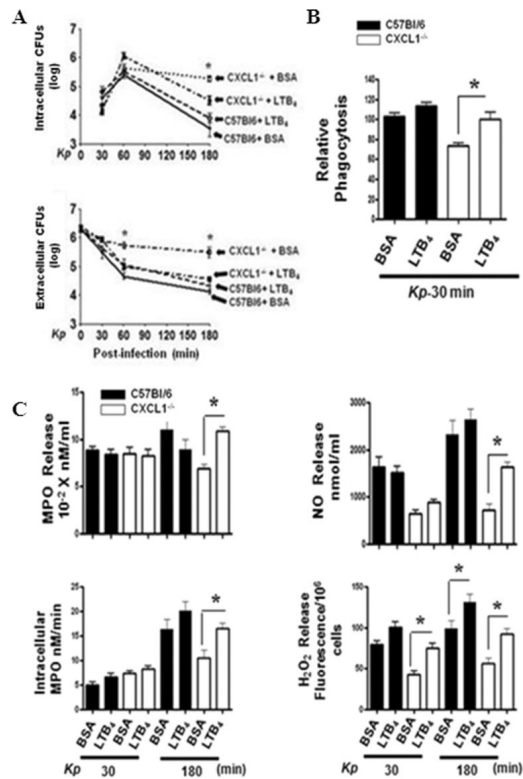


Figure 6. Impaired bacterial killing, ROS and RNS generation, and phagocytosis in *K. pneumoniae*-stimulated CXCL1^{-/-} neutrophils is restored by LTB₄
 (A) Bacterial killing capacity of *K. pneumoniae*-infected, LTB₄-treated neutrophils from WT and CXCL1^{-/-} deficient mice was determined at 30, 60, and 180 mins by assessing intracellular and extracellular CFUs. Relative phagocytosis (B) of *K. pneumoniae*-infected WT and CXCL1^{-/-} neutrophils at 30 min post-treatment (1 MOI). Relative phagocytosis index was calculated as described in *Materials and Methods*. MPO release, intracellular MPO, NO, and H₂O₂ production (C) in *K. pneumoniae*-infected WT and CXCL1^{-/-} neutrophils at 30 and 180 mins post-LTB₄ treatment. For experiments A-C, a total of 7-9 mice/group were used. (* indicates p<0.05 as compared to BSA treated neutrophils).

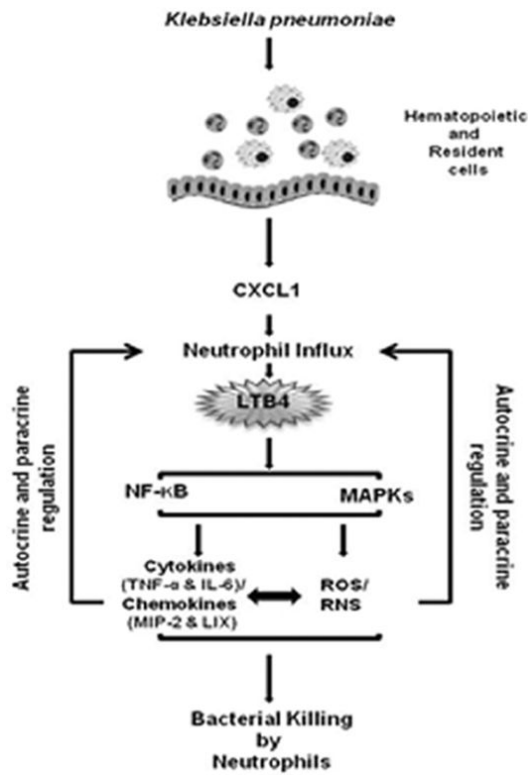


Figure 7. Scheme depicting CXCL1-dependent bacterial clearance in the lung following *K. pneumoniae* infection

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-κB 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.