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NLRC4 Inflammasome-Mediated Production of IL-1β Modulates Mucosal Immunity in the Lung against Gram-Negative Bacterial Infection

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Bacterial flagellin is critical to mediate NLRC4 inflammasome-dependent caspase-1 activation. However, Shigella flexneri, a non-flagellated bacterium, and a flagellin (fliC) knockout strain of Pseudomonas aeruginosa are known to activate NLRC4 in bone marrow-derived macrophages. Furthermore, the flagellin-deficient fliC strain of P. aeruginosa was used in a mouse model of peritonitis to show the requirement of NLRC4. In a model of pulmonary P. aeruginosa infection, flagellin was shown to be essential for the induction of NLRC4-dependent caspase-1 activation. Moreover, in all P. aeruginosa studies, IL-1β production was attenuated in NLRC4−/− mice; however, the role of IL-1β in NLRC4-mediated innate immunity in the lungs against a non-flagellated bacterium was not explored. In this article, we report that NLRC4 is important for host survival and bacterial clearance, as well as neutrophil-mediated inflammation in the lungs following Klebsiella pneumoniae infection. NLRC4 is essential for K. pneumoniae-induced production of IL-1β, IL-17A, and neutrophil chemoattractants (keratinocyte cell-derived chemokines, MIP-2, and LPS-induced CXC chemokines) in the lungs. NLRC4 signaling in hematopoietic cells contributes to K. pneumoniae-induced lung inflammation. Furthermore, exogenous IL-1β, but not IL-18 or IL-17A, partially rescued survival, neutrophil accumulation, and cytokine/chemokine expression in the lungs of NLRC4−/− mice following infectious challenge. Furthermore, IL-1R1−/− mice displayed a decrease in neutrophilic inflammation in the lungs postinfection. Taken together, these findings provide novel insights into the role of NLRC4 in host defense against K. pneumoniae infection. The Journal of Immunology, 2012, 188: 000–000.

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ower respiratory tract infections remain a major burden of disease in the United States and the world, as measured by disability-adjusted life-years (1). The Gram-negative bacterium, Klebsiella pneumoniae, causes severe pneumonia along with extensive lung damage, even with small inoculums. In the last decade, the extensive spread of multiple drug-resistant K. pneumoniae strains has become a severe problem (2–4). In this regard, carbapenem-resistant/β-lactamase-producing K. pneumoniae causes ≥50% mortality in the United States and worldwide (2–4). Furthermore, K. pneumoniae is known to induce life-threatening pneumonia in alcoholics and patients with diabetes (5–8).

Abbreviations used in this article: BALF, bronchoalveolar lavage fluid; BM, bone marrow; DKO, double knockout; i.t., intratracheal(ly); KC, keratinocyte cell-derived chemokine; LIX, LPS-induced CXC chemokine; MOI, multiplicity of infection; MPO, myeloperoxidase; NALP3, NACHT, leucine-rich repeat, and PYD domains-containing protein 3; PI, propidium iodide; PRR, pattern recognition receptor; siRNA, small interfering RNA; WT, wild-type.

The development of pneumonia depends on a complex interplay between mucosal colonization by the infectious organism and mucosal immunity (8–10). The host innate immune response against infection involves pathogen recognition by pattern recognition receptors (PRRs) expressed on host cells (11–14). In this regard, ligation of both extracellular and intracellular PRRs can induce the expression of cytokines/chemokines and neutrophil migration to the lungs during infection. The recognition of a microbe’s specific molecular structures, also called pattern-associated molecular patterns, by PRRs leads to cascades of events ultimately resulting in neutrophil infiltration into the lungs, followed by monocyte/macrophage migration to the site of infection (8, 11–14). Nucleotide-binding domain, leucine rich containing (NLR) proteins modulate host immunity via inflammation and apoptosis and are involved in the recognition of pattern-associated molecular patterns and damage-associated molecular patterns, such as endogenous ligands released from infected tissues or tissues undergoing destruction (15–19). The recognition of such ligands by NLRs can lead to activation of caspase-1 (previously known as ICE) through the assembly of a cytosolic protein complex known as the inflammasome (15–19).

NLRC4 belongs to the NLR family containing an N-terminal CARD domain, a central NOD domain, and a C-terminal leucine-rich repeat domain and is involved in assembly of the inflammasome complex (20). Several lines of evidence suggest an important function for NLRC4 in caspase-1 activation in response to Salmonella typhimurium (21), Shigella flexneri (22), and Legionella pneumophila (23, 24). In these investigations, animals infected with these pathogens exhibited flagellin-induced activation of NLRC4, leading to the induction of IL-1β, IL-18, and macrophage cell death. In particular, a C-terminal region of L.
pneumonia flagellin was shown to activate caspase-1 through NLRC4 (23, 24).

More recently, NLRC4 has been identified as a receptor for bacterial flagellin, as well as type III secretion-system components (25, 26). With regard to pulmonary pathogens, although a flagellin-deficient strain of Pseudomonas aeruginosa caused caspase-1 activation in macrophages via NLRC4, demonstrating that NLRC4 activation can be flagellin independent, the type III secretion system was indispensable for NLRC4-mediated caspase-1 activation (27). Furthermore, NACHT, leucine-rich-repeat, and PYD domains-containing protein 3 (NALP3) and apoptosis-associated speck-like protein containing a caspase recruitment domain were shown to be indispensable to protect mice against a high inoculum of K. pneumoniae (7.4 × 10^7/mouse) (28). To this end, we studied the role of NLRC4 in neutrophil-dependent immunity in the lungs against K. pneumoniae and found that NLRC4 is essential for host survival, bacterial clearance, and neutrophilic inflammation in the lungs in response to K. pneumoniae infection. NLRC4 signaling in hematopoietic, but not resident cells, predominantly contributes to K. pneumoniae-induced neutrophilic inflammation in the lungs. Moreover, exogenous IL-1β reversed host defense defects in NLRC4−/− mice following infectious challenge. Our data define NLRC4-dependent caspase-1 activation following K. pneumoniae infection resulting in production of inflammatory cytokines, recruitment of neutrophils into the lungs, and pathogen clearance/survival.

Materials and Methods

Animals

Eight- to ten-week-old female mice, genetically deficient for Nlrc4 (NLRC4−/−) (27) or Il1r1 (IL-1R1−/−) (The Jackson Laboratory, Bar Harbor, ME) and weighing 20–25 g were used for experiments. NLRC4−/− and IL-1R1−/− mice were backcrossed 10 times with age-matched C57BL/6 mice. NLRC4−/−IL-1R1−/− mice were generated by intercrossing NLRC4−/− and IL-1R1−/− mice. Mice were kept on a 12:12 h light/dark cycle with free access to food and water and were maintained under specific pathogen-free conditions. Animal experiments were approved by the Louisiana State University Animal Research Committee.

Human monocyte-derived macrophage stimulation with K. pneumoniae

Frozen human monocytes were obtained from Astarte Biologics (Redmond, WA). Cells were thawed at 37°C and resuspended in RPMI 1640 containing 5% FBS. The population of monocytes was, on average, 90% pure, as determined by flow cytometry using an anti-CD14 marker and the resulting homogenates was plated in 10-fold serial dilutions onto a tryptic soy agar plate and onto a MacConkey agar plate. Similarly, for enumerating bacterial CFU in lungs and spleen, whole tissues were homogenized in PBS for 15 and 30 s, respectively, and 20 µl homogenates was plated in 10-fold serial dilutions onto tryptic soy agar and MacConkey agar plates. After inoculation of NLRC4−/− and NLRC4+/+ mice with K. pneumoniae, their survival was monitored for up to 15 d.

Collection of BALF

The animals were euthanized and exsanguinated by cardiac puncture at the designated time points. The trachea was exposed and cannulated with a 20-gauge catheter, as described earlier (31–34). BALF was collected by instilling 0.8 ml PBS containing heparin and dextrin four times. Total leukocytes in BALF were enumerated by counting on a hemocytometer. BALF cells were subsequently subjected to cytospin and stained with Diff-Quick, and differential leukocyte cell counts were determined by standard light microscopy. The remainder (2 ml) of the undiluted cell-free BALF was passed through a 0.22-µm filter and used for the estimation of cytokines/chemokines.

Harvesting lungs

At the designated time points postinfection, the whole (nonlavaged) lungs were excised from mice and snap frozen. Further, for long-term storage, these lung tissues were stored at −70°C and used for cytokine/chemokine determination. Western blots, and myeloperoxidase (MPO) activity assay. Briefly, lung tissue was homogenized in 2 ml PBS supplemented with 0.1% Triton X-100 and complete protease inhibitor (1 tablet/50 ml media), and the resulting homogenates were centrifuged at 12,000 × g for 20 min. The supernatants were harvested, passed through a 0.22-µm filter, and used as required.

Measuring MPO activity

MPO is an enzyme found in the cells of the myeloid lineage, and it has been used largely as a marker for neutrophil migration into the lungs. Briefly, the lung samples were weighed, homogenized, and centrifuged, and the pellet obtained was resuspended in 50 mM potassium phosphate buffer (pH 6) supplemented with 0.5% hexadecyltrimethylammonium bromide, as described earlier (31–38). Samples were then sonicated, incubated at 60°C for 2 h, and assayed for MPO activity in an H2O2/0-dianisidine buffer at 460 nm. Change in absorbance was measured every 5 min at 460 nm using a spectrophotometer. The activity was calculated between 0 and 90 s.

Mouse cytokine ELISA

Cytokines/chemokines in the BALF, lung homogenates, and culture media of alveolar macrophages were determined at different time points by sandwich ELISA, as described earlier (31–38). The minimum detection limit of the assay was 2 pg/ml protein. For mouse lungs, TNF-α, IL-6,
LPS-induced CXC chemokine (LIX), and MIP-2 concentrations were normalized to the total protein concentration in the samples measured by Bradford assay (Bio-Rad, Hercules, CA). Data are expressed as pg/mg total protein for lung tissue and pg/ml for BALF.

Lung histology

The lungs were perfused from the right ventricle of the heart with 10 ml isotonic saline 24 h postinfection and harvested. For H&E staining, lungs were fixed in 4% phosphate-buffered formalin, processed in paraffin blocks, and fine sections (5 µm in thickness) were cut. Semiquantitative histology was performed by a veterinary pathologist in blinded fashion, according to the following scoring scale used in our earlier publications (32, 33): 0, no inflammatory cells (macrophages or neutrophils) present in section; 1, <5% of section is infiltrated by inflammatory cells; 2, 5–10% of section is infiltrated by inflammatory cells; and 3, >10% of section is infiltrated by inflammatory cells.

NF-κB DNA-binding assay

An ELISA-based NF-κB DNA-binding assay in the lungs of saline-treated and K. pneumoniae-infected NLRC4−/− and NLRC4+/+ mice was performed to detect the activation of the p65 subunit of NF-κB in the nucleus, per the manufacturer’s protocol (Active Motif, Carlsbad, CA). Nuclear extracts were prepared using the Nuclear Extraction Kit (Active Motif). Nuclear extracts containing equal amounts of protein from each lung sample were added to the precoated (NF-κB–specific oligonucleotide) 96-well plate. The plate was incubated for 1 h at room temperature. After washing the plate three times, a primary Ab specific for NF-κB/p65 was added, and the plate was incubated for 1 h at room temperature. After three washings to remove excess primary Ab, an anti-HRP conjugate was added to the plate, and the OD was measured at 450 nm (31–34).

BM chimeras

BM transplantation experiments were performed, as described in earlier publications (31, 35). BM was flushed from tibias and femurs from donor mice, and a total of 8 × 10⁶ BM cells was injected into the tail veins of lethally irradiated (two 525-rad doses separated by 3 h) recipient mice. Mice were transplanted 8 wk postBM reinfusion. Experiments were performed 8 wk after BM reconstitution. We found that 84% of blood leukocytes were derived from donor marrow at the time when mice were used for experiments (6–8 wk posttransplantation).

Immunoblotting

The harvested lungs from NLRC4−/− and NLRC4+/+ mice were homogenized in 1 ml PBS containing 0.1% Triton X-100 and complete protease and phosphatase inhibitor mixture (Roche, Indianapolis, IN) in chilled conditions for 2.5 min. TissueLyser II (QIAGEN, Valencia, CA) was used for the homogenization, and the samples were subsequently centrifuged at maximum speed in a microfuge for 20 min at 4°C to remove cellular debris. The resulting supernatant was used for immunoblotting. Bradford protein assay (Bio-Rad) was performed to ensure equal quantity loading of protein on the gel. The samples were resolved on 8–15% Tris-glycine gels, and the proteins were transferred onto a polyvinylidene fluoride membrane using standard protocols. Abs recognizing phospho-IκBα/β (Ser176/180), phospho-NF-κB/p65 (Ser536), phospho-IκBα/β (Ser32/36), IKKβ, NF-κB/p65, IkBα, VCAM-1, ICAM-1, phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204), phospho-p38 MAPK (Thr180/Tyr182), phospho-SAPK/INK (Thr183/Tyr185), cleaved caspase-1, cleaved IL-1β, and cleaved IL-18 were added at 1:1000 dilution. Abs to total p38 and GAPDH were used at 1:5000 (Cell Signaling, Danvers, MA; Santa Cruz Biotechnology). Secondary Abs consisted of HRP-conjugated goat anti-mouse (for total and phospho-Abs, IKKα and IKKβ) and rabbit anti-goat (for VCAM-1 and ICAM-1; Biocodex, San Diego, CA). The membranes were processed with an ECL chemiluminescence kit (ThermoFisher Scientific, Waltham, MA), and the signal was detected by exposing the processed blots to x-ray film (GeneMate, Autorad Film, BioExpress, Kaysville, UT) (31–34).

Cell surface and intracellular staining

The method used to detect IL-17A–producing cells in the lungs was described in earlier publications (34, 39, 40). Briefly, lungs were minced, digested with collagenase for 90 min, and made into a single-cell suspension. Cells were stimulated with PMA and ionomycin in the presence of GolgiStop (BD Biosciences, San Diego, CA) for 5 h. Following stimulation, cells were washed with PBS and surface stained with markers for IL-17A–producing T cells (γδ, NK1.1, CD4, and CD8α) for 30 min. Cells were washed, fixed, and permeabilized for intracellular staining with IL-17A and IFN-γ Abs. Finally, cells were washed and resuspended for flow cytometric analysis. FlowJo software (Tree Star, Ashland, OR) was used to analyze the data.

Administration of recombinant murine IL-1β, IL-17A, IL-18, and IL-1α

A single dose of 1 µg active recombinant murine proteins in 50 µl (IL-1β, IL-1α, IL-17A and IL-18 [R&D Systems, Minneapolis, MN]) in 0.1% BSA containing saline or vehicle alone (0.1% BSA in saline) was administered i.t. 1 h after i.t. administration of K. pneumoniae (1 × 10⁶ CFU/50 µl) to NLRC4−/− mice. This i.t. dose of IL-1β (1 µg/50 µl) was shown in previous models to be biologically active in vivo (41–43).

Determination of pyroptosis by flow cytometry

Lung or spleen digests from C57BL/6 or NLRC4−/− mice challenged with K. pneumoniae for 24 or 48 h were used to determine cells undergoing pyroptosis. Briefly, lungs were minced and digested with collagenase filtered through a 0.70-µm filter to make single-cell suspensions. Furthermore, spleens were mashed, and the resulting cell suspension was passed through a 0.70-µm filter. Following two washings with PBS, cells were blocked with FcR and aliquoted for surface staining with conjugated PerCP anti-mouse Gr-1/Ly6G and EMR1 and allophycocyanin–anti-mouse CCR2 or CXCR2. RBCs were lysed by adding NH₄Cl lysing buffer. Samples were centrifuged and resuspended twice with 1× PBS. Cells were resuspended in 1× binding buffer and 5 µl Annexin V eFluor and 5 µl propidium iodide (PI) were added, according to the manufacturer’s protocol (Annexin V Apoptosis Detection Kit; BD Pharmingen). The cell suspension was vortexed and incubated for 15 min in the dark at room temperature. A total of 100 µl 1× binding buffer was added, and the cells were analyzed by flow cytometry. CCR2 or CXCR2–Gr-1/Ly6G (neutrophils) and EMR1 (macrophages) that were positive for Annexin V eFluor and negative for PI are shown in graphs.

Statistical analysis

Data are expressed as mean ± SE. The intensity of immunoreactive bands was determined using gel-digitizing software (UN-SCAN-IT; Silk Scientific, Orem, Utah). Data were analyzed by ANOVA, followed by the Bonferroni post hoc analysis for multiple comparisons. All statistical calculations were performed using InStat software and GraphPad Prism 4.0. Differences were considered statistically significant at p < 0.05, compared with control. Survival curves were compared using the Wilcoxon signed-rank test.

Results

NLRC4 regulates K. pneumoniae–induced proinflammatory mediators in human and murine macrophages

Macrophages are the sentinel cells of pathogen recognition in the lung and are a critical component of the innate immune response to microbes (44, 45). The NLRC4 inflammasome has been implicated in influencing the production of proinflammatory mediators in BM-derived macrophages (23, 25). We first compared the differences in the levels of inflammatory cytokines in human blood monocyte-derived macrophages following NLRC4 siRNA knockdown and in murine alveolar macrophages obtained from NLRC4−/− mice following infection with the Gram-negative bacterium, K. pneumoniae. In siRNA-transfected human monocyte-derived macrophages, we found reduced levels of IL-6, IL-1β, and IL-18 (Fig. 1A), along with attenuated activation of NF-κBα/β and neutrophil chemokines (keratinocyte cell-derived) post infection (Fig. 1B, 1C). Similarly, in NLRC4−/− murine alveolar macrophages, we observed decreased levels of cytokines (TNF-α, IL-6, and IL-1β) and neutrophil chemokines (keratinocyte cell-derived chemokine [KC] and MIP-2) (Fig. 1D), as well as reduced activation of NF-κBα and MAPKs 6 h post-K. pneumoniae infection compared with NLRC4+/+ macrophages (Fig. 1E, 1F).

Together, these data suggest that NLRC4 is an important factor in the K. pneumoniae–mediated inflammatory response in macrophages.

NLRC4−/− mice show reduced survival, enhanced bacterial burden in the lungs, and attenuated neutrophil influx and cytokine production following K. pneumoniae infection

To assess the importance of NLRC4 in the lungs during Gram-negative bacterial infection, NLRC4−/− and NLRC4+/+ (WT) mice were infected i.t. with two doses of K. pneumoniae (10⁵ or
10^4 CFU/mouse), and survival was monitored up to 15 d post-infection. The NLRC4^−/− group showed reduced survival in response to both of the infectious doses. Only 16.7% of NLRC4^−/− animals survived to day 15 postinfection with the higher dose of K. pneumoniae compared with 50% of WT mice. Similarly, in response to the lower dose of K. pneumoniae, only 40% of NLRC4^−/− animals survived, whereas 70% of WT mice survived to day 15 (Fig. 2A, 2B).

To determine whether survival of NLRC4-deficient mice was associated with a defect in bacterial clearance in the lungs and/or bacterial dissemination, we determined bacterial counts 24 and 48 h post-K. pneumoniae infection. NLRC4^−/− mice exhibited higher lung and spleen CFU compared with their WT counterparts at 48 h (Fig. 2C, 2D). We also observed enhanced dissemination of K. pneumoniae in blood, kidneys, and liver of NLRC4^−/− mice compared with their littermate controls (data not shown). Thus, NLRC4^−/− mice have a defect in bacterial clearance and dissemination, which likely explains the decreased survival of NLRC4^−/− mice following K. pneumoniae infection.

To elucidate the mechanisms associated with enhanced bacterial CFU in the lungs and spleens, we assessed pulmonary neutrophil recruitment following K. pneumoniae challenge. Total WBC and neutrophil accumulation in the airspaces of NLRC4^−/− mice were reduced at 48 h compared with WT controls (Fig. 2E, 2F).
FIGURE 2. Role of NLRC4 in host defense against pulmonary *K. pneumoniae* infection. (A and B) NLRC4 deficiency enhances mortality induced by i.t. *K. pneumoniae*. NLRC4<sup>−/−</sup> and C57BL/6 (NLRC4<sup>+/+</sup>) mice were inoculated i.t. with 1,000 (A) or 10,000 CFU (B) of *K. pneumoniae*. Mortality was monitored. *p < 0.05, log-rank test. Data shown represent n = 12 mice/group from one of two representative independent experiments. (C and D) NLRC4<sup>−/−</sup> mice show greater bacterial burden in the lungs and dissemination in response to *K. pneumoniae* infection. Mice were treated with 1 × 10<sup>3</sup> CFU of *K. pneumoniae* i.t., and lung and spleen homogenates were cultured for 24 or 48 h. Data shown represent mean parenchymal CFU ± SE. *p < 0.05, NLRC4<sup>+/+</sup> versus NLRC4<sup>−/−</sup> mice. (E-G) NLRC4 deficiency reduces influx of PMNs to the *K. pneumoniae*-exposed lung. NLRC4<sup>−/−</sup> or C57BL/6 (NLRC4<sup>+/+</sup>) mice (n = 4–7/group) were exposed to 1 × 10<sup>3</sup> CFU of *K. pneumoniae* i.t. Total WBCs (E) and neutrophils (F) in BALF were enumerated 24 and 48 h after exposure. *p < 0.05, NLRC4<sup>+/+</sup> versus NLRC4<sup>−/−</sup> mice. (G) MPO activity, a quantitative measure of PMNs, was assessed in lung homogenates 24 and 48 h after *K. pneumoniae* challenge. *p < 0.05. Data shown are representative of three independent experiments (n = 4–6/group). (H) Reduced lung pathology in NLRC4<sup>−/−</sup> mice following *K. pneumoniae* inoculation. Mice were inoculated with 1 × 10<sup>3</sup> CFU of *K. pneumoniae*/mouse i.t. Lungs were obtained 24 and 48 h postinfection and stained with H&E, and inflammatory changes in histological sections were scored. Shown are representative sections from four mice under each condition with identical results (original magnification ×200). Semiquantitative inflammation score is a quantification of four lung sections in each group. (I) NLRC4 deficiency inhibits *K. pneumoniae*-induced airspace cytokine and chemokine expression. TNF-α, IL-1β, IL-17A, KC, MIP-2, and LIX in BALF (pg/ml) and lung homogenates (pg/mg) were measured by ELISA 24 and 48 h after *K. pneumoniae* challenge (n = 4–7 mice/group). *p < 0.05, NLRC4<sup>+/+</sup> versus NLRC4<sup>−/−</sup> mice.

Because we observed a reduction in IL-17A in the lungs of NLRC4<sup>−/−</sup> mice following *K. pneumoniae* infection, we decided to assess the presence of IL-17A–producing T cell subsets in the lungs during *K. pneumoniae* infection by flow cytometry, using intracellular and surface staining. In an earlier report, we found that NK cells and γδ T cells were the predominant sources of IL-17A production in the lungs 6 h post-*E. coli* infection (34). In control (saline-challenged) groups, neutrophil accumulation in the lungs was not observed in NLRC4<sup>−/−</sup> or NLRC4<sup>+/+</sup> groups (Fig. 2E, 2F). Consistent with reduced neutrophil recruitment into the lungs, we also observed attenuated MPO activity in lung homogenates from NLRC4<sup>−/−</sup> mice (Fig. 2G). NLRC4<sup>+/+</sup> mice demonstrated severe suppurative bronchopneumonia (score of 3.0), whereas NLRC4<sup>−/−</sup> mice displayed moderate suppurative pneumonia (score of 2.0) 48 h after *K. pneumoniae* infection. In contrast, no pathological changes were observed in saline-challenged (control) lungs obtained from either NLRC4<sup>−/−</sup> or NLRC4<sup>+/+</sup> animals (Fig. 2H).

Because the impairment in *K. pneumoniae*-induced neutrophil accumulation observed in NLRC4<sup>−/−</sup> mice likely reflects a decrease in the production of cytokines/chemokines in the lungs upon infection, we quantified the expression of cytokines (TNF-α, IL-1β, and IL-17A) and neutrophil chemoattractants (KC, MIP-2, and LIX) in BALF and lung homogenates 24 and 48 h after *K. pneumoniae* challenge. We found that the levels of TNF-α, KC, MIP-2, and LIX were attenuated in NLRC4<sup>−/−</sup> mice 48 h following *K. pneumoniae* infection. Strikingly, IL-1β and IL-17A levels in NLRC4<sup>−/−</sup> mice were attenuated as early as 24 h following *K. pneumoniae* challenge (Fig. 2I).
contrast, upon *K. pneumoniae* infection we found that γδ T cells, CD4+ T cells, CD8+ T cells, and NK/NKT cells all produce IL-17A in the lung 48 h postinfection; however, the proportion of T cell subsets that produce IL-17A and/or IFN-γ was reduced in the lungs of NLRC4−/− mice compared with controls (Fig. 3).

**NLRC4 deficiency impairs activation of NF-κB and MAPK, as well as expression of ICAM-1 and VCAM-1**

To investigate the cause of the reduced cytokine/chemokine expression in NLRC4−/− mice, we examined the activation of transcription factors, including NF-κB/p65, in the lungs following *K. pneumoniae* infection. In this regard, we focused on the activation of NF-κB, because this is the most extensively studied transcription factor known to regulate a variety of genes encoding proinflammatory factors (46, 47). To determine activation of NF-κB/p65 in NLRC4−/− mice after *K. pneumoniae* infection, we determined p65 DNA binding using nuclear extracts from the lungs. Our findings revealed a decrease in NF-κB/p65 DNA binding in cells isolated from NLRC4−/− mice compared with their WT counterparts 48 h after *K. pneumoniae* infection (Fig. 4A). We used Western blotting to further examine NF-κB activation in the lungs following *K. pneumoniae* challenge. In NLRC4−/− mice, we observed a decrease in NF-κB/p65 phosphorylation at Ser536 24 and 48 h post-*K. pneumoniae* infection. The decrease in p65 phosphorylation correlated with a decrease in the phosphorylation of IkBa (Ser53) in the lungs of NLRC4−/− mice starting at 24 h postinfection with *K. pneumoniae* and persisting for up to 48 h after infection. Concomitantly, we observed increased accumulation of IkBa protein in NLRC4−/− mice 48 h after *K. pneumoniae* infection. However, in control (saline-challenged) mice, we detected extremely low levels of phosphorylated IKKa/b or NF-κB/p65 in NLRC4−/− and NLRC4+/+ (WT) animals (Fig. 4B, 4C).

Neutrophil recruitment from the bloodstream into lungs involves numerous sequential steps mediated by interactions between cell adhesion molecules on leukocytes and endothelium (8–10, 48). Because it was shown that VCAM-1 and ICAM-1 play key roles in neutrophil extravasation, we determined their expression levels in lung homogenates after *K. pneumoniae* infection. In NLRC4−/− mice, the expression of both ICAM-1 and VCAM-1 was reduced 48 h following *K. pneumoniae* infection compared with control mice (Fig. 4B, 4C).

Decreased neutrophil accumulation in the lungs observed in response to *K. pneumoniae* in NLRC4−/− mice could also be the

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**FIGURE 3.** Importance of NLRC4 in IL-17A–producing cells in the lung in response to *K. pneumoniae* infection. (A–D) Representative FACS plots of lung digests after *K. pneumoniae* infection showing IFN-γ– and IL-17A–producing γδ, CD4, CD8, and NKT cells. Lung T cells were isolated 48 h after *K. pneumoniae* infection and immunostained for surface with γδ, CD4, CD8, NKT, and intracellular IFN-γ and IL-17A. Shown is a representative of three separate experiments. (E) NLRC4 deficiency decreases the number of IL-17A–expressing T cells. Data shown are representative of three separate experiments (n = 4–6 mice per group). *p < 0.05.
result of attenuated activation of MAPKs, which regulate transcription factors, such as AP-1 and STAT-1 (49, 50). In this context, we studied the activation of ERK, JNK, and p38. Unlike WT controls, NLRC4−/− mice displayed attenuated activation of ERK and JNK at 24 and 48 h after K. pneumoniae infection. Not surprisingly, activation of MAPKs was not observed in WT or NLRC4−/− mice following saline challenge (Fig. 4D, 4E).

NLRC4−/− mice demonstrate reduced cleavage of caspase-1, IL-1β, and IL-18 following K. pneumoniae infection

In general, inflammasome activation triggers autotaxic activation or cleavage of caspase-1 (16, 19). Caspase-1, in turn, cleaves pro–IL-1β and pro–IL-18, producing mature IL-1β and IL-18 (16, 19, 27). In an effort to clarify whether NLRC4 has functions in K. pneumoniae-induced cleavage of caspase-1, pro–IL-1β, or pro–IL-18, we measured their cleavage products in the lungs following K. pneumoniae infection. We observed reduced cleavage of caspase-1, IL-1β, and IL-18 in NLRC4-deficient mice compared with control mice (Fig. 5). This suggests that activation of caspase-1 and cleavage of IL-1β and IL-18 are dependent upon NLRC4 activation following infection with K. pneumoniae.

NLRC4 does not induce pyroptosis in pulmonary macrophages and neutrophils following K. pneumoniae infection

NLRC4 was shown to mediate pyroptosis in flagellated bacterial infection models caused by Burkholderia pseudomallei (51) and S. typhimurium (22). Caspase-1 activation can trigger a form of cell death called pyroptosis that requires the proteolytic cleavage of caspase-1 (52). Pyroptosis is characterized by the insertion of pores into the plasma membrane of myeloid cells, including macrophages and neutrophils, which can be detected by annexin V binding. To investigate the role of NLRC4-mediated caspase-1–
dependent pyroptosis in host defense following *K. pneumoniae*-mediated caspase-1 activation, we used flow cytometry in cells isolated from the lung and spleen. Our results suggest that caspase-1 activation by BM-derived cells and not resident lung cells plays a role in promoting neutrophil accumulation and innate immunity against *K. pneumoniae* infection.

**Neutrophil recruitment to the lungs in response to *K. pneumoniae* infection is dependent upon NLRC4 signaling by BM-derived cells and not resident lung cells**

Because lung inflammation in *K. pneumoniae*-infected mice could be due to recruited BM cells or resident alveolar cells, we determined which population of cell-derived NLRC4 signaling was important in promoting neutrophil recruitment and host immunity. To address this issue, lethally irradiated WT or NLRC4−/− mice were reconstituted with BM from donor WT or NLRC4−/− mice to generate four groups: WT mice reconstituted with WT marrow (WT→WT), WT mice reconstituted with NLRC4−/− marrow (NLRC4−/−→WT), NLRC4−/− mice reconstituted with WT marrow (WT→NLRC4−/−), and NLRC4−/− mice reconstituted with NLRC4−/− marrow (NLRC4−/−→NLRC4−/−). Eight weeks postreconstitution, these BM chimera mice were inoculated i.t. with *K. pneumoniae*, and neutrophil accumulation in the BALF was determined. We found that *K. pneumoniae*-induced neutrophil influx into the lungs was attenuated in NLRC4−/−→NLRC4−/− and NLRC4−/−→WT chimera mice compared with WT→WT chimera animals (Fig. 7A). Neutrophil recruitment to the lung was not observed in any chimeric mice in response to saline challenge (data not shown). Together, these data suggest that NLRC4 signaling by BM cells is required for neutrophil recruitment and innate immunity against *K. pneumoniae* infection.

**Administration of rIL-1β, but not rIL-17A or rIL-18, following *K. pneumoniae* infection rescues neutrophil recruitment in NLRC4−/− mice**

Because reduced IL-1β, IL-17A, and IL-18 levels were observed in NLRC4−/− mice following *K. pneumoniae* infection, we wanted to determine whether exogenous IL-1β, IL-17A, or IL-18 could rescue neutrophil accumulation and cytokine/chemokine production in the lungs of NLRC4−/− mice following *K. pneumoniae* infection. To this end, NLRC4−/− mice were administered a single i.t. dose of 1 μg of IL-1β, IL-17A, or IL-18 1 h post-*K. pneumoniae* infection. Administration of IL-1β in NLRC4−/− mice after *K. pneumoniae* infection resulted in increased neutrophil recruitment to the lungs (Fig. 7B) compared with the administration of IL-18 or IL-17A, which did not augment neutrophil recruitment (Fig. 7B). Notably, unlike IL-1β, administration of exogenous IL-1α in NLRC4−/− mice after *K. pneumoniae* challenge did not enhance neutrophil recruitment to the lungs (data not shown). We also performed survival experiments in response to *K. pneumoniae* administration after i.t. IL-1β treatment. Our data indicated that administration of IL-1β to NLRC4−/− mice after *K. pneumoniae* infection resulted in enhanced survival (Fig. 7C). Given the critical role of IL-1β in promoting neutrophil accumulation against i.t. *K. pneumoniae* challenge, we administered three concentrations of IL-1β to NLRC4−/− mice to demonstrate concentration-dependent rescue after *K. pneumoniae* infection. In this regard, rIL-1β partially rescued neutrophil influx in NLRC4−/− mice in a dose-dependent manner (Fig. 7D, 7E). In addition, we found that the production of KC, MIP-2, LIX, and IL-17A in the lungs of NLRC4−/− mice was augmented in *K. pneumoniae*-challenged mice following administration of exogenous IL-1β (Fig. 7F). Together, these findings...
demonstrate that rIL-1β plays an important role in NLRC4-dependent neutrophil-mediated host innate immunity against *K. pneumoniae*.

**IL-1RI<sup>-/-</sup>**-deficient mice exhibit defects in bacterial clearance, neutrophil recruitment, and chemokine/chemokine production after exposure to *K. pneumoniae*.  

To investigate the contribution of NLRC4-dependent IL-1β in promoting neutrophil accumulation against i.t. *K. pneumoniae* challenge, we used IL-1RI<sup>-/-</sup> mice to evaluate survival, bacterial CFU, neutrophil recruitment, and neutrophil chemotactic levels. Compared with WT controls, IL-1RI<sup>-/-</sup> mice displayed reduced survival, enhanced bacterial burden and dissemination in the lungs, as well as reduced neutrophil chemotactic expression (KC and MIP-2) and neutrophil accumulation in the lungs following *K. pneumoniae* infection (Fig. 8A–G). Similarly, activation of NF-κB and MAPKs, as well as expression of ICAM-1 and VCAM-1, was reduced in IL-1RI<sup>-/-</sup> mice following *K. pneumoniae* infection (Fig. 8H, 8I). To determine whether exogenous IL-1β alone could reconstitute neutrophil recruitment, NLRC4<sup>-/-</sup>/IL-1RI<sup>-/-</sup> (double knockout [DKO]) and IL-1RI<sup>-/-</sup> (knockout) mice were administered a single i.t. dose of 1 μg of IL-1β 1 h postinfection; however, we found that IL-1β administration did not rescue neutrophil influx in DKO mice (Fig. 8J). Collectively, these results support the role for the NLRC4-IL-1β axis in *K. pneumoniae*-induced innate immunity.

**Discussion**

The NLRC4 inflammasome is important for the host immune response against intracellular pathogens, such as *S. typhimurium* (21) and *L. pneumophila* (23, 24). Further, these studies demonstrated that cytosolic bacterial flagellin is the major trigger that activates the NLRC4 inflammasome (21–24). Although NLRC4 is essential for host immunity against extracellular pathogens, such as *P. aeruginosa* (53, 54), *P. aeruginosa* flagellin is not required to signal via NLRC4, despite the fact that proteins secreted by the type III secretion system are essential to induce activation of caspase-1 via NLRC4 (27). For *K. pneumoniae*, a nonflagellated extracellular bacterium, apoptosis-associated speck-like protein containing a caspase recruitment domain and NALP3 were shown to be important in promoting caspase-1 activation and IL-1β release in murine macrophages following an extremely high dose of *K. pneumoniae* challenge (7.4 × 10<sup>9</sup> CFU/mouse) (28). In this investigation (28), there was no difference in survival between WT and NLRC4<sup>-/-</sup> mice following an extremely high dose of *K. pneumoniae* infection. Furthermore, no detailed mechanistic studies were performed to understand the role of NLRC4 in the host defense against *K. pneumoniae* infection. In the current study, we explore the functional consequence of NLRC4 loss on host immunity in the lung following infection with *K. pneumoniae* using inoculum (1 × 10<sup>8</sup> and 1 × 10<sup>6</sup> CFU/mouse) based on the LD<sub>50</sub> concentration.

The antibacterial defenses of the lung include activity of resident alveolar macrophages (55, 56). Any defect in host defense func-

**FIGURE 7.** Relative contribution of NLRC4-expressing BM-derived cells versus resident cells to *K. pneumoniae*-induced neutrophil recruitment. (A) BM chimeras were generated with WT and NLRC4<sup>-/-</sup> mice. Mice were then infected with 1 × 10<sup>3</sup> CFU of *K. pneumoniae*/mouse i.t., and BALF neutrophils were enumerated 48 h after exposure (n = 4–6/group). *p < 0.05, WT/NLRC4<sup>+/+</sup> versus NLRC4<sup>-/-</sup> mice. (B) Administration of rIL-7A, rIL-1β, or rIL-18 after *K. pneumoniae* infection rescues NLRC4 deficiency. NLRC4<sup>-/-</sup> mice and WT controls were inoculated i.t. with *K. pneumoniae* (1 × 10<sup>3</sup> CFU in 50 μl of PBS) and administered a single dose of recombinant murine IL-17A, IL-1β, IL-18, or vehicle. BALF neutrophils were enumerated 48 h after exposure (n = 5–7/group). *p < 0.05, NLRC4<sup>+/+</sup> versus NLRC4<sup>-/-</sup> mice. (C) Survival was monitored up to 15 d postexposure (n = 20/group). *p < 0.05, NLRC4<sup>+/+</sup> versus NLRC4<sup>-/-</sup> mice. (D-F) NLRC4<sup>-/-</sup> mice and WT controls were inoculated i.t. with *K. pneumoniae* (1 × 10<sup>3</sup> CFU in 50 μl of PBS), followed by administration of different doses of recombinant murine IL-1β or vehicle (saline) alone to NLRC4<sup>-/-</sup> mice. Total WBCs (D), neutrophils (E), and cytokine/chemokines levels (F) in BALF were measured 48 h after *K. pneumoniae* exposure (n = 4–6/group). *p < 0.05, NLRC4<sup>+/+</sup> versus NLRC4<sup>-/-</sup> mice.
The role of NLRC4 was assessed in various studies, including those related to bacterial infections. For instance, in a study involving K. pneumoniae infection, NLRC4 was shown to limit bacterial colonization and disease severity (Fig. 4). This is supported by the observation that NLRC4 regulates NF-κB in human macrophages, that NLRC4 contributes to IL-18, TNF-α, and MAPK activation, as well as expression of ICAM-1 and VCAM-1 (8, 10).

The initial host response to bacterial infection is the recruitment of leukocytes from the bloodstream to the site of infection, where they phagocytose and eliminate the infectious microbes (8, 10). In this study, we show that the absence of NLRC4 results in enhanced susceptibility to intrapulmonary K. pneumoniae infection. Reduced inflammatory cell recruitment, as well as expression of KC, MIP-2, and LIX during bacterial infection, was observed in NLRC4-deficient mice (Fig. 4). These findings underscore the importance of NLRC4 in the human immune response to infection with a nonflagellated bacterium.

Infiltrating leukocytes from the BALF of IL-1R1−/− and WT (C57BL/6) mice were enumerated on days 1 and 2 after K. pneumoniae infection (n = 4–6/group). Total neutrophils in BALF were enumerated 48 h after exposure. IL-1R1 ablation results in reduced NF-κB and MAPK activation, as well as expression of ICAM-1 and VCAM-1 in the lung, after K. pneumoniae exposure. IL-1R1+/+ and IL-1R1−/− mice were infected with 1 × 10⁵ CFU of K. pneumoniae/mouse i.t., and lungs were obtained 24 and 48 h postinfection. Lung homogenates were used to assess activation of NF-κB and MAPK, as well as expression of ICAM-1 and VCAM-1 by immunoblotting. A representative blot is shown from 3 blots/experiment with identical results. Protein immunoblot bands were quantified by densitometry and normalized to GAPDH or total p38. Data are mean ± SE (n = 4–6/group). *p < 0.05, IL-1R1+/+ versus IL-1R1−/− mice.

Administration of rIL-1β after K. pneumoniae infection did not rescue neutrophil recruitment in IL-1R1−/− mice. IL-1R1−/− (knockout) and IL-1R1/NLRC4−/− (DKO) mice were inoculated i.t. with K. pneumoniae (1 × 10⁵ CFU in 50 μl of PBS) and then administered a single dose of recombinant murine IL-1β or vehicle (0.1% BSA). *p < 0.05, WT versus KO and DKO mice.

The data cannot rule out the possibility that decreased activation of NF-κB and MAPKs in the lungs following K. pneumoniae infection could be due to attenuated neutrophil influx following K. pneumoniae infection. It is possible that the production of cytokines/chemokines and adhesion molecules via activation of NF-κB and MAPK could be secondary to the reduced IL-1β production.

IL-17A was shown to recruit lung neutrophils both directly and indirectly by stimulating the production of chemokines, such as KC, MIP-2, and G-CSF (55, 57). Although IL-23 was shown to induce IL-17A and IL-17F expression in CD4⁺ T cells (57, 58), and it is required for the maximum and sustained differentiation of Th17 cells (58, 59), additional mediators, including IL-6, IL-21, and IL-22, were shown to promote Th17 differentiation as well (56, 60, 61). Hyper IgE syndrome patients have low levels of IL-17 and have recurrent bacterial infections due to attenuated neutrophil recruitment to tissues (62). Importantly, mice with constitutively active NALP3 inflammasome display enhanced IL-1β activity, increased Th17 cell numbers, and neutrophil-dominated inflammation in skin (63). Our data show that NLRC4 is important for the differentiation or recruitment of IL-17A producing T cells.
in the lungs following *K. pneumoniae* challenge (Fig. 3). The contribution of innate immune cells to IL-17 production cannot be ruled out by our studies, because a recent study demonstrated that neutrophils produce IL-17A in the lung in a dectin-1– and IL-23–dependent manner during *Aspergillus fumigatus* infection (64). Our finding that IL-1β is important for neutrophil-mediated host immunity against *K. pneumoniae* infection in the lung is consistent with earlier findings showing the requirement of IL-1R activation in host immunity against systemic infections, brain abscesses, septic arthritis, and skin infections (65–67). It was demonstrated in acute inflammatory arthritis models in rabbits that IL-1β is required for the production of neutrophil CXC chemokines, such as IL-8 (41). In a murine model of cutaneous infection caused by *Staphylococcus aureus*, it was shown that IL-1β is required for neutrophil recruitment to the skin and for the production of KC and MIP-2 (41).

Recent studies provided evidence that both intracellular and extracellular bacteria can activate the NLRC4 inflammasome (23, 24, 27, 68). Although WT *S. typhimurium* activates caspase-1 in an NLRC4-dependent fashion, flagellin-deficient mutants showed impaired caspase-1 activation except at high MOI, which can induce caspase-1–dependent IL-1β secretion in macrophages (22). Furthermore, *L. pneumophila* can signal through the NLRC4 inflammasome to activate caspase-1 in the presence of a coactivator, NAIP-5 (69, 70). The activation of caspase-1 was evident in WT *L. pneumophila*, whereas a flagellin knockout mutant of *L. pneumophila* failed to cause caspase-1 activation. In studies involving *P. aeruginosa*, both the nonflagellated strain, PA103ΔU, and the flagellin-deficient mutant strain, PAKΔfc, induced activation of caspase-1, as well as secretion of IL-1β (27). Notably, the translocon proteins of the type III secretion system, such as PopB and PopD, were required for the induction of caspase-1 activation and secretion of IL-1β (27). Caspase-1 activation requires intact type III secretion system of other bacterial pathogens as well, including *S. typhimurium* and *S. flexneri* (21, 22). Although structurally different, the functionally analogous type IV secretion system is necessary to activate caspase-1 in murine macrophages following *L. pneumophila* infection, because the Dot mutants of *L. pneumophila*, which lack a functional type IV secretion system, failed to cause caspase-1 activation (71). However, we found that a nonflagellated bacterium, *K. pneumoniae*, can induce caspase-1 activation and secretion of IL-1β in macrophages via NLRC4, although *K. pneumoniae*-induced caspase-1 activation via NLRC4 does not induce pyroptosis. Because IL-1R1−/− mice show much more pronounced shortening of survival rates compared with NLRC4−/− mice upon *K. pneumoniae* infection, these findings suggest a role for other NLRs/inflammasomes that could be determined by future investigations.

Although hematopoietic cells in the lung produce several neutrophil chemotactic substances, such as KC (72, 73) and MIP-2 (74, 75), resident cells, including alveolar epithelial type II cells, produce other neutrophil chemoattractants, such as LIX (38). Intriguingly, we found that a requirement for NLRC4 signaling predominantly via hematopoietic cells for neutrophil accumulation in the lung in response to *K. pneumoniae* infection. Furthermore, our findings are consistent with earlier reports for the role of hematopoietic cells or resident cells in lung inflammation. First, MyD88 derived from hematopoietic cells is more important for LPS-induced expression of TNF-α and IL-12p40 (76), although both hematopoietic and resident cell-derived MyD88 signaling are essential for LPS-induced neutrophil influx (77–79). In addition, myeloid differentiation-2 receptor signaling in both hematopoietic and resident cells is essential for neutrophil-mediated inflammation, and the expression of MIP-2, TNF-α, and IL-6 is mediated by both cell types in the lungs after LPS challenge (35). Finally, KC produced by both hematopoietic and resident cells is important for bacterial clearance and neutrophil recruitment to the lung upon *K. pneumoniae* infection (31).

The mechanism(s) by which NLRC4 senses *K. pneumoniae* and the components of *K. pneumoniae* that induce NLRC4 activation are two unknowns highlighted by our studies. Several structurally and functionally diverse stimuli can activate the NLRP3 inflammasome, such as bacteria, virus, pore-forming toxins, extracellular ATP, silica crystals, and amyloid (80–85), although it is not clear how a single inflammasome, such as NLRP3, can recognize multiple stimuli. Thus, it is possible that NLRC4 can recognize endogenous ligands during bacteria-induced inflammation in addition to directly recognizing pathogens; however, the endogenous/bacterial ligands that can activate NLRC4 and the mechanisms responsible for NLRC4 activation during *K. pneumoniae* infection in the lung remain to be elucidated. Based upon the complete genome sequencing of a *K. pneumoniae* isolate, the genome of Kp342 has 10 of 11 established protein-secretion systems, including types I, II, IV, V, and VI secretion systems (86). It is not known whether these protein-secretion systems are important for bacterial virulence and whether the serotype 2 used in this investigation encodes these protein-secretion systems.

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**Disclosures**

The authors have no financial conflicts of interest.

**References**


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Differential requirements for NAIP5 in activation of the NLRC4 inflammasome.


