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NLRP12 Modulates Host Defense through IL-17A-CXCL1 Axis

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Abstract

We used an extracellular pathogen *Klebsiella pneumoniae* to determine the role of NLRP12 since this bacterium is associated with devastating pulmonary infections. We found human myeloid cells (neutrophils and macrophages) and non-myeloid cells (epithelial cells) show upregulation of NLRP12 in human pneumonic lungs. NLRP12 silenced human macrophages and murine *Nlrp12*$^{-/-}$ macrophages displayed reduced activation of NF-κB and MAPK and expression of HDACs following *K. pneumoniae* infection. NLRP12 is important for the production of IL-1β in human and murine macrophages following *K. pneumoniae* infection. Furthermore, host survival, bacterial clearance and neutrophil recruitment are dependent on NLRP12 following *K. pneumoniae* infection. Using bone marrow chimeras, we showed that hematopoietic cell driven NLRP12 signaling predominantly contributes to host defense against *K. pneumoniae*. Intratracheal administration of either IL-17A+ CD4 T cells or CXCL1+ macrophages rescues host survival, bacterial clearance, and neutrophil recruitment in *Nlrp12*$^{-/-}$ mice following *K. pneumoniae* infection. These novel findings reveal the critical role of NLRP12-IL-17A-CXCL1 axis in host defense via modulating neutrophil recruitment against this extracellular pathogen.

Introduction

Lower respiratory tract infections remain the most significant cause of worldwide infectious disease morbidity and health care costs [1]. The Gram-negative extracellular bacterium, *Klebsiella pneumoniae*, induces lung destruction and multiple abscesses in the lung even with small inoculums. In the recent years, the extensive spread of multi-drug resistant *K. pneumoniae* strains has caused ≥50% mortality in the U.S. and world [2, 3].

Although over 20 members of the NLR family have been identified, the function of most of their members in contributing to host resistance against microbial infection has not been

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determined. Nevertheless, reports suggest a function of some NLRs or inflammasomes in sensing bacterial pathogens [4, 5]. Regarding *K. pneumoniae* infection, thus far, only two types of inflammasomes have been reported to regulate host immunity to this aflagellated bacterium: NLR family pyrin domain containing 3 (NLRP3) [6] and NLRC4 inflammasomes [7, 8].

NLRP12 (aka NALP12/MONARCH-1/PYPAF-7) was shown as the first NLR to induce IL-1β maturation via the interaction with ASC [9](. Recent studies suggest that the NLRP12 inflammasome plays a role in intestinal homeostasis [10, 11] and tumorigenesis [11, 12]. Regarding the role of NLRP12 in bacterial recognition, a recent report has shown that NLRP12 is important to contribute to antibacterial defense against *Yesinia pestis* following subcutaneous or intravenous challenge [13]. The results also show that NLRP12-deficient (*Nlrp12*$^{-/-}$) animals had reduced survival and enhanced bacterial burden in the spleen, along with attenuated production of IL-18, IL-1β, and IFN-γ after *Y. pestis* infection [13]. In another study using a very high dose of *K. pneumoniae*, results show reduced macrophage and lymphocyte influx and attenuated TNF-α levels in the lungs following the infection although neutrophil influx and bacterial clearance were not different between WT and *Nlrp12*$^{-/-}$ mice [7].

The goal of our current investigation was to delineate the unique role of NLRP12 by invoking innate immunity against *K. pneumoniae* by using a human cell system and a mouse model of infection.

Results

NLRP12 expression is increased in human pneumonic lungs

To examine whether NLRP12 expression is increased in human lungs, we used lung sections from patients with pneumonia and lung injury due to bacterial infection using immunofluorescence for NLRP12 in pneumonic and uninjured (control) human lungs. Diffused NLRP12 staining was detected in pneumonic lungs whereas limited NLRP12 staining was observed in these cell types in the lung sections from control patients (Fig. 1). In particular, both myeloid cells [neutrophils (lipocalin+) and macrophages (CD68+)] and non-myeloid cells [alveolar type II epithelial cells (ProSPC+)] express NLRP12 in pneumonic/injured lungs (Fig. 1A). Furthermore, we used *K. pneumoniae*-infected mouse lungs to examine the expression of NLRP12 in neutrophils (Gr-1+), type II epithelial cells (Pro-SPC+) and macrohphages (F4/80+) (Fig 1B). Our findings together indicate that myeloid cells (neutrophils and macrophages) and non-myeloid cells (alveolar type II epithelial cells) show upregulation of NLRP12 in infected lungs.

NLRP12 regulates proinflammatory cytokines/chemokines in human and mouse macrophages

To determine the function of NLRP12 in human cells, we measured cytokines/chemokines in human peripheral blood monocyte-derived macrophages, following NLRP12 siRNA knockdown, and in *Nlrp12*$^{-/-}$ alveolar macrophages, following the infection with the Gram-negative bacterium, *K. pneumoniae*. Expression of proinflammatory mediators is regulated

by NF-κB and MAP Kinases[14]. In addition, Histone deacetylases (HDACs) constitute a family of enzymes that play important roles in the epigenetic regulation of gene expression [15]. Treatment with the specific HDAC inhibitor (trichostatin A) is shown to be essential to induce cytokines in human alveolar epithelial cell line A549 response to bacterial infection[16]. In siRNA-transfected human monocyte-derived macrophages, we found reduced NF-κB activation and attenuated levels of IL-6, IL-1β, and IL-18 (Figs. 2A-B) 3 or 6 hours after *K. pneumoniae* infection (Fig. 2A-B). Moreover, we found, reduced HDAC 2, 3 and 5 upregulation and decreased activation of MAPKs (p38, ERK and JNK) in NLRP12 siRNA-transfected human cells (Fig. 2C-D). In a similar fashion in murine alveolar macrophages, we observed decreased cytokines (TNF-α, IL-6, and IL-1β) and neutrophil chemokine (CXCL2/MIP-2) in *Nlrp12*$^{-/-}$ cells, 3 and 6 h after *K. pneumoniae* infection (supplemental data; Figs. 1A-B). Attenuated NF-κB activation, reduced HDAC 1, 2 and 3 upregulation and decreased activation of MAPKs (p38, ERK and JNK) was also observed in *Nlrp12*$^{-/-}$ cells (supplemental data; Figs. 1C-D).

NLRP12 regulates host defense against K. pneumoniae infection

To assess the effect of NLRP12 activation in pulmonary defense during extracellular Gram-negative bacterial infection, WT mice and *Nlrp12*$^{-/-}$ mice were infected intratracheally (i.t.) with two (higher and lower) doses of *K. pneumoniae* (10^3 or 10^4 CFUs/mouse), and survival was monitored up to 15 days after the infection. The *Nlrp12*$^{-/-}$ mouse group displayed attenuated survival to both higher and lower infectious doses (Fig. 3A). To determine if reduced survival of *Nlrp12*$^{-/-}$ mice after *K. pneumoniae* infection is dependent on impaired bacterial clearance in the lungs or bacterial dissemination in *Nlrp12*$^{-/-}$ mice, *K. pneumoniae* CFU were quantified from whole lung and spleen at 24 and 48 h post-infection (lower dose). *Nlrp12*$^{-/-}$ mice demonstrated higher lung, liver and spleen CFUs as compared to their WT littermates at 48 hours (Fig. 3B).

To identify mechanisms that contribute to higher bacterial CFUs in lungs and extrapulmonary organs, we assessed neutrophil recruitment to the airspaces following *K. pneumoniae* challenge because neutrophil influx is shown to be critical to clear *K. pneumoniae* infection in tissues [8, 17]. Total WBC and neutrophil accumulation in the airspaces of *Nlrp12*$^{-/-}$ mice was attenuated at 48 hours compared with WT controls (Fig. 3C). Consistent with reduced neutrophil recruitment into the airspaces, we also observed reduced neutrophil influx in lung parenchyma (MPO activity in lung homogenates) of *Nlrp12*$^{-/-}$ mice as compared to WT (Fig. 3C). To determine if the decreased neutrophil influx is dependent on the production of cytokines/chemokines following *K. pneumoniae* infection, we measured the expression of cytokines (IL-23, IL-17A, IL-1β) and neutrophil chemoattractants (CXCL1/KC, CXCL2/MIP-2, and CXCL5/LIX) in bronchoalveolar lavage (BAL) fluid and lung homogenates 24 and 48 h following *K. pneumoniae* challenge (Fig. 3D). Intriguingly, IL-1β, IL-23, IL-17A and G-CSF levels in *Nlrp12*$^{-/-}$ mice in bronchoalveolar lavage fluid (BALF) or lung homogenates were attenuated at 48 h following the *K. pneumoniae* challenge (Fig. 3D).

Because multiple proinflammatory genes are regulated by NF-κB, HDAC and MAPKs [18, 19], we next investigated the activation of NF-κB, expression of HDACs and

activation of MAPKs in the lungs of *Nlrp12*$^{-/-}$ mice following *K. pneumoniae* infection. As shown in supplemental data; Fig. 2A-C, NF-κB activation was reduced in *Nlrp12*$^{-/-}$ mice at 24 and 48 h following *K. pneumoniae* challenge. Additionally, *Nlrp12*$^{-/-}$ mice infected with *K. pneumoniae* exhibit reduced expression of HDAC2 (supplemental data; Fig. 2D-E) although no change in activation of p38, ERK, and JNK was observed at 24 and 48 h post-infection (supplemental data; 2F-G).

Using semiquantitative histology, WT mice showed severe suppurative bronchopneumonia, whereas *Nlrp12*$^{-/-}$ mice displayed moderate suppurative pneumonia 48 h after *K. pneumoniae* infection (Fig. 3E). In contrast, no significant cellular influx and alveolar edema was observed in saline-challenged (control) lungs obtained from either *Nlrp12*$^{-/-}$ or WT animals (data not shown). To demonstrate whether the effect of NLRP12 gene deficiency is mouse strain specific, we used WT and KO mice on an A/J background (after 10 generations of backcrossing) and we observed that infected *Nlrp12*$^{-/-}$ mice showed 1) more bacterial burden in the lungs and spleens; and 2) reduced neutrophil influx in BALF following the infection (Fig. 3F), suggesting that NLRP12 effects are not mouse background specific.

In prior studies, NLRP12 has been suggested to form inflammasomes in order to enhance caspase-1 activation and IL-1β and IL-18 maturation [9, 13]. Therefore, despite measuring IL-1β levels in BALF following the infection, we evaluated caspase-1 activation in the infected lungs using fluorometry and western blotting. We detected a decrease in caspase-1 activity and caspase-1 cleavage in *Nlrp12*$^{-/-}$ lungs following the infection (Fig. 3G). Moreover, western blot results demonstrated that a significant amount of procaspase-1 was present in the lungs infected with *K. pneumoniae* (Fig. 3G).

In mice, ELR+ CXC chemokines, such as CXCL1/KC, CXCL2/MIP-2, and CXCL5/GCP-2/LIX are known neutrophil chemoattractants[20]. In a recent investigation, it has been shown that NLRP12 modulates dendritic cell (DC) and myeloid cell migration in a mouse model of contact hypersensitivity[21]. In this study, DCs and neutrophils obtained from the *Nlrp12*$^{-/-}$ mice show attenuated migration towards DC chemoattractants, such as CCL19, CCL21 and CXCL12 and neutrophil chemoattractant, such as CXCL1/KC. Our results also demonstrated attenuated neutrophil migration towards CXCL2/MIP-2, but reduced migration towards KC, which is not statistically significant (Fig. 3H).

NLRP12 mediates IL-17A differentiation of CD4+ T cells

The amount of IL-17A production is reduced in the lungs of *Nlrp12*$^{-/-}$ mice in response to *K. pneumoniae* (Fig. 4). Reduced number of IL-17 producing cells may be due to 1) attenuated recruitment from the bloodstream, and/or 2) augmented differentiation of Th0 cells to become Th17 cells locally. Therefore, we did an *in vitro* T cell differentiation assay to determine these possibilities. Our results show diminished differentiation of Th0 cells to Th17 and Th1 cells in *Nlrp12*$^{-/-}$ mice but no difference between CD4+ T cells of WT and *Nlrp12*$^{-/-}$ mice in Th2 differentiation (Fig. 4) highlighting the role of NLRP12 in Th17/Th1 differentiation.

Bone marrow-derived NLRP12 is important for host defense

Next, we asked if host defense against *K. pneumoniae* could be due to recruited bone marrow (hematopoietic) cells and/or resident alveolar cells. To address this hypothesis, WT or *Nlrp12*$^{-/-}$ mice were lethally-irradiated and reconstituted with bone marrow cells from donor WT or *Nlrp12*$^{-/-}$ mice to generate four groups: 1) WT mice reconstituted with WT marrow (WT→WT); 2) WT mice reconstituted with *Nlrp12*$^{-/-}$ marrow (*Nlrp12*$^{-/-}$ →WT); 3) *Nlrp12*$^{-/-}$ mice reconstituted with WT marrow (WT→ *Nlrp12*$^{-/-}$); and 4) *Nlrp12*$^{-/-}$ mice reconstituted with *Nlrp12*$^{-/-}$ marrow (*Nlrp12*$^{-/-}$ → *Nlrp12*$^{-/-}$). Eight weeks post-reconstitution, these bone marrow chimera mice were i.t. inoculated with *K. pneumoniae,* and bacterial burden in the lungs and spleens was determined. We found more bacterial burden in *Nlrp12*$^{-/-}$→ *Nlrp12*$^{-/-}$ and *Nlrp12*$^{-/-}$→WT chimera mice as compared with WT→WT or WT→ *Nlrp12*$^{-/-}$chimera animals (Fig. 5A-B). Leukocye/neutrophil recruitment to the lung was attenuated in *Nlrp12*$^{-/-}$→ *Nlrp12*$^{-/-}$ and *Nlrp12*$^{-/-}$→WT chimera mice as compared to (WT→WT or WT → *Nlrp12*$^{-/-}$chimera animals (Fig. 5C-D).

NLRP12 is dispensable for pyroptosis

NLRP12 has previously been shown to induce pyroptosis in response to flagellated bacterial infection, such as *Burkholderia pseudomallei* [22] and *Salmonella typhimurium* [23]. Pyroptosis is induced by caspase-1 activation by proteolytic cleavage of caspase-1 [24]. However, annexin V stains not only pyroptotic cells but also apoptotic cells[25]. We detected pyroptosis and/or apoptosis using flow cytometry-based annexin V binding. As shown in Fig. 6A-B, pyroptosis or apoptosis in neutrophils (Ly6G+) or macrophages (EMR1+) in *K. pneumoniae*-infected lungs and spleens between WT and *Nlrp12*$^{-/-}$ mice was not dfifferent.

Adoptive transfer of CD4+ T cells rescues host defense in NLRP12$^{-/-}$ mice

Our findings demonstrated that 1) IL-17A levels were reduced in *Nlrp12*$^{-/-}$ mice following *K. pneumoniae* infection (Figs. 3); and 2) CD4+ T cells from *Nlrp12*$^{-/-}$ mice show attenuated Th17 differentiation (Fig. 4). Studies unequivocally demonstrated that CD4+ T cells are an important source of IL-17A [26, 27]. To examine if an increase in the number of T cells could rescue NLRP12 deficiency, we adoptively transferred 0.5 million IL-17+ CD4 T cells i.t. to *Nlrp12*$^{-/-}$ mice at the time of the infection. Transfer of mature wild-type (IL-17$^{+/+}$) CD4+ T cells, but not transfer of *Il-17*$^{-/-}$ CD4+ T cells, rescued survival, bacterial load in the lungs and spleen, neutrophil influx and cytokine/chemokine expression in lungs (Fig. 7A-D).

Adoptive transfer of CXCL1+ macrophages restores host resistance in NLRP12$^{-/-}$ mice to K. pneumonia

Our data showed that CXCL1, a neutrophil chemoattractant, levels were attenuated in *Nlrp12*$^{-/-}$ mice after *K. pneumoniae* infection (Fig. 3). We have shown that that macrophages are an important source of CXCL1 (supplemental data; Fig. 1A). To examine if an increase in the number of CXCL1+ (WT) macrophages could rescue NLRP12 deficiency, we adoptively transferred 0.5×10^6 bone marrow macrophages i.t. to *Nlrp12*$^{-/-}$ mice at the time of the infection. Transfer of mature WT, but not transfer of *Cxcl1*$^{-/-}$

macrophages, rescued survival, bacterial burden in the lungs and spleen, neutrophil influx and cytokine/chemokine expression in lungs (Fig. 8A-D).

Discussion

In humans, mutations in gene encoding NLRP12 are linked to skin rashes, fevers, and joint pains that are similar to what is seen in patients with Familial Cold Autoinflammatory Syndrome (FCAS) [28, 29]. These mutations seem to decrease the ability of NLRP12 protein to attenuate the inflammation, leading to the intermittent episodes of fever since these mutations may lead to increased caspase-1 activity [30, 31]. The host defenses of the human lung include successful elimination of the microbes by resident alveolar macrophages [32, 33]. Any defects in host defense functions in macrophages ultimately lead to infectious complications in the host by excessive bacterial colonization. Our findings conclude that NLRP12 in human macrophages has an important host defense function against a non-flagellated bacterium. This was unexpected and represents the first report of NLRP12-dependent protein induction in these unique human cells in response to *K. pneumoniae* infection.

In mice, both proinflammatory and anti-inflammatory functions of NLRP12 have been demonstrated [9, 11, 12]. Initial findings indicate that NLRP12 regulates dendritic cell (DC) and neutrophil migration both *in vitro* and *in vivo*: (a) Isolated bone marrow dendritic cells obtained from *Nlrp12*$^{-/-}$ mice show decreased migration toward CCR7 and CXCR4 ligands; (b) the migration of *Nlrp12*$^{-/-}$ bone marrow neutrophils to CXCL1/KC was also attenuated by ~50% *in vitro*; and (c) experiments also show that *Nlrp12*$^{-/-}$ DCs display a significantly reduced capacity to migrate to draining lymph nodes in a mouse model of contact hypersensitivity [21]. Regarding investigations dealing with bacterial pathogens, findings reveal that NLRP12 contributes to host defense against *Yesinia pestis* in a mouse model of systemic (s.c and i.p) infection [13]. Nevertheless, only a single report has addressed the role of NLRP12 in host resistance to infectious agents via the intrapulmonary route [7]. Using a very high inoculum of *K. pneumoniae* (74,000 CFU/mouse), the authors show reduced macrophage and lymphocyte influx and attenuated TNF-α levels in the lungs following the infection although neutrophil influx and bacterial clearance were not different between WT (control) and *Nlrp12*$^{-/-}$ mice. However, we observed that host survival, bacterial clearance and neutrophil recruitment to the lungs are dependent on NLRP12 following *K. pneumoniae* infection. The discrepancy between the published study and our study could be explained by the fact that 1) both studies used different *K. pneumoniae* strains; 2) Allen *et al.* have used an extremely high dose (74,000 CFUs/mouse) and we used a low dose (1,000 CFUs/mouse) in all experiments, but used both lower and higher doses of *K. pneumoniae* (1000 and 10,000 CFUs/mouse) for survival experiments.

The current study is the first to demonstrate a role for NLRP12 in host resistance against both extracellular pulmonary bacterial pathogen. Redundant roles for inflammasomes may occur for optimal innate immune responses against bacteria. Thus far, two other inflammasomes have been implicated in the regulation of host immunity to *K. pneumoniae*: NLR family pyrin domain containing 3 (NLRP3) [6] and NLR family CARD domain containing 4 (NLRC4 or IPAF) [8] . The involvement of multiple inflammasomes for host

defense against *K. pneumoniae* supports the emerging concept and relates to the cooperative interactions between different inflammasomes during bacterial infection in the host. Although cooperative interactions have not been explored in the lungs, interaction among different inflammasomes has been demonstrated in the gut [34, 35]. Future studies using double- or triple-KO mice should explore these interactions in more detail.

It has been demonstrated that neutrophil influx is a critical event to clear *K. pneumoniae* in the lungs as neutrophil depletion prior to the infection enhanced susceptibility to the infection [8]. Here, we demonstrate that deletion of NLRP12 leads to augmented susceptibility to intrapulmonary *K. pneumoniae* infection. Additional data illustrate that NLRP12 inhibits bacterial colonization in the lungs and dissemination of *K. pneumoniae*. This increase CFUs correlates with decreased neutrophil influx and the production of neutrophil chemoattractants, such as KC, MIP-2, or LIX in the lungs. This is also the first demonstration of an important role for NLRP12 in host resistance against pulmonary infection. Although attenuated production of neutrophil chemoattractants have contributed to reduced neutrophil influx in the lungs, our results also reveal that NLRP12$^{-/-}$ neutrophils have an inherent defect towards migrating neutrophil chemoattractants, such as CXCL2/MIP-2. Consistent with this speculation, a previous study shows that the migration of *Nlrp12*$^{-/-}$ bone marrow neutrophils towards CXCL1/KC was reduced by ~50% compared with the neutrophils from control mice [21].

While hematopoietic cells in the lung produce several neutrophil chemokines − including KC [36] and MIP-2 [37] − resident cells, such as alveolar epithelial type II cells, produce other neutrophil chemoattractants, such as LIX [38]. We show that a requirement for NLRP12 signaling predominantly via hematopoietic cells for bacterial clearance and neutrophil accumulation in the lungs. Our findings are also consistent with earlier reports of the role of either hematopoietic cells or resident cells in infectious or noninfectious lung inflammation: (a) MyD88 derived from hematopoietic cells is more important for LPS-induced expression of TNF-α and IL-12p40 [39], although both hematopoietic and resident cell-derived MyD88 signaling are essential for LPS-induced neutrophil influx [40, 41]; (b) MD-2 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 [42]; and (c) CXCL1/KC produced by both hematopoietic and resident cells is important for bacterial clearance and neutrophil recruitment to the lung upon *K. pneumoniae* infection [43]. Since both hematopoietic cells and resident cells show upregulated expression of NLRP12, these results may lead to the prediction that both cell types to contribute to host defense. However, our results highlight the contribution of hematopoietic cell driven NLRP12 in host defense. Therefore, the inducible expression levels of NLRP12 in hematopoietic cells versus stromal cells in the lungs following the infection and cell-type specific responses following *K. pneumoniae* infection need to be determined by future studies to resolve this discrepancy.

A rapid immune response is critical to augmenting host defense. The innate immune system is crucial to rapidly detect infection. The emerging role of inflammasomes as mediators of innate immunity positions them as therapeutic targets. One of the known inflammasome activators, alum, is in widespread use as vaccine adjuvants in humans. The finding that

inhibition of NLRP12 can paralyze pulmonary defense could have a deep impact on the strategies to treating and/or preventing bacterial pneumonias.

Methods

Immunohistochemistry

For immunofluorescence staining human lung sections from lung without evidence of infection, injury or other diseases (n=3) or from patients who died off bacterial infection with ALI/ARDs were obtained from a commercial source (Biochain, CA). These sections were analyzed for NLRP12 immunostaining. Briefly, deparaffinized fixed lung sections were permeabilized with the buffer containing Triton X-100 (0.1%) and then blocked with serum. Lung sections were incubated with anti-NLRP12 (ABGENT) and surface markers including anti-lipocalin Ab for PMNs (R&D), anti-proSPC Ab for type II epithelial cells (Millipore; US Biological) or anti-CD68 Ab for macrophages (BioLegend). For mouse lung sections, we used anti-Gr1 for PMNs (US Biological), anti-proSPC for type II epithelial cells or anti-F4/80 (Biolegend) as surface markers along with anti-NLRP12 Ab (ABGENT). Sections were washed and incubated with Alexa-conjugated secondary antibodies (Invitrogen, Carlsbad, CA). Tissue sections were washed and mounted using Vectashield mounting medium (Vector Laboratories, Inc., CA 94010) containing DAPI stain for nuclear staining. Images were acquired using an Axiocam digital camera (Zeiss, Thornwood, NY) connected to a Zeiss Axioskop 2 Plus research microscope.

Human macrophages

Frozen human peripheral blood mononuclear cells were obtained from Astarte Biologics (Redmond, WA) and were used as described in our previous publication [8]. For monocyte/macrophage differentiation, monocytes were cultured on plates for up to 7 days in RPMI 1640, containing 5% FBS, 1% penicillin-streptomycin, and 100 ng/mL M-CSF. For knockdown experiments, a pre-validated pool of siRNA (a cocktail of 4 siRNAs) for human *NLRP12* was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Cells (0.5×10^6) were transfected with either 40 nM siRNA or a control siRNA (Santa Cruz Biotechnology Inc) using TransIT-TKO Transfection Reagent from MIRUS (Madison, WI) for 48 hours. Cells were then infected with 1 MOI of *K. pneumoniae* for 6 hours. For cytokine/chemokine assays, supernatants were collected 24 hours post-infection. For immunoblotting experiments, macrophages were washed 3 times with PBS before lysing with Urea/Chaps/Tris buffer supplemented with protease and phosphatase inhibitors.

Mouse macrophages

All mice were on C57BL/6 background were bred in specific pathogen–free rooms within animal facilities at the Louisiana State University (LSU). Controls for each experiment were gender and age matched. Murine alveolar macrophages were isolated from BALF fluid from WT or *Nlrp12*$^{-/-}$ mice as previously described [43, 44]. Mice were anesthetized and then sacrificed by cardiac exsanguination. Lungs were lavaged with 0.8 mL sterile saline each time through an intratracheal catheter as described previously [43, 45], and a total of 8 mL saline was instilled and recovered from each mouse. The lavage fluid was spun at $300\times g$ for 10 minutes to pellet alveolar macrophages. Cells were cultured in 12-well culture plates at

37°C with 5% CO_2 at a concentration of 0.5×10^6 cells per well in 1 ml RPMI 1640 medium (Sigma Chemical Co., St. Louis, MO) supplemented with 10% FBS, 1 mM pyruvate, 100 U/ml penicillin, and 0.1 mg/ml streptomycin. After 2 hours of incubation, non-adherent cells were removed with phosphate buffered saline (PBS), and the medium was replaced. Cells were then infected with 1 multiplicity of infection (MOI) of *K. pneumoniae* (ATCC 43816) for designated time points. For cytokine studies, media was collected at 3 and 6 hours following the infection. For western blotting, cells were washed three times with PBS and lysed with Urea/Chaps/Tris buffer containing protease and phosphatase inhibitors.

Mice

8- to-10-week-old female mice, genetically deficient for NLRP12 (*Nlrp12*$^{-/-}$) [46] or age and gendet-matched WT mice weighing 22-26 grams, were used for *in vivo* experiments. *Nlrp12*$^{-/-}$ mice were backcrossed 10 times with age-matched C57Bl/6 or A/J mice. Mice were kept on 12:12 hour light:dark cycle with free access to food and water. All animal experiments were approved by the Louisiana State University IACUC.

Pneumonia model

K. pneumoniae serotype 2 strain (ATCC 43816) was used for a intrapulmonary challenge as described earlier [17, 43, 45]. The bacteria were grown for 8 hours at 37°C in 50 mL tryptic soy broth (TSB) with shaking at 225 rpm. Bacteria were harvested by centrifuging the culture at 1200×*g* for 2 min and washed twice in sterile saline. The cells were resuspended in an isotonic saline at a concentration of 10^3 CFUs/50 μL/mouse. After anesthesia, *K. pneumoniae* suspension (10^3 CFUs in 50 μL) in 0.9% saline was inoculated via the intratracheal (intrapulmonary) route. The CFUs were enumerated by serially diluting the suspension of initial inoculums and subsequently plating 20 μL aliquots of each dilution onto a tryptic soy agar (TSA) plate and a MacConkey agar plate. Similarly, for counting bacterial CFUs in lungs and spleen, tissues were homogenized in PBS for 15 and 30 sec respectively, and 20 μL of homogenates were plated in 10-fold serial dilutions onto TSA and MacConkey agar plates. The survival of NLRP12$^{-/-}$ and WT mice was monitored for up to 15 days following inoculation with *K. pneumoniae*. For adoptive transfer experiments, 0.5×10^6 IL-17A+/IL-17A- CD4+ T cells or CXCL1+/CXCL1- bone marrow macrophages were i.t administered at the time of the infection.

BALF collection

The animals were euthanized, and the trachea was exposed and subsequently cannulated with a 20-gauge catheter as described earlier [43, 45]. BAL fluid was collected 4 times by instilling 0.8 mL of PBS containing heparin and dextrose. Total leukocytes in BAL fluid were enumerated by counting on a hemocytometer, whereas BAL differential leukocyte cell counts were determined by standard light microscopy. The remaining (2 mL) of the undiluted cell-free bronchoalveolar lavage fluid (BALF) was passed through a 0.22 μm filter and used for the determination of cytokine/chemokine levels.

Lung isolation

Following the infection, the whole (non-lavaged) lungs were excised and snap frozen. For long-term storage, these lung tissues were stored at -70°C and used for cytokine/chemokine determination, western blots, and MPO activity assay. Lung tissue was briefly 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*/20 min. The supernatants were harvested, passed through a 0.22 μm filter, and used as required.

MPO activity

The lung homogenates were resuspended in 50 mM potassium phosphate buffer (pH 6.0) supplemented with 0.5% hexadecyltrimethylammonium bromide (HTAB), as described in previous publications [17, 38, 42, 43, 45]. Samples were then sonicated, incubated at 60°C for 2 hours, and assayed for MPO activity in a hydrogen peroxide/*O*-dianisidine buffer at 460 nm. Absorbance was measured at 460 nm using a spectrophotometer. The increase activity was calculated between 0 sec and 90 sec.

Cytokine/chemokine determination

Cytokines/chemokines were determined by sandwich ELISA as described earlier [17, 42, 43, 45]. The minimum detection limit of the assay was 2 pg/mL of protein. For mouse lungs, TNF-α, IL-6, LIX, MIP-2, IL-23, IL-17A, IL-1β, and IL-18 concentrations were normalized to the total protein concentration in the samples measured. Results are expressed as pg/mg of total protein for lung tissue and in pg/mL for BALF.

Semiquantitative histology

The lungs were perfused from the right ventricle of heart with an isotonic saline, 24 and 48 hours post-infection, and harvested. For hematoxylin and eosin staining, lungs were fixed in 4% phosphate-buffered formalin, processed in paraffin blocks, and cut into fine sections (5 μm in thickness). Semiquantative histology was performed by a Veterinary Pathologist in a blinded fashion according to the following scoring scale: 0, no inflammatory cells (macrophages or neutrophils) are 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, as indicated in our earlier publications [45, 47].

Caspase-1 activation

(A) Fluorometry: Infected or control lungs were used to make single cell suspension. A total of 50 cells/well were used in a 96 well plate to measure caspase-1 activity according to manufacturer's recommendation (Biovision, CA). At the end of incubation, samples were measured at a 400-nm excitation filter and 505-nm emission. Increase in florescence activity was plotted as relative fluorescence units (RFU). (B). Western blotting: The lungs were harvested at the designated times and homogenized in 1 mL of phosphate buffered saline (PBS) containing 0.1% Triton X-100 supplemented with the cocktail of complete protease and phosphatase inhibitors as described in earlier publications[48]. Mouse anti-caspase-1 Ab (AdipoGen, CA) at a concentration of 1 μg/ml was used.

Chemotaxis

Neutrophil transmigration assay was performed in a Transwell system using 24-well tissue culture plates with a pore size of 3.0 μm as described in a prior publication[49]. Chemoattractants, either recombinant KC (1 μg/0.5 ml) or MIP-2 (1 μg/0.5ml), and phosphate-buffered saline (PBS) supplemented with bovine serum albumin (BSA; 2 μg/ml) was added to each of the lower wells in the chamber. A total of 0.1×10^6 LPS-activated neutrophils (PMNs) in Dulbecco modified Eagle medium (DMEM) supplemented with 0.1% BSA was added to every well of the upper chamber. Following incubation at at 37°C 5% CO_2 for 3 h, the cells from 10 fields in the lower plate were counted using an inverted microscope. The number of PMNs in lower chamber is indicated as relative chemotaxis.

Th1/Th2/Th17 differentiation

Th1/Th2/Th17 differentiation has been performed as previously described [50, 51] . Cells were washed and resuspended in PBS followed by blocking with Fc receptor blocking reagent. Cells were surface stained with anti-CD4 and intracellular with anti- IFN-γ, -IL-4 or -IL-17A. Flowjo software was used for data analysis.

Bone marrow (BM) transplantation

BM chimera experiments were performed as described in earlier publications [17, 42, 43]. Recipient groups were gamma irradiated from a cesium source in two 525-rad doses separated by 3 hours. BM was flushed from tibias and femurs from donor mice, and a total of 8×10^6 BM cells were injected into the tail veins of lethally irradiated recipient mice. Reconstituted mice were treated with 0.2% neomycin sulfate for the first 2 weeks post-transplantation. Bacterial challenge experiments were performed 8 weeks after BM reconstitution. In another set of experiments, we used donor cells expressing green fluorescent protein. Blood sample was collected from recipient mice at 6 and 8 weeks after reconstitution, and hematological parameters, such as total WBC counts and differential counts were measured. Using flow cytometry, we found that more than 75-85% of blood leukocytes were derived from donor marrow at the time the mice were used for experiments (6–8 weeks post-transplantation.

Pyroptosis or apotosis

Lung or spleen digests from C57BL/6 (WT) or *Nlrp12*$^{-/-}$ mice challenged with *K. pneumoniae* for 24 or 48 h were used to determine cells undergoing pyroptosis as outlined in previous publication [8]. Briefly, lung and spleens cell suspensions were passed through a 0.70 μ filter. Following 2 PBS washings, cells were FcR blocked and aliquoted for surface staining with conjugated PerCP anti-mouse Gr-1/Ly6G or EMR1 and APC anti-mouse CCR2 or CXCR2. Red blood cells (RBCs) were lysed by adding NH_4Cl lysing buffer. Cells were resuspended in 1× binding buffer containing 5 μl of Annexin V-FITC and 5 μl of PI according to the manufacturer's protocol (Annexin V apoptosis detection kit from BD Pharmingen). The cell suspension was vortexed and incubated for 15 mins in the dark at room temperature. A total of 100 μl 1× binding buffer was added, and cells were analyzed by flow cytometry. CCR2 or CXCR2 positive Gr-1/Ly6G (neutrophils) and EMR1

(macrophages) that were positive for Annexin V-FITC and negative for PI are shown in histograms.

Adoptive transfer of CD4$^+$ T cells and macrophages

Splenic CD4$^+$ T cells were isolated and made single cell suspensions. Cell suspensions were washed, RBCs were lysed and CD4+ T cells were isolated by negative selection from single cell suspension using the EasySep cell separation procedure (StemCell Technologies, Vancouver, Canada). Resulting cell preparations were resuspended to a final density of 0.5×10^6 cells per 50 μL PBS for i.t. administration. BM from femur and tibia was flushed and marrow was passed through a 21G needle 4-6 times to dissociate the cells. RBCs were lysed using 1X RBC lysis buffer. Cell suspension was washed with PBS twice and cells were resuspended in DMEM +5% FBS+P/S containing 2 million cells/ml with M-CSF 25 ng/ml and seeded for 6-7 days. Fresh BMDM growth medium was added on day 3 and 5, and the formation of mature BMDM was evaluated after 7 days using flow cytometry analysis to detect cells expressing CD11b and F4/80. Resulting cell preparations were resuspended to a final density of 0.5×10^6 cells per 50 μL PBS for i.t. administration.

Statistics

Data are expressed as mean ± SEM. ANOVA, followed by Bonferroni's post hoc analysis, was performed for comparisons among multiple groups. All statistical calculations were performed using InStat software and GraphPad Prizm 4.0 (GraphPad Sotware, La Jolla, CA). All experiments were performed 3 times, with the exception of the survival experiments, which were performed twice. The survival results were compared by Wilcoxon rank sign test. A p value *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$ was considered significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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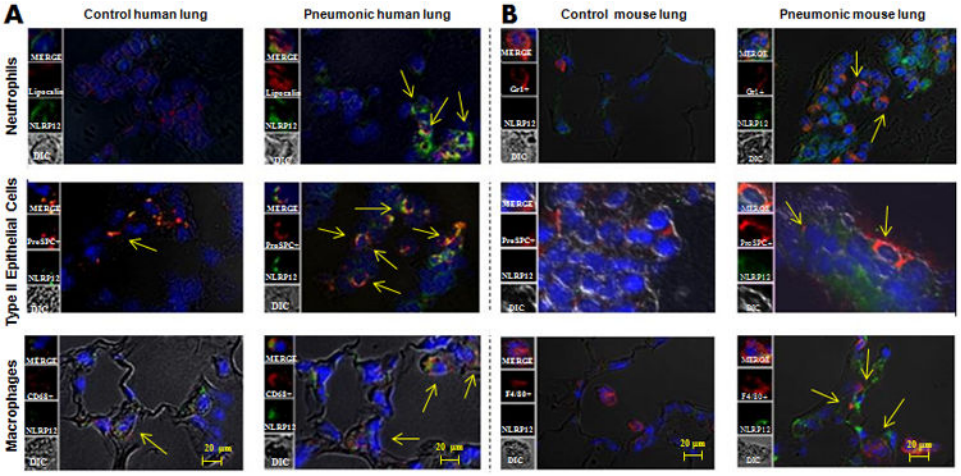

Figure 1. A. NLRP12 expression is increased in myeloid cells (neutrophils and macrophages) and epithelial cells in the lung during ALI/pneumonia

A. Immunofluorescence microscopy was performed for NLRP12 expression in normal human (control) lung tissue and human lung tissue from bacterial pneumonia. NLRP12 is indicated by green staining, neutrophils are shown by lipocalin staining, epithelial cells are shown by proSPC staining, whereas macrophages are shown by CD68 staining. **B** NLRP12 expression is enhanced in myeloid cells (neutrophils and macrophages) and epithelial cells in mouse lungs during pneumonia. NLRP12 is indicated by green staining, neutrophils are shown by Gr1 staining, epithelial cells are stained by proSPC staining and macrophages are shown by F4/80 staining. This is a representative image of 5 sections with similar results. Original magnification × 200.

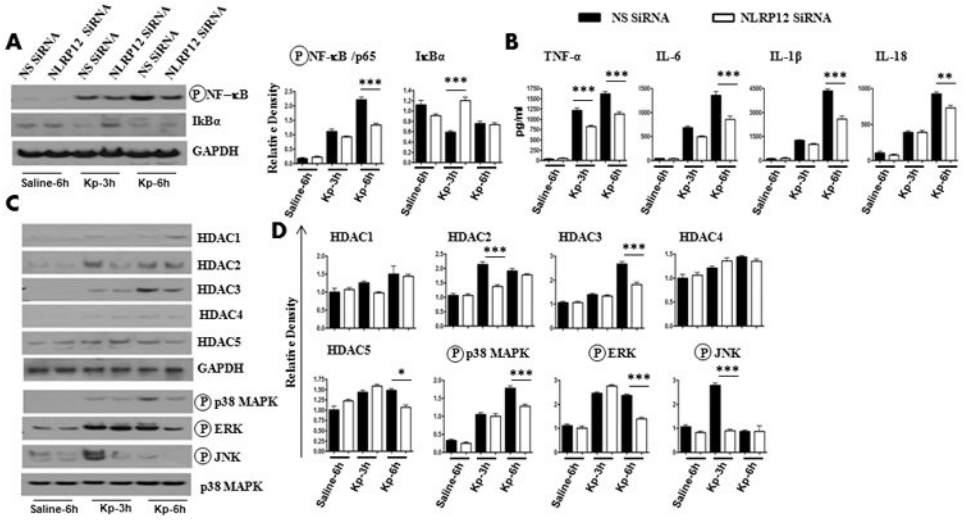

Figure 2. NLRP12 is important for NF-κB activation, cytokine/chemokine production, HDAC expression and MAPK activation in human macrophages following *K. pneumoniae* infection

A-B. Human monocyte-derived macrophages (0.5×10^6) were transfected with 40 nM NLRP12 siRNA or scrambled siRNA (nonspecific; NS) for 48 h. Cells were then infected with 1 MOI of *K. pneumoniae* for 3 or 6 h and NF-κB activation was measured by western blotting (A), and cytokine/chemokine levels in supernatants (B) were measured by sandwich ELISA. Representative blots are shown from three independent experiments with identical results. Densitometry was performed using Gel Digitizing Software and normalized to GAPDH. **C-D**. Human macrophages were transfected with *siRNA and* then infected with *K. pneumoniae* in a similar manner, and cell lysates were used to determine HDAC expression and MAPK activation by western blotting. Representative blots are shown from three independent experiments with identical results. Densitometry was performed using Gel Digitizing Software and normalized to GAPDH. Means ± SE values were obtained from three separate experiments. NS, non-specific. *, $p<0.05$; **, $p<0.01$;***, $p<0.001$. Error bars represent SE.

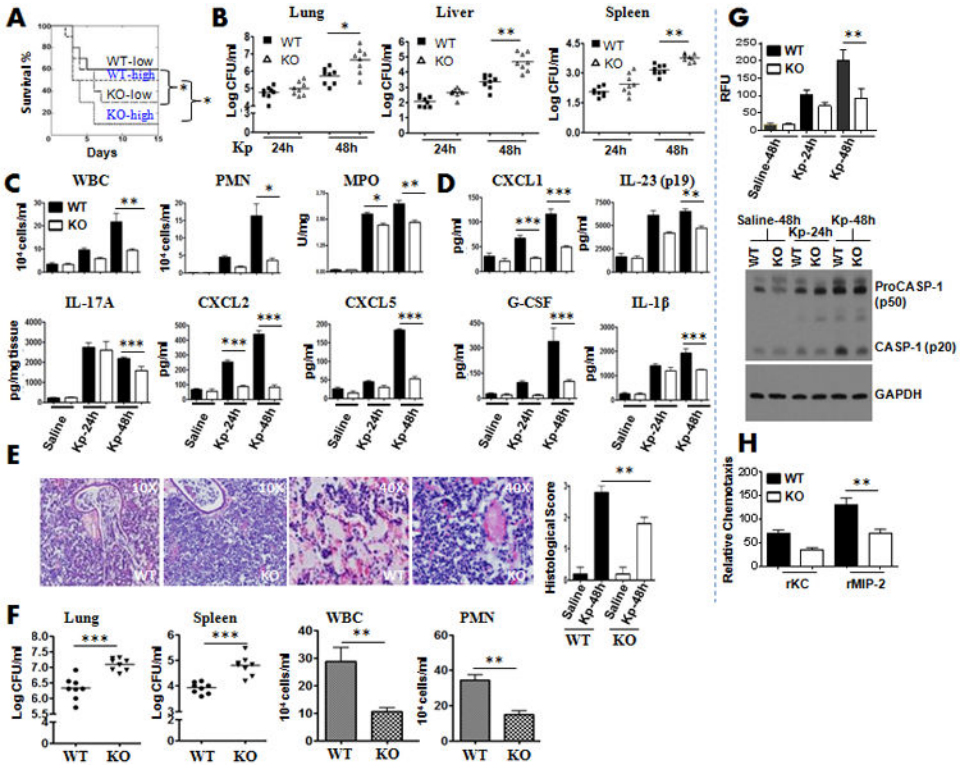

Figure 3. NLRP12 modulates host defense against *K. pneumoniae* infection

A. *Nlrp12*$^{-/-}$ and C57BL/6 (control) mice were inoculated i.t. with 10^3 or 10^4 CFUs of *K. pneumoniae and m*ortality was monitored up to 15 days (∗, $p < 0.05$ by log rank test). Data shown represent $n = 20$ mice/group from two representative independent experiments. **B**. Mice were infected with 1×10^3 CFUs of *K. pneumoniae* i.t. and lung and spleen homogenates were cultured at 24 h or 48 h for bacterial enumeration. Data shown represent mean parenchymal CFUs ± SE (**P* < 0.05 comparing *WT with Nlrp12*$^{-/-}$ *mice*). **C-D**. *Nlrp12*$^{-/-}$ *or* C57BL/6 mice were exposed to 1×10^3 CFUs of *K. pneumoniae* i.t. bronchoalveolar lavage fluid (BALF) was collected and total white blood cells (C), neutrophils (C), parenchymal neutrophil influx (C) or cytokine/chemokine levels (D) were enumerated 24 h and 48 h after challenge (n = 7-10/group). **E**. Mice were inoculated with 1×10^3 CFUs of *K. pneumoniae*/mouse i.t. Lungs were obtained at 48 h post-infection 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 (magnification, ×200). Semiquantitative Inflammation score is a quantification of four lung sections in each group. **F.** Mice on an A/J background were infected with 1×10^3 CFUs of *K. pneumoniae* i.t. and lung and spleen homogenates were used to enumerate bacterial burden whereas BALF was used to enumerate neutrophil influx at 48 h post-infection. Data shown represent mean ± SE (n=7-9 mice/group). **G**. Mice were i.t. infected with 1×10^3 CFU of *K. pneumoniae* and lung homogenates were used to measure caspase-1 activity at 24 and 48 h post-infection (n=4-5 mice/group). Representative blots are shown from 4-5 mice with similar results. **H**. Migration of neutrophils toward KC and MIP-2. Neutrophil numbers in the lower chamber of a transwell were enumerated after 3 h of incubation. Data are a

representation of 4 to 6 mice/group). *, $p<0.05$; **, $p<0.01$;***, $p<0.001$. Error bars represent SE.

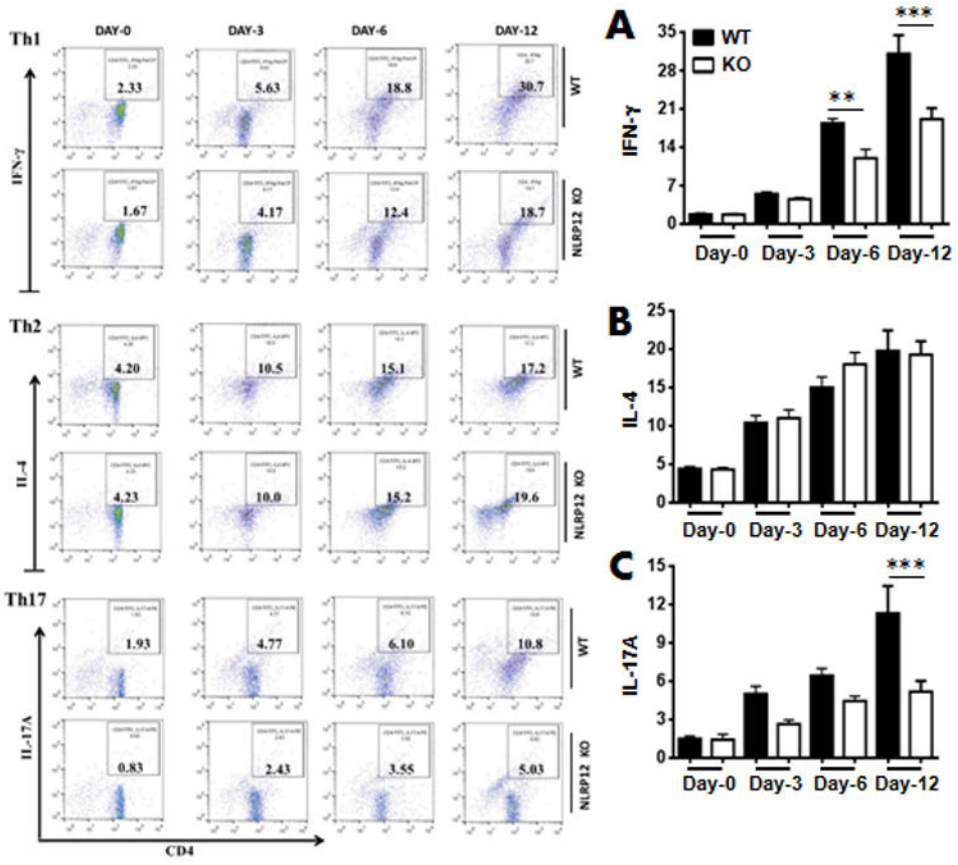

Figure 4. ***Nlrp12*$^{-/-}$** ***mice*** **display attenuated Th17/Th1 differentiation of CD4 T cell population**

A-C right panel. Naive CD4+ T cells from WT and KO mice were isolated and stimulated with PMA as described in Materials and Methods and a representative dot blot has shown. **A-C left panel.** Quantitation of IL-17A producing CD4+ T cells from 3 independent experiments (n=5-8 mice/group). *, p<0.05; **, p<0.01;***, p<0.001. Error bars represent SE.

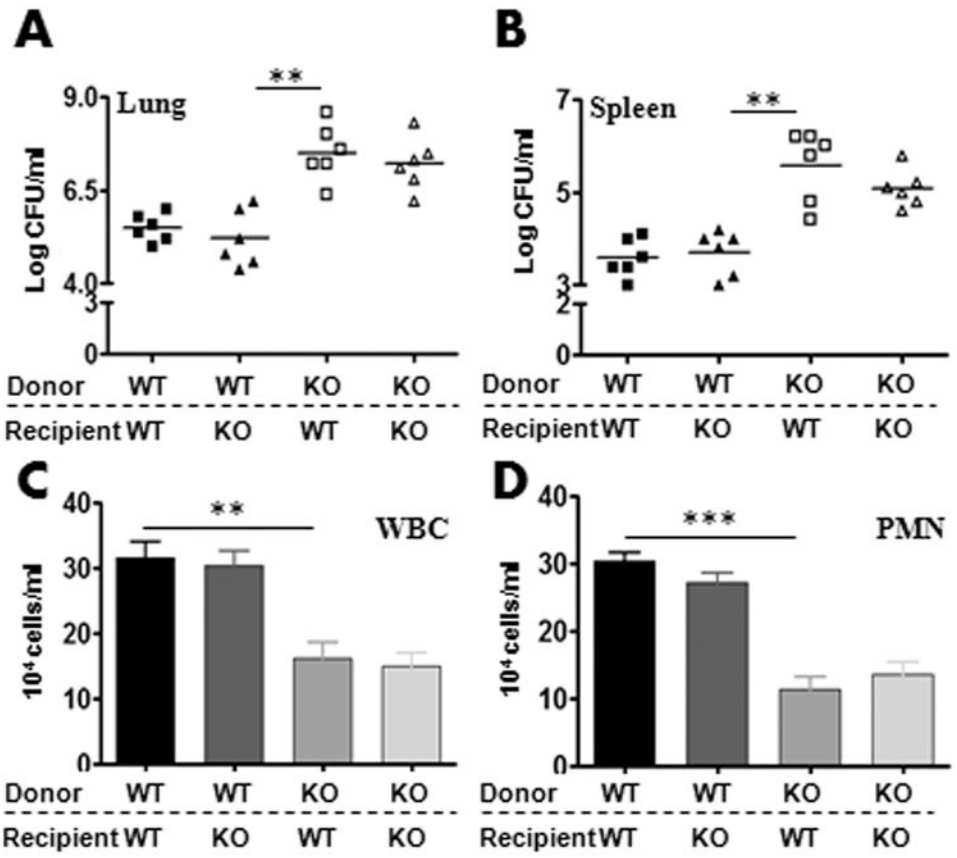

Figure 5. NLRP12-expressing bone marrow-derived cells are important to clear *K. pneumoniae* infection

A-B. Bone marrow chimeras were generated with WT and NLRP12$^{-/-}$ mice. Mice were then infected with 1 × 10^3 CFUs of *K. pneumoniae*/mouse i.t. and bacterial burden in the lungs (A) and spleens (B) were assessed at 48 h post-infection (A-B). In another set of experiments, bronchoalveolar lavage fluid (BALF) was collected and total white blood cell (C) and neutrophil numbers (D) were enumerated 48 h after the infection (n = 5-8/group). *, $p<0.05$; **, $p<0.01$;***, $p<0.001$. Error bars represent SE.

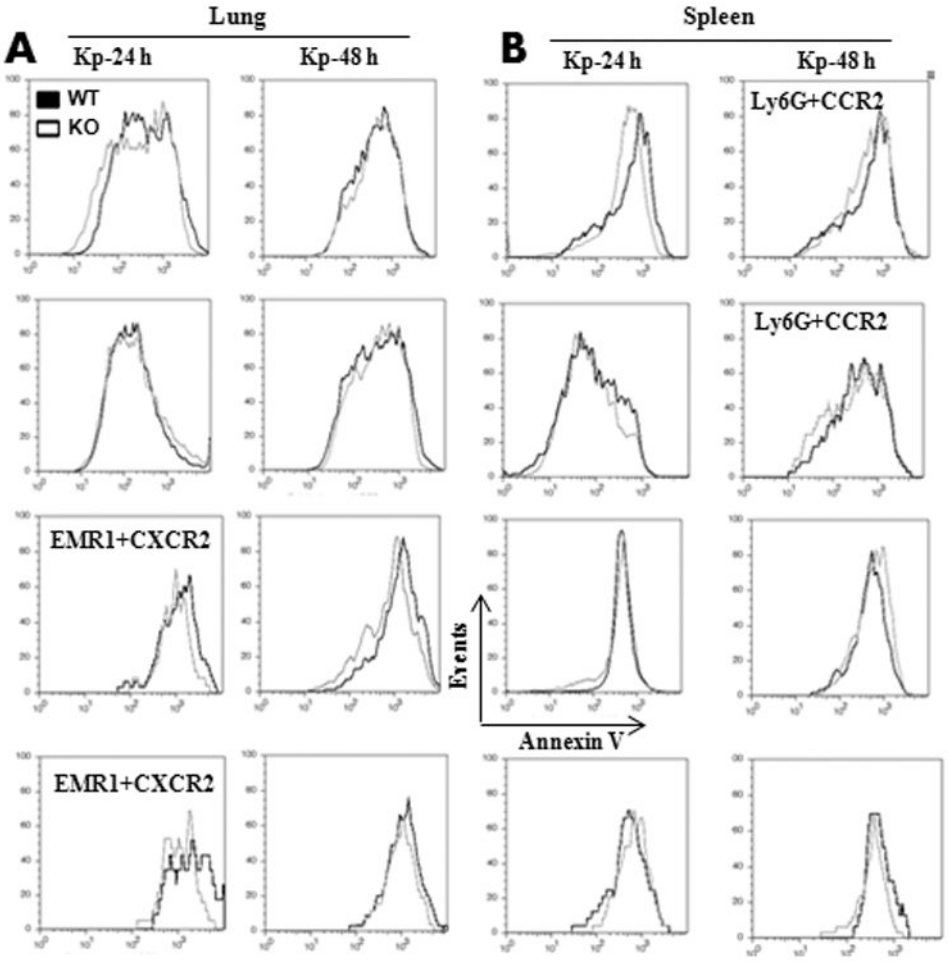

Figure 6. NLRP12 does not control pyroptosis in macrophages and neutrophils in the lung and spleen following *K. pneumoniae* infection

Flow cytometry was performed on $CCR2^+$ or $CXCR2^+$ neutrophils (Ly6G+) and macrophages (EMR1) obtained from lung and spleen homogenates at 24 and 48 h following *K. pneumoniae* infection as described in Materials and Methods. Representative plots from CCR2- or CXCR2-expressing neutrophils and macrophages from three independent experiments ($n = 5$ mice per group). Error bars represent SE.

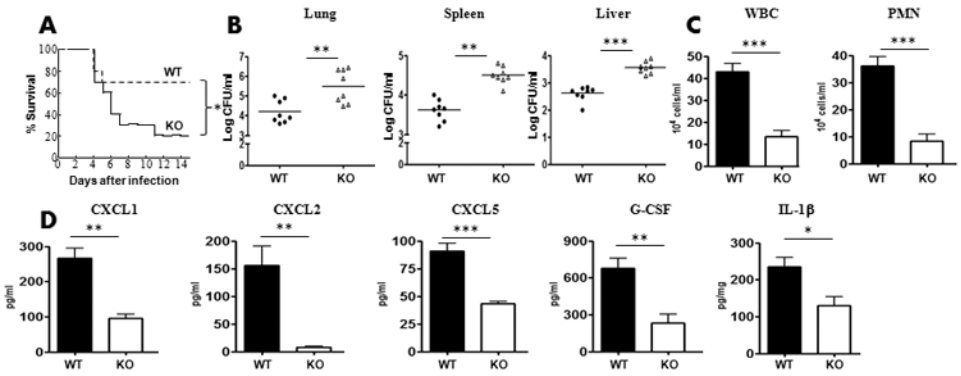

Figure 7. IL-17A producing CD4+ T cells rescues survival, bacterial clearance, cellular recruitment and cytokine expression following *K. pneumonia* infection

A-B. *Nlrp12*$^{-/-}$ mice administered with splenic CD4^{+} T cells (0.5 ×10^{6}/mouse) cells i.t. at the time of infection. Survival (A), Bacterial CFUs in the lung (B), spleen (B) and liver (B) at 48 h post-*K. pneumoniae* infection were enumerated. (n=8 mice/group). **C-D**. Leukocyte/neutrophil influx (C) and cytokine expression (D) in BALF or lung homogenates was measured at 48 h post-infection (For survival, n=20 mice/group whereas for other experiments n=6-9 mice/group). *, p<0.05; **, p<0.01;***, p<0.001. Error bars represent SE.

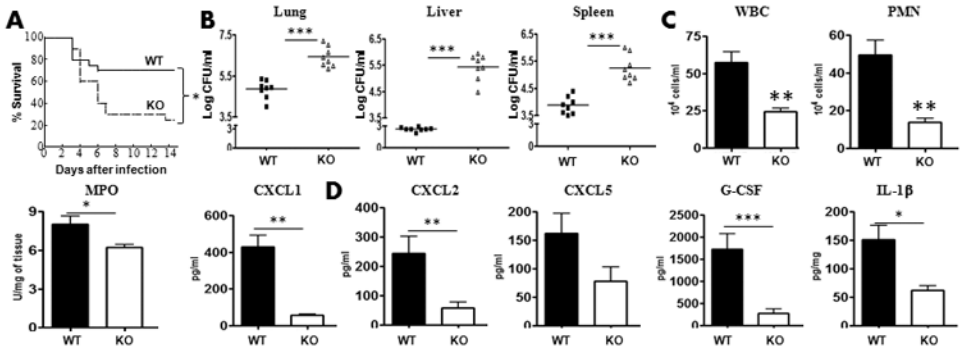

Figure 8. CXCL1 producing macrophages restores survival, bacterial clearance, leukocyte recruitment and cytokine production in response to *K. pneumoniae* infection

A-B. *Nlrp12*$^{-/-}$ mice administered with WT or KO bone marrow macrophages (0.5 ×10^{6}/ mouse) i.t. at the time of the infection. At 48 h post-*K. pneumoniae* infection, survival (A), Bacterial CFUs in the lung (B), spleen (B) and liver (B) were enumerated (n=8 mice/group). **C-D**. Leukocyte/neutrophil recruitment (C) and cytokine production (D) in BALF or lung homogenates was measured at 48 h post-infection (For survival, n=20 mice/group whereas for other experiments n=6-10 mice/group). *, p<0.05; **, p<0.01;***, p<0.001. Error bars represent SE.