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ARTICLE

NLRC4 suppresses IL-17A-mediated neutrophil-dependent host defense through upregulation of IL-18 and induction of necroptosis during Gram-positive pneumonia

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Gram-positive pathogens, including *Staphylococcus aureus*, cause necrotizing pneumonia. The central feature of *S. aureus* pneumonia is toxin-induced necroptosis of immune and resident cells, which impedes host defense. However, the role of the NLRC4 in the lung following *S. aureus* infection remains elusive. Here, we demonstrate that *S. aureus* activates the NLRC4 to drive necroptosis and IL-18 production, which impaired IL-17A-dependent neutrophil-mediated host susceptibility. In particular, *Nlrc4*$^{-/-}$ mice exhibit reduced necroptosis, enhanced neutrophil influx into the lungs, decreased bacterial burden, and improved host survival. Loss of NLRC4 signaling in both hematopoietic and non-hematopoietic cells contributes to the host protection against *S. aureus* pneumonia. Secretion of IL-17A by γδ T cells is essential for neutrophil recruitment into the lungs of *Nlrc4*$^{-/-}$ mice following infection. Moreover, treatment of wild-type mice with necroptosis inhibitors or genetic ablation of MLKL and IL-18 improves host defense against *S. aureus* infection, which is associated with increased IL-17A+γδ T cells and neutrophils. Taken together, these novel findings reveal that *S. aureus* activates the NLRC4 to dampen IL-17A-dependent neutrophil accumulation through induction of necroptosis and IL-18. Thus, modulating the function of the NLRC4 may be an attractive therapeutic approach for treating *S. aureus* infections.

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INTRODUCTION

Gram-positive pathogens, including *Staphylococcus aureus,* are the major cause of healthcare-associated pneumonia, sepsis, and mortality in post-influenza infection patients.[1,2] In particular, the emergence of methicillin-resistant *S. aureus* (MRSA) as an endemic dominant strain poses a menacing therapeutic challenge in the United States.[3] Unlike other hospital-acquired *S. aureus* strains, MRSA causes a broad spectrum of necrotizing infections of skin and soft tissues, including pneumonia, and these infections are associated with high morbidity and mortality, even in immunocompetent individuals.[3,4] *S. aureus* pneumonia is characterized by extensive inflammation and localized necrosis leading to loss of alveolar architecture, hemorrhage, and consolidation of lungs.[5] Emerging evidence suggests that pathogenesis of *S. aureus* pneumonia is largely mediated by over-activation of host inflammatory signaling cascades, although the precise mechanisms underlying this remain elusive.

Despite its antibiotic resistance, the success of *S. aureus* as a highly invasive pathogen is largely attributed to its arsenal of virulence factors, including cytolytic and pore-forming toxins.[6] All *S. aureus* strains produce exotoxins or surface proteins, which have diverse targets in multiple cell types. *S. aureus* has been shown to activate NLRP3 and NOD2, and to target CD11b and ADAM10.[7–10] Unlike Gram-negative pathogen-induced infections, mice deficient in innate immune molecules including ADAM10, NLRP3, NOD2, IFNR-1, and TNFR have improved outcomes in *S. aureus* (Gram positive) pneumonia.[10–14] In general, these gene-deficient mice show enhanced bacterial clearance, attenuated pro-inflammatory cytokine production, and decreased lung injury/pathology contributing to increased survival against pulmonary *S. aureus* infection. These observations suggest *S. aureus* may have evolved to utilize host innate immune molecules to dampen immune responses. However, the precise mechanism through which *S. aureus* manipulates the host inflammatory machinery to favor the pathogen is not clear.

Necroptosis is a recently identified, pro-inflammatory mode of cell death regulated by receptor-interacting serine-threonine kinases (RIP1/3) and executed by mixed lineage kinase domain-like protein (MLKL).[15,16] Recently, toxin-induced rapid loss of macrophages via necroptosis has been suggested as a major mechanism of lung damage in *S. aureus* pneumonia.[7,17] Similarly, alveolar macrophages exposed to pore-forming toxins from *Serratia marcescens* undergo necroptosis, which contributes to the severity of pneumonia through substantial loss of critical immune cells and extensive bystander tissue injury.[18] Genetic ablation of *Rip3* or treatment with inhibitors of necroptosis protects alveolar macrophages, which is associated with reduced bacterial burden and improved pneumonia outcomes in mice.[17,18] However, the mechanism responsible for the necroptosis-mediated depletion of immune cells that

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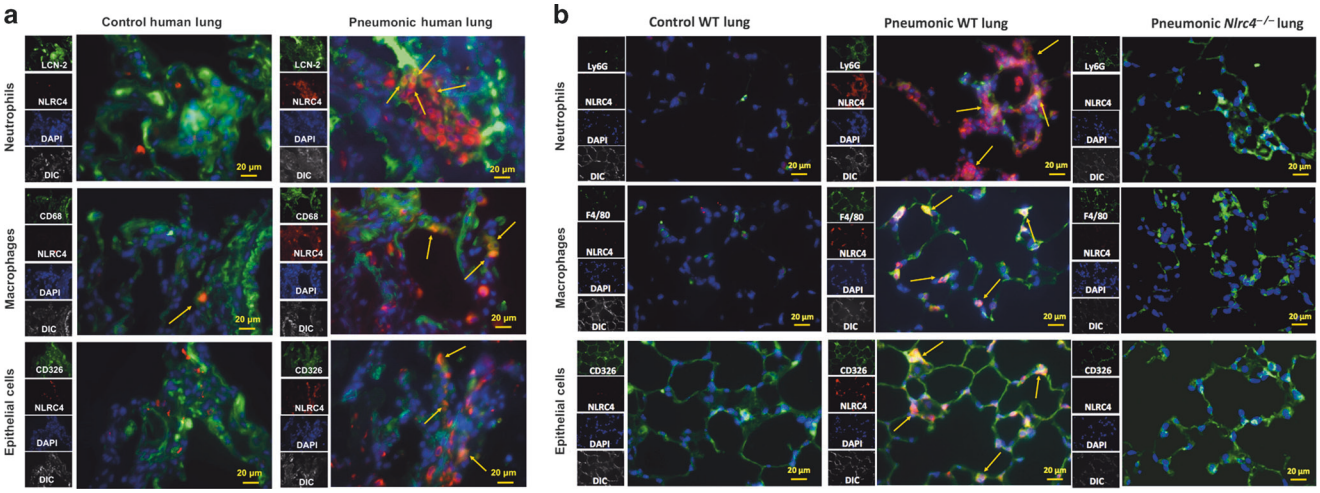

Fig. 1 NLRC4 expression is enhanced in human and murine pneumonic lungs. (**a**) NLRC4 expression was assessed using immunofluorescence in lung sections from healthy controls and from patients with clinical diagnosis of unspecified bacterial pneumonia. Red staining indicates NLRC4 expression. Neutrophils (Lipocalin-2^{+}), macrophages (CD68^{+}), and epithelial cells (CD326^{+}) were stained green. Nuclei were stained with DAPI (blue). Arrowheads (yellow) indicate co-localization of expression of NLRC4 with specific cell markers. Images are representative of five different sections with similar results. (**b**) NLRC4 expression in lung sections from control and *S. aureus*-infected WT and *Nlrc4*$^{-/-}$ mice at 24 hpi. Red staining indicates NLRC4 expression. Neutrophils (Ly6G^{+}), macrophages (F4/80^{+}), and epithelial cells (CD326^{+}) are stained green. Nuclei were stained with DAPI (blue). Arrowheads (yellow) indicate co-localization of expression of NLRC4 with specific cell markers. Images are representative of five different sections with similar results. Original magnification, ×40. DAPI, 4′,6-diamidino-2-phenylindole; LCN2, lipocalin-2; hpi, hours post infection

impedes bacterial clearance in *S. aureus* pneumonia largely remains unknown.

Regarding inflammasomes, NLRC4 belongs to the NLR family of proteins containing an N-terminal CARD domain, a central NACHT domain, and a C-terminal leucine-rich repeat domain and is involved in assembly of the inflammasome complex. It was identified as a cytosolic sensor for bacterial flagellin and type III secretion system (T3SS).[19,20] However, *Klebsiella pneumoniae*, a non-flagellated bacterium lacking T3SS, and a flagellin-deficient strain of *Pseudomonas aeruginosa* have been shown to activate the NLRC4, suggesting its activation by other bacterial or endogenous host ligands.[21,22] Nonetheless, the role of NLRC4 in bacterial pneumonia caused by a Gram-positive organism still remains elusive.[21,23] Although recent studies linked NLRP3 and ASC to *S. aureus* toxin-induced necroptosis, the precise mechanism for this has not been elucidated either.[7,18] Furthermore, *Nlrp3*$^{-/-}$ mice do not show a completely protective phenotype in *S. aureus* pneumonia, indicating both NLRP3-dependent and independent mechanisms may be important in eliciting lung damage.[13] To this end, we investigated whether the activation of the NLRC4 is important in the pathogenesis of *S. aureus* pneumonia in a mouse model. We found that NLRC4-driven necroptosis and interleukin-18 (IL-18) suppress IL-17A signaling from γδ T cells leading to attenuated neutrophil recruitment, which is necessary to eradicate *S. aureus* from the lung. Thus, these findings provide novel insight into how necroptosis and IL-18 dampens host defense in *S. aureus* pneumonia and suggests the inhibition of NLRC4 as a potential therapeutic approach for control of *S. aureus* infection in the lungs.

RESULTS

NLRC4 expression is enhanced in human and murine pneumonic lungs

To determine whether NLRC4 expression is upregulated in human pneumonic lungs, we performed immunofluorescence staining of NLRC4 in lung sections from pneumonic patients and healthy controls. As virulence factors from *S. aureus* are known to target both myeloid and non-myeloid cells,[8,10] we assessed NLRC4 expression in myeloid cells (neutrophils, macrophages) and non-myeloid cells (epithelial cells) and found increased expression of NLRC4 in Lipocalin-2^{+} neutrophils, CD68^{+} macrophages, and CD326^{+} epithelial cells (Fig. 1a). To explore if NLRC4 expression is increased during Gram-positive pneumonia in mouse lungs, we performed immunofluorescence staining in lung sections at 24 h post-*S. aureus* infection. We observed enhanced expression of NLRC4 in Ly6G^{+} neutrophils, F4/80^{+} macrophages, and CD326^{+} epithelial cells in infected wild-type (WT) mouse lungs compared to that in control lungs (Fig. 1b). Collectively, our results indicate that both myeloid and non-myeloid cells show upregulation of NLRC4 during bacterial pneumonia.

NLRC4 deficiency contributes to host protection against *S. aureus* pneumonia

To characterize the importance of NLRC4 in *S. aureus* pneumonia, WT and *Nlrc4*$^{-/-}$ mice were infected intratracheally (i.t.) with a lethal inoculum of *S. aureus* (2×10^{8} CFU/mouse) and survival was monitored up to 100 h. Compared to WT counterparts, *Nlrc4*$^{-/-}$ mice showed enhanced survival (Fig. 2a). To determine if the enhanced survival in *Nlrc4*$^{-/-}$ mice was due to increased bacterial clearance, WT and *Nlrc4*$^{-/-}$ mice were infected i.t. with a sub-lethal inoculum of *S. aureus* (5×10^{7} CFU/mouse). Compared to WT mice at 12 and 24 h post infection (hpi), *Nlrc4*$^{-/-}$ mice showed a diminished bacterial burden in the lung and bronchoalveolar lavage fluid (BALF) along with decreased dissemination in the spleen (Fig. 2b–d). We next determined whether the enhanced bacterial clearance was due to augmented neutrophil accumulation in the lungs. Our results revealed that *Nlrc4*$^{-/-}$mice had increased numbers of total white blood cells, neutrophil, and macrophage recruited to the lungs (Fig. 2e–g). Furthermore, unlike *Nlrc4*$^{-/-}$ mice, WT mice had increased protein leakage, an indicator of lung permeability (Fig. 2h). To assess if the detrimental role of NLRC4 activation is MRSA strain-specific, we infected WT and *Nlrc4*$^{-/-}$ mice with a methicillin-susceptible *S. aureus* (MSSA) Newman strain. The protective phenotype seen in *Nlrc4*$^{-/-}$ mice infected with *S. aureus* was also seen in pneumonia caused by the MSSA strain (Fig. 2i, j).

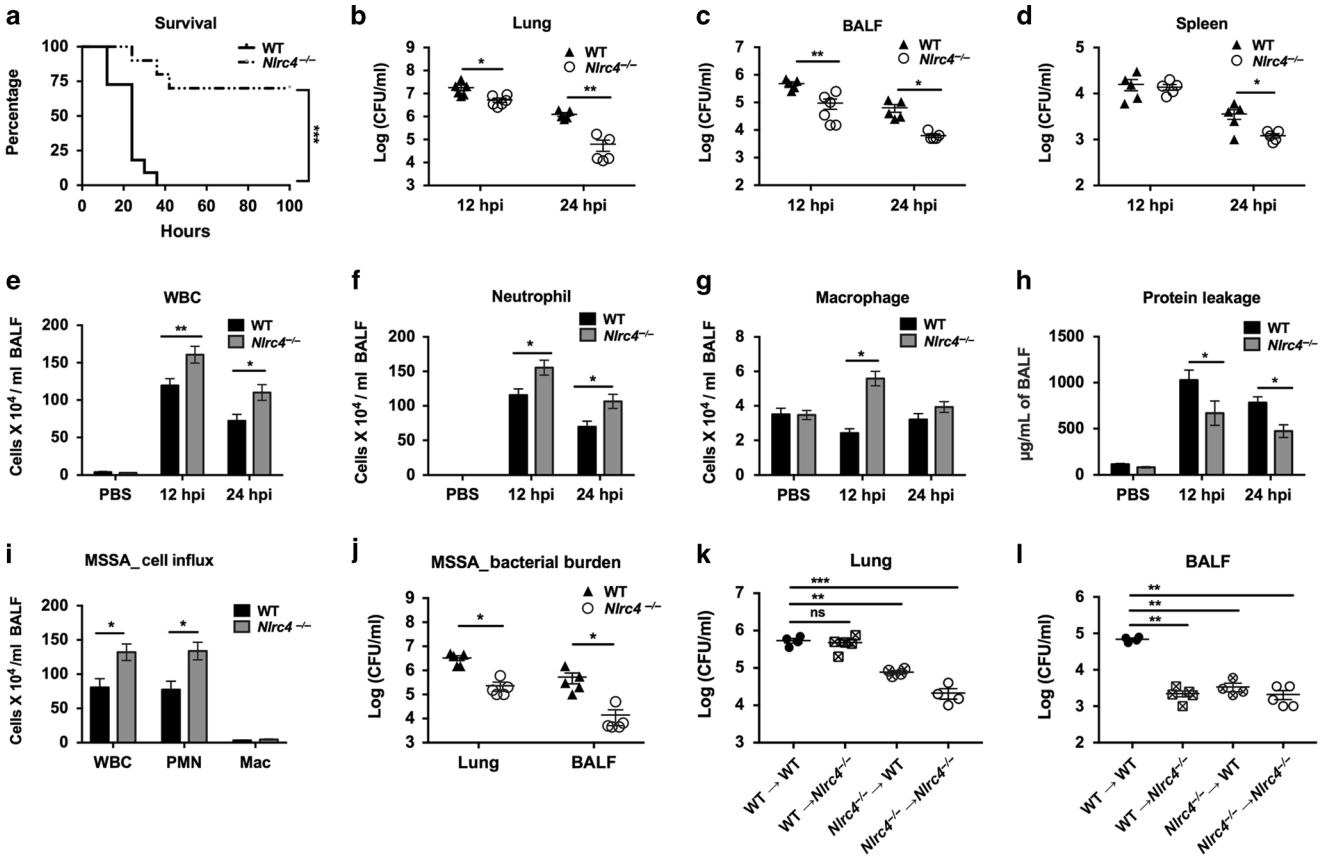

Fig. 2 NLRC4 deficiency confers host resistance against *S. aureus* pneumonia. (**a**) WT (control) and *Nlrc4*$^{-/-}$ mice were inoculated intratracheally with 2×10^8 CFU of *S. aureus* and survival was monitored up to 100 h. A Kaplan–Meier plot is used to show survival of mice from each group. ($n = 10$ mice/group). (**b–h**) WT and *Nlrc4*$^{-/-}$ mice were inoculated intratracheally with 5×10^7 CFU of *S. aureus* or PBS (control). BALF and organs were harvested at the designated time points. Control mice (PBS treated) were sacrificed at 24 hpi. The bacterial burden in the (**b**) lungs, (**c**) BALF, and (**d**) spleen was quantitated at 12 and 24 hpi.. Number of (**e**) total white blood cells, (**f**) neutrophils, (**g**) macrophages, and (**h**) level of total protein were quantified in BALF at 12 and 24 hpi ($n = 5$–6 mice/pneumonia group, $n = 3$ mice/control group). (**i, j**) WT and *Nlrc4*$^{-/-}$ mice were inoculated intratracheally with 5×10^7 CFU of *S. aureus Newman strain* (MSSA). (**i**) Cellular influx in BALF and (**j**) bacterial burden in lungs and BALF were determined at 24 hpi. (**k, l**) Bone marrow reconstituted mice were inoculated intratracheally with 5×10^7 CFU of *S. aureus*. The bacterial burden in (**k**) lung and (**l**) BALF was quantitated at 24 hpi. Each symbol represents a single mouse (**b–d, j–l**). Data from a representative experiment are shown. All experiments were performed three times, but survival and chimera study were performed twice. Statistical significance was determined by log-rank (**a**), Mann–Whitney (**b–d, j**), unpaired *t*-test (**e–i**), one-way ANOVA (followed by Bonferroni's post hoc comparisons) (**k, l**). $^*p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$. BALF, bronchoalveolar lavage fluid; CFU, colony-forming unit; WBC, white blood cell; nd, non-detectable

S. aureus targets both myeloid and non-myeloid cells.[7,10,11] In Fig. 1a, b, we demonstrate increased expression of NLRC4 in both hematopoietic (myeloid cells) and non-hematopoietic (epithelial cells) cells in human and murine pneumonic lungs. Therefore, we next investigated the contribution of hematopoietic versus non-hematopoietic cells to host protection using bone marrow chimeras. In this context, lethally irradiated WT or *Nlrc4*$^{-/-}$ mice were reconstituted with RBC-lysed bone marrow cells (4×10^6/mouse) from donor WT or *Nlrc4*$^{-/-}$ mice. Two months after reconstitution, chimeric mice were infected with *S. aureus*. Interestingly, the *Nlrc4*$^{-/-}$ → *Nlrc4*$^{-/-}$, *Nlrc4*$^{-/-}$ → WT but not WT → *Nlrc4*$^{-/-}$ chimeric groups had decreased bacterial burden in lungs as compared with the WT → WT group (Fig. 2k). However, the *Nlrc4*$^{-/-}$ → *Nlrc4*$^{-/-}$, *Nlrc4*$^{-/-}$ → WT, and WT → *Nlrc4*$^{-/-}$groups had reduced bacterial burden in BALF as compared to the WT → WT group (Fig. 2i). Taken together, our results suggest NLRC4 activation, in both hematopoietic and non-hematopoietic compartments, is detrimental, and their deficiency in mice displays robust protective phenotype in *S. aureus* pneumonia.

S. aureus activates NLRC4 through PKC-δ to induce IL-1β, IL-18, and necroptosis

S. aureus α-hemolysin has been shown to activate NLPR3 in human and mouse monocytes.[7] Along the same line, to examine the virulence factors associated with NLRC4 activation, we used an isogenic α-hemolysin-deficient *S. aureus* mutant strain, in human and murine cells. Direct evidence of increased NLRC4 expression in humans was obtained using lung lysates from pneumonic patients and comparing them to lysates from healthy individuals (Supplementary Figure 1a). In a similar manner, *S. aureus* activated NLRC4 in both human monocyte-like (THP-1) and neutrophil-like (HL-60) cells (Supplementary Figure 1a). To further characterize the role of NLRC4 in response to *S. aureus* infection, we used THP-1 and THP1-NLRC4 cells (stably overexpressing NLRC4). Compared to THP-1 cells, THP1-NLRC4 produced greater amounts of IL-1β and IL-18 in response to *S. aureus* (Fig. 3a, b). In addition, THP1-NLRC4 cells showed increased caspase-1 activity, as evidenced by their marked staining with the fluorescently labeled inhibitor of caspases reagent (Fig. 3c and Supplementary Figure 1b). We also observed increased expression and phosphorylation of NLRC4 in a

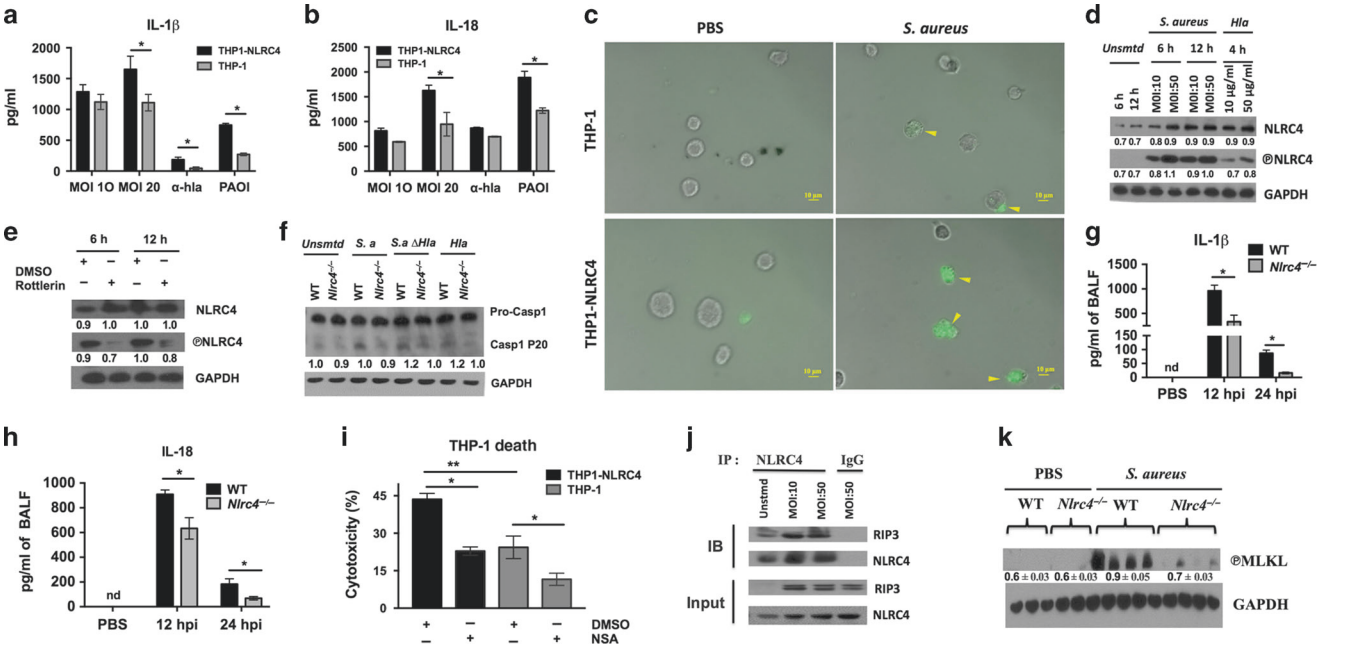

Fig. 3 *S. aureus* activates NLRC4 through PKC-δ to induce IL-1β, IL-18, and necroptosis. Levels of (**a**) IL-1β and (**b**) IL-18 in the supernatant of THP-1 and THP1-NLRC4 cells incubated with *S. aureus* (MOI 10 or 20), purified α-hemolysin (25 μg/ml), or *Pseudomonas aeruginosa* PAO1 (MOI 10) for 8 h. (**c**) Active FLICA-caspase-1 (green) was detected in THP-1 and THP1-NLRC4 cells infected with *S. aureus* (MOI 50) or PBS for 6 h. Original magnification, ×40. (**d**) Immunoblot analysis of total NLRC4 and phospho-NLRC4 in cell lysates from WT-BMDMs infected with *S. aureus* (MOI 10 or 50) for 6 or 12 h or stimulated with commercially purified α-hemolysin (10 or 50 μg/ml) for 4 h. (**e**) Immunoblot analysis of total NLRC4 and phospho-NLRC4 in cell lysates from WT-BMDMs infected with *S. aureus* (MOI 10) for 6 or 12 h with or without 1-h pre-treatment with 10 μM Rottlerin (PKC-δ inhibitor). (**f**) Immunoblot analysis of pro-caspase-1 and cleaved caspase-1 (p-20) in cell lysates from WT and *Nlrc4*$^{-/-}$-BMDMs infected with MOI 10 of *S. aureus* or its isogenic α-hemolysin-deficient mutant or stimulated with purified α-hemolysin (50 μg/ml) for 6 h. (**g**, **h**) WT and *Nlrc4*$^{-/-}$ mice were inoculated with *S. aureus* (5×10^7 CFU/mouse). Level of (**g**) IL-1β and (**h**) IL-18 measured BALF collected at 12 and 24 hpi ($n = 4$–6 mice/pneumonia group, $n = 3$ mice/control group). (**i**) Cytotoxicity in THP-1 and THP1-NLRC4 cells stimulated with *S. aureus* (MOI 100) for 2 h with or without 1 h pre-treatment with 100 μM of necrosulfonamide (NSA). (**j**) BMDMs from WT mice were left untreated or were incubated with *S. aureus* (MOI 10 or 50) for 6 h. Cell lysates were immunoprecipitated with ant-NLRC4 Ab or IgG, and then immunoblotted for RIP3 and NLRC4. Whole-cell lysates are shown as the input. (**k**) Immunoblot analysis of phospho-MLKL in the lung homogenates of WT and *Nlrc4*$^{-/-}$ mice infected with *S. aureus* (5×10^7 CFU/mouse). Data from a representative experiment are shown. All experiments were performed three times. In vitro experiments have at least four biological replicates. Numeric values in immunoblot indicate ratio of mean gray scale value of protein of interest to its GAPDH control. Statistical significance was determined by unpaired *t*-test in all experiment except in cell death assay (**i**), by one-way ANOVA (followed by Bonferroni's post hoc comparisons). $^*p < 0.05$; $^{**}p < 0.01$. *Hla*, α-hemolysin; *ΔHla*, α-hemolysin-deficient; MOI, multiplicity of infection; DMSO, dimethyl sulfoxide; IP, immunoprecipitation; IB, immunoblotting

dose- and time-dependent manner with *S. aureus* and purified α-hemolysin in bone marrow-derived macrophages (BMDMs) from WT mice (Fig. 3d and Supplementary Figure 1c). Recently, protein kinase C-δ (PKC-δ) mediated phosphorylation of NLRC4 was shown to be critical for inflammasome activation.[24] As anticipated, *S. aureus*-induced NLRC4 phosphorylation was dependent on PKC-δ in macrophages, as Rottlerin (PKC-δ inhibitor) reduced expression of phospho-NLRC4 (Fig. 3e). We also tested whether *S. aureus*-induced NLRC4 activation leads to caspase-1 processing and IL-1β production, key features of inflammasome activation. To this end, we stimulated BMDMs from WT and *Nlrc4*$^{-/-}$ mice with *S. aureus*, its isogenic α- hemolysin-deficient mutant, or purified α-hemolysin toxin. As expected, the cleavage of pro-caspase-1 to its active (phospho-20) form was reduced in *Nlrc4*$^{-/-}$ BMDMs, which was independent of the expression of α- hemolysin by *S. aureus* (Fig. 3f). This observation indicates *S. aureus* may expresses virulence factors other than α-hemolysin that may serve as activators of NLRC4-dependent caspase-1 processing. In addition, NLRC4 deficiency modestly reduced IL-1*β* production in *S. aureus*-infected BMDMs, so as the pre-treatment with Rottlerin (Supplementary Figure 1d, e). Furthermore, *Nlrc4*$^{-/-}$ mice had significant reduction in key inflammasome-dependent cytokines, such as IL-1β and IL-18 (Fig. 3g, h).

S. aureus has been shown to induce necroptosis in lungs.[17] Therefore, in order to assess the role of NLRC4 in context of necroptosis, we performed cytotoxicity assay in THP-1 and BMDM following *S. aureus* infection. We detected increased cytotoxicity of THP1-NLRC4 and WT-BMDM compared to THP-1 and *Nlrc4*$^{-/-}$-BMDMs (Fig. 3i and Supplementary Figure 1f), providing direct evidence of the involvement of NLRC4 in *S. aureus*-induced cell death. In addition, pre-treatment with necrostatin-1 (Nec-1: RIP1 inhibitor) or necrosulfonamide (MLKL inhibitor) protected THP1-NLRC4 and WT-BMDM from *S. aureus*-induced necroptosis respectively (Fig. 3i and Supplementary Figure 1f). Furthermore, we observed increased co-localization of RIP3 and phospho-MLKL in *S. aureus*-infected WT-BMDMs compared to *Nlrc4*$^{-/-}$-BMDMs (Supplementary Figures 1g, h) as well as the co-immunoprecipitation of RIP3 and NLRC4, indicating their potential interaction in the necrosome complex following *S. aureus* infection (Fig. 3j). Given that MLKL pore formation is a critical step in necroptosis, we sought to detect the level of phospho-MLKL in *S. aureus*-infected lungs. Corroborating our in vitro results, *Nlrc4*$^{-/-}$ mice show reduced MLKL phosphorylation in their lungs compared to WT controls (Fig. 3k). In a similar manner, WT mice displayed enhanced LDH release and secretion of necroptosis-related alarmins, such as HMGB-1 and IL-1 α, in BALF at 12 and 24 hpi (Supplementary Figures 1i–k). Taken together, our results indicate that *S. aureus* phosphorylates NLRC4 in PKC-δ-dependent manner and activation of which leads to production of IL-1β, IL-18, and necroptotic cell death.

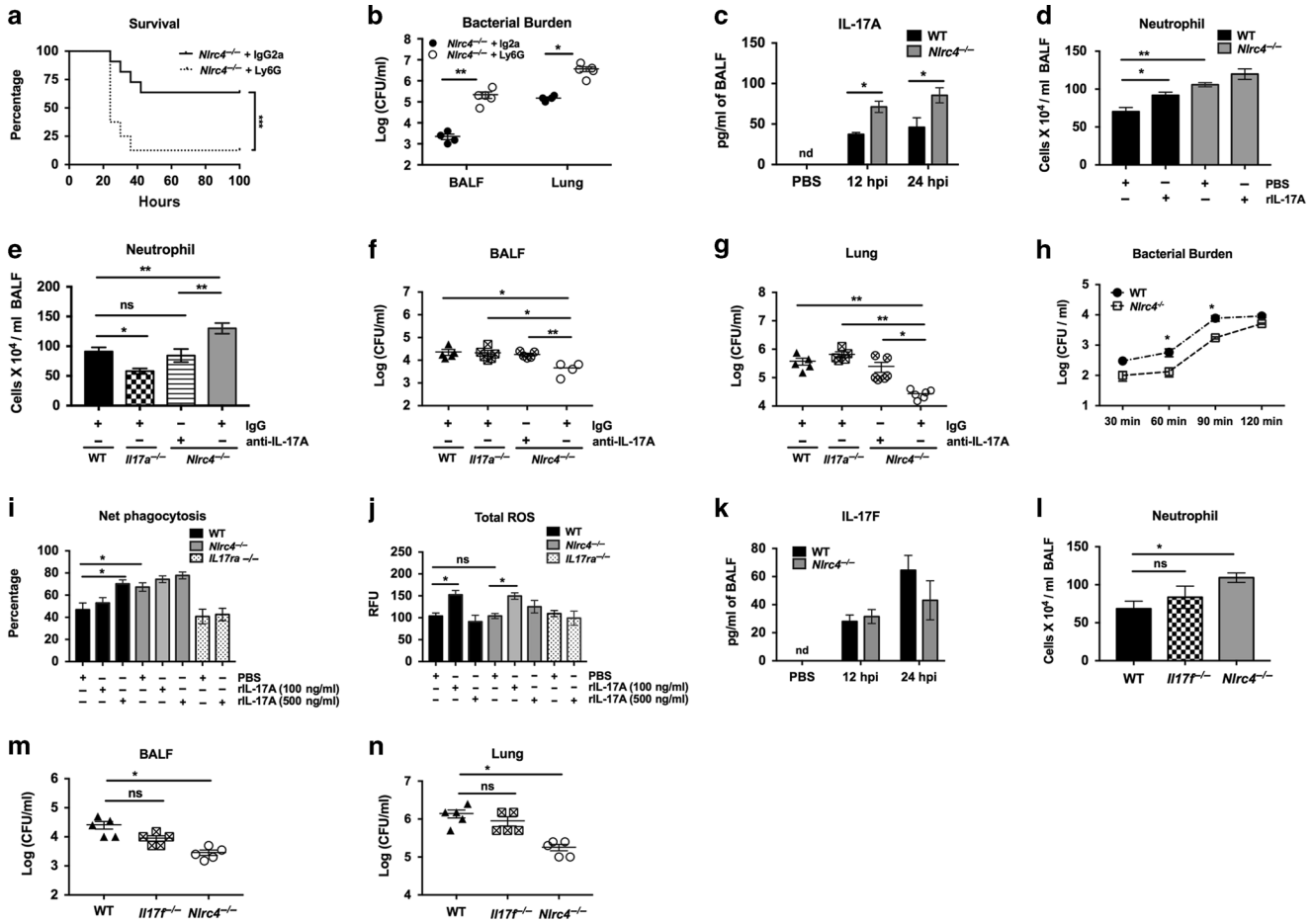

Fig. 4 NLRC4 deficiency promotes IL-17A-mediated neutrophil recruitment and bacterial clearance. (**a**, **b**) *Nlrc4*$^{-/-}$ mice were injected with anti-Ly6G (IA8) Ab intraperitoneally at 24 and 2 h prior to intratracheal infection with 2×10^8 CFU for survival and 5×10^7 CFU for pneumonia. (**a**) A Kaplan–Meier plot is used to show survival of mice from each group. ($n = 10$ mice/group). (**b**) Bacterial burden was determined at 24 hpi. Each symbol represents a single mouse. (**c**,**d**) WT and *Nlrc4*$^{-/-}$ mice were inoculated intratracheally with 5×10^7 CFU of *S. aureus* or PBS followed by administration of recombinant murine IL-17A or PBS 1 h later. (**c**) Level of IL-17A at 12 and 24 hpi and (**d**) number of neutrophils at 24 hpi in the BALF were enumerated. (**e–g**) WT, *Il-17a*$^{-/-}$ and *Nlrc4*$^{-/-}$ mice were inoculated intratracheally with 5×10^7 CFU of *S. aureus* or PBS. *Nlrc4*$^{-/-}$ mice received a single dose of anti-IL17A Ab or IgG at 12 h prior to infection. (**e**) Number of neutrophils and (**f**) bacterial burden in BALF and (**g**) lungs were determined at 24 hpi. Each symbol represents a single mouse ($n = 4$–6 mice/pneumonia group, $n = 3$ mice/control group). (**h–j**) BMDNs were isolated from WT, *Nlrc4*$^{-/-}$, and *IL-17ra*$^{-/-}$ mice and stimulated with *S. aureus* (MOI 1) in the presence or absence of recombinant murine IL-17A (100 or 500 ng/ml) or PBS. (**h**) Intracellular killing of neutrophils was determined at indicated time points by assessing the intracellular bacterial burden as described in Supplementary methods. (**i**) Net phagocytosis and (**j**) total ROS production at 1 hpi was determined as described in Supplementary Methods. (**k–n**) Wild type, *Il-17f*$^{-/-}$, and *Nlrc4*$^{-/-}$ mice were inoculated intratracheally with 5×10^7 CFU of *S. aureus*. BALF and lungs were collected at 12 and 24 hpi. (**k**) Level of IL-17F, (**l**) number of neutrophils in BALF, and bacterial burden in (**m**) BALF and (**n**) lung were determined at 24 hpi. Each symbol represents a single mouse ($n = 5$ mice/pneumonia group, $n = 3$ mice/control group). Data from a representative experiment are shown. All experiments were performed three times. In vitro experiments have at least four biological replicates. Statistical significance was determined by log-rank (**a**), Man–Whittney (**b**, **h**), unpaired *t*-test (**c**, **k**), and one-way ANOVA (followed by Bonferroni's *post hoc* comparisons) (**d–g**, **i**, **j**, **l–n**). *$p < 0.05$; **$p < 0.01$. IgG, immunoglobulin G; ROS, reactive oxygen species

NLRC4 deficiency promotes IL-17A-mediated neutrophil recruitment and bacterial clearance

Neutrophil recruitment is a critical event for bacterial clearance during *S. aureus* infection.[25] *Nlrc4*$^{-/-}$ mice displayed enhanced neutrophil recruitment (Fig. 2f). Furthermore, neutrophil depletion with anti-Ly6G antibody (IA8) increased the mortality and bacterial burden in *Nlrc4*$^{-/-}$mice (Fig. 4a, b). However, lower levels of pro-inflammatory cytokines in BALF of *Nlrc4*$^{-/-}$ mice did not explain the higher neutrophil counts, indicating these cytokines (TNF-α, MCP-1, Il-6) may not be associated with neutrophil recruitment in *S. aureus* infection (Supplementary Figures S2a–c). It should be noted that IL-17A has been shown to be critical for neutrophil influx and bacterial clearance at the site of *S. aureus* infection.[26] Thus, we measured the level of IL-17A in BALF and found that IL-17A was increased in *Nlrc4*$^{-/-}$ mice at 12 and 24 hpi (Fig. 4c). To further implicate IL-17A-mediated neutrophil recruitment in *Nlrc4*$^{-/-}$ mice, administration of recombinant IL-17A in WT mice one-hpi increased neutrophil influx to a level comparable to *Nlrc4*$^{-/-}$ mice (Fig. 4d). Further, ablation of *Il17a* or blockade with anti-IL-17A antibody in *Nlrc4*$^{-/-}$ mice abrogated neutrophil influx, which was associated with an increased bacterial burden in BALF and lungs (Fig. 4e–g). Since IL-17A is not directly involved in neutrophil recruitment, we explored whether enhanced recruitment may be due to increased level of CXC chemokines and neutrophil mobilization through IL-17A/G-CSF axis. Accordingly, *Nlrc4*$^{-/-}$ mice also showed increased production of CXCL5, but not CXCL1 and CXCL2, at 12 hpi and higher trend of G-CSF at both 12 and 24 hpi (Supplementary Figures S2d–g). To examine if NLRC4 deficiency also alters the phagocytic function of neutrophils, we utilized a phagocytosis

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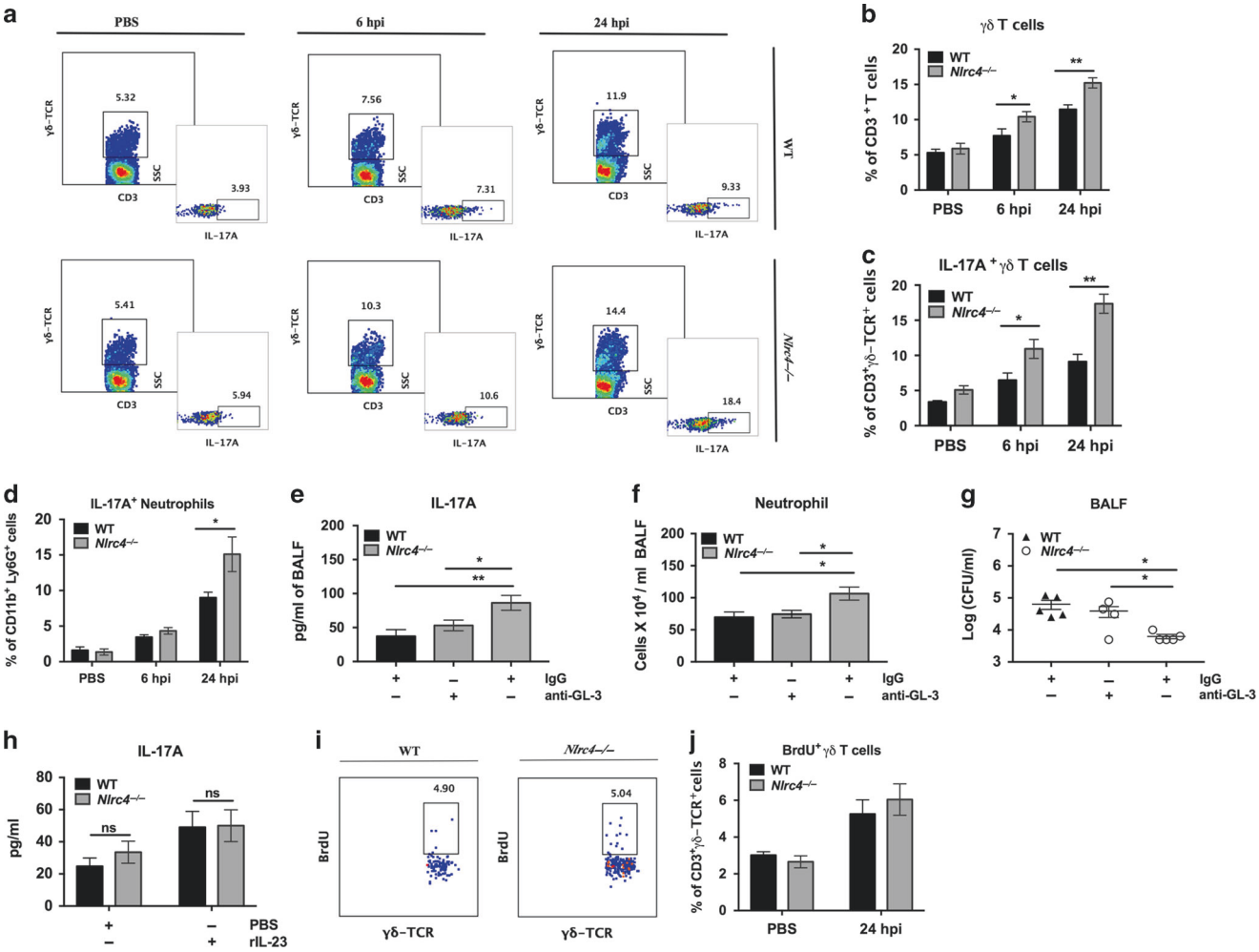

Fig. 5 NLRC4 deficiency augments IL-17A$^+$γδ T cells and neutrophils to regulate neutrophil influx. (**a–d**) Flow cytometric analysis of the lungs from WT and *Nlrc4*$^{-/-}$ mice either uninfected or intratracheally infected with 5×10^7 CFU of *S. aureus*. (**a**) FACS plots of CD3$^+$ γδ-TCR$^+$ cells (larger) and IL-17A$^+$ CD3$^+$ γδ-TCR$^+$ cells (smaller) at 6 and 24 hpi. Mice receiving PBS were sacrificed at 24-h time points. (**b**) The percentage of total γδ T cells, (**c**) IL-17A-producing γδ T cells, and (**d**) IL-17A producing CD11b$^+$ Ly6G-1$^+$ neutrophils enumerated by flow cytometry in lungs at 6 and 24 hpi ($n = 5$ mice/pneumonia group, $n = 3$ mice/control group). (**e–g**) WT and *Nlrc4*$^{-/-}$ mice were inoculated intratracheally with 5×10^7 CFU of *S. aureus*. *Nlrc4*$^{-/-}$ mice received an intraperitoneal injection of anti-γδ-TCR ab (GL-3) or IgG at 12 h prior to infection. (**e**) Level of IL-17A, (**f**) number of neutrophils, and (**g**) bacterial burden in BALF was determined at 24 hpi ($n = 5$ mice/group). (**h**) The CD3$^+$ γδ-TCR$^+$ cells were sorted from the spleen of the uninfected wild-type and *Nlrc4*$^{-/-}$ mice and incubated with *S. aureus* (MOI 10) for 18 h in the presence or absence of IL-23 (40 ng/ml). (**h**) IL-17A was measured in the supernatant. (**i, j**) WT and *Nlrc4*$^{-/-}$ mice were inoculated intratracheally with 5×10^7 CFU of *S. aureus* or PBS. These mice received a single injection of BrdU (1 mg/mouse) intraperitoneally 1-h prior to infection. (**i**) FACS plot showing BrdU$^+$ CD3$^+$ γδ-TCR$^+$ cells and (**j**) their percentage in the lungs of mice 18 hpi ($n = 5$ mice/pneumonia group, $n = 3$ mice/control group). Data from a representative experiment are shown. All experiments were performed three times. In vitro experiments have at least four biological replicates. Statistical significance was determined by unpaired *t*-test (**b–d**) and one-way ANOVA (followed by Bonferroni's post hoc comparisons) (**e–g**) $^*p < 0.05$; $^{**}p < 0.01$. BrdU, bromodeoxyuridine

assay in bone marrow-derived neutrophils (BMDNs). To our surprise, WT-BMDNs show higher intracellular bacterial survival than *Nlrc4*$^{-/-}$- BMDNs following *S. aureus* infection (Fig. 4h). Further, *Nlrc4*$^{-/-}$- BMDNs were also effective at particle uptake (Fig. 4i), suggesting NLRC4 deficiency improved both uptake and clearance in BMDN. Furthermore, the defect in uptake/phagocytosis of WT-BMDN was rescued by pre-treatment with IL-17A, which was associated with increased production of intracellular reactive oxygen species (Fig. 4i, j). Unlike in BMDNs, NLRC4 was dispensable for the phagocytic function of BMDMs (Supplementary Figure S3a). Moreover, deficiency of IL-17F, another IL-17 homodimeric cytokine complex that binds to the same receptors (IL-17RA or IL-17RC),[27] did not alter neutrophil recruitment or bacterial clearance in *S. aureus* pneumonia (Fig. 4k–n). Collectively, our data indicate that the increased level of IL-17A drives the influx and improves the phagocytic abilities of recruited neutrophil in *Nlrc4*$^{-/-}$ mice.

NLRC4 deficiency augments IL-17A$^+$γδ T cells and neutrophils to regulate neutrophil influx

Although IL-17A is known as a signature cytokine for CD4$^+$ T cells (Th17 subsets), emerging evidence has revealed that multiple cell types, such as γδ T cells, NK/NKT cells, neutrophils, innate lymphoid cells, CD8$^+$ T cells, and colonic epithelial cells, also secrete IL-17A.[27] To investigate the primary source of IL-17A during the early stage of infection, we utilized intracellular flow cytometry on lung single cell suspensions at 6 and 24 hpi. There was an increase in total and IL-17A producing CD3$^+$ γδ-TCR$^+$ cells in the *Nlrc4* $^{-/-}$ mice at 6 and 24 hpi (Fig. 5a–c). While neutrophils (CD11b$^+$ Ly6G$^+$), CD4$^+$ T cells, CD8$^+$ T cells, and NK1.1$^+$ cells also

produce IL-17A during *S. aureus* infection, but NLRC4 deficiency did not alter their number in our model (Supplementary Figures S4a–d). However, IL-17A-producing neutrophils were higher at 24 hpi in *Nlrc4*$^{-/-}$ mice, indicating recruited neutrophils help sustain IL-17A levels and recruit more inflammatory cells (Fig. 5d and Supplementary Figure 4a). Human neutrophils activated with *S. aureus* also produced IL-17A (Supplementary Figure 4e). To further implicate γδ T cells in IL-17A-mediated antimicrobial signaling in *Nlrc4*$^{-/-}$ mice, we depleted γδ T cells using an anti-γδ-TCR antibody (GL-3). Administration of the GL-3 antibody reduced the level of IL-17A in *Nlrc4*$^{-/-}$ mice to a level comparable with WT mice receiving isotype-matched IgG control Ab (Fig. 5e). Furthermore, depletion of γδ T cells impaired host defense in *Nlrc4*$^{-/-}$ mice, as there was decreased neutrophil influx and increased bacterial burden in the BALF of these mice at 24 hpi (Fig. 5f, g). We next asked if genetic deficiency of NLRC4 alters the production of IL-17A from γδ T cells in addition to their recruitment to the lungs. To this end, we FACS sorted CD3^{+} γδ-TCR^{+} cells from spleens of naïve mice and stimulated them with *S. aureus* in the presence or absence of IL-23 in vitro. Surprisingly, the γδ T cells from WT and *Nlrc4*$^{-/-}$ mice did not show altered production of IL-17A following IL-23 stimulation (Fig. 5h). To examine the possibility of increased γδ T cell proliferation in lungs of *Nlrc4*$^{-/-}$ mice, we injected BrdU intraperitoneally before *S. aureus* infection and stained for BrdU-positive γδ T cells. We found that NLRC4 deficiency did not regulate the proliferation of this cell type in the lungs (Fig. 5i, j). Thus, these data indicate increased frequency of γδ T cells from early stage and neutrophil at late stage in *Nlrc4*$^{-/-}$ mice produce IL-17A that is critical of neutrophil recruitment to the lungs in *S. aureus* pneumonia.

NLRC4-driven necroptosis and IL-18 suppress IL-17A-dependent neutrophil recruitment by limiting γδ T cell expansion

We showed that *S. aureus*-induced NLRC4 activation results in the production of IL-1β, IL-18, and necroptosis in macrophages and lungs (Fig. 3 and Supplementary Fig 1). Necroptosis-mediated loss of immune cells in WT mice, but not in the *Rip3*$^{-/-}$ mice, has been shown to worsen the outcome of *S. aureus* pneumonia.[17] Here, we sought to elucidate whether NLRC4-coupled necroptosis suppress IL-17A-mediated antibacterial defense in pneumonia. To confirm necroptosis in vivo, we administered Nec-1 intraperitoneally to block RIP1 and found that it inhibited LDH release in BALF of WT mice at 24 hpi (Fig. 6a). To identify if necroptosis alters γδ T cell-derived IL-17A signaling axis, we enumerated percentage of total and IL-17A producing γδ T cells in the lungs of *S.* aureus-infected *Mlkl*$^{-/-}$ mice. As anticipated, *Mlkl*$^{-/-}$ mice had a higher percentage of IL-17A^{+} γδ T cells, but not total γδ T cells, at 24 hpi (Fig. 6b, c, and Supplementary Figure S5a). Furthermore, the genetic ablation or blockade of MLKL with GW806742X impaired IL-17A production, neutrophil recruitment, and bacterial clearance from BALF and lungs of *Nlrc4*$^{-/-}$ mice (Fig. 6d–g). Previous reports have shown that NLRC4 processed IL-18 is deleterious in *P. aeruginosa*-induced pneumonia.[23,28] In this regard, mice lacking expression of *Il18* had increased total and IL-17A-producing γδ T cells compared to WT controls, with numbers comparable to those of *Nlrc4*$^{-/-}$ mice (Fig. 6h, i, and Supplementary Figure 5b). Similarly, administration of recombinant IL-18 in *Nlrc4*$^{-/-}$ mice decreased both total and IL-17A producing γδ T cells in the lungs at 24 hpi (Fig. 6h, i, and Supplementary Figure 5b). However, deficiency of IL-1β, a key NLRC4-dependent cytokine, did not alter the frequency of γδ T cells in *S. aureus* pneumonia (Fig. 6j, k, and Supplementary Figure 5c). Intriguingly, IL-1β$^{-/-}$ mice did augment host defense with enhanced neutrophil recruitment and bacterial clearance, similar to *Nlrc4*$^{-/-}$ and *IL-18*$^{-/-}$ (Figures 8l, m). Taken together, our results indicate that NLRC4-mediated necroptosis and IL-18 act synergistically, independent of IL-1β, to suppress the recruitment of γδ T cells, which dampens IL-17A-mediated antibacterial defense in *S. aureus* pneumonia.

Blockade of NLRC4 signaling or necroptosis improves host defense in *S. aureus* pneumonia

Like other inflammasomes, activation of NLRC4 results in cleavage of caspase-1 leading to production of IL-1β and IL-18 during *S. aureus* infection (Fig. 3). To determine if deficiency of caspase-1 (downstream of NLRC4) results in a similar outcome in *S. aureus* pneumonia, mice lacking expression of *Casp1/11* or pre-treated with Ac-yvad-cmk (caspase-1 inhibitor) were infected with *S. aureus*. As compared to WT controls, mice lacking *Casp1/11* or WT mice pre-treated with Ac-yvad-cmk demonstrate reduced bacterial burden in the BALF and lungs as well as improved survival (Supplementary Figures S6a–c). To determine if blockade of necrosome components also had a similar effect in *S. aureus* pneumonia, we infected mice pre-treated with Nec-1 with *S. aureus*. As anticipated, blockade of RIP1 with Nec-1 enhanced neutrophil recruitment and subsequent bacterial clearance in the BALF, which was associated with improved host survival rates (Supplementary Figures S6d–f). As *S. aureus*-induced NLRC4 activation was dependent on PKC-δ (Fig. 3e), we investigated the role of PKC-δ inhibition in host defense. To this end, mice were pre-treated with Rottlerin or dimethyl sulfoxide (DMSO) at 12 h prior to infection. As expected, Rottlerin pre-treated mice show reduced IL-1β production, increased neutrophil influx, and bacterial clearance at 24 hpi (Supplementary Figures S6g–i). Collectively, our results indicate that the inhibition of the NLRC4 signaling or necroptosis signaling cascades improves host defense against *S. aureus* infection.

DISCUSSION

The pathogenesis of Gram-positive (*S. aureus*) pneumonia is mediated by its several virulence factors, such as exotoxins and surface proteins. These virulence factors target different innate signaling molecules in infiltrating leukocytes and resident epithelial cells. Moreover, growing evidence indicates that *S. aureus* is able to exploit innate signaling pathways, as mice deficient in *Adam10, Nlrp3, Nod2, Ifnr-1*, and *Tnfr* expression showed augmented host defense against this pathogen.[10–14] Although toxin-induced necroptotic lung damage has been implicated in the pathogenesis of *S. aureus* pneumonia,[17] the precise mechanism underlying the subversion of host immunity is largely unknown. We report that NLRC4 activation leads to necroptotic cell death and IL-18 production, which in turn impedes host defense in *S. aureus* pneumonia. Furthermore, we demonstrate a novel mechanism by which NLRC4-driven necroptosis and IL-18 production suppress γδ T cell-derived IL-17A-dependent neutrophil recruitment during *S. aureus* infection. Finally, we show that blockade or genetic deficiency in NLRC4 signaling cascade and necroptosis pathway improve host defense in the context of *S. aureus* pneumonia.

Pulmonary host defense regulated by the NLRC4 is protective against Gram-negative pathogens, such as *K. pneumoniae*,[21] *L. pneumophila*,[29,30] and *B. pseudomallei*,[31] but deleterious against *P. aeruginosa*.[23,32] In contrast, our study reports NLRC4 activation is deleterious in the context of Gram-positive pathogens, as *Nlrc4*$^{-/-}$ mice show improved host defense and survival. In accordance with this, activation of other NOD-like receptors has also been shown to be detrimental during *S. aureus* infection. For example, *Nod2*$^{-/-}$ and *Nlrp3*$^{-/-}$ mice are less susceptible to *S. aureus* pneumonia.[12,13] Because NLRP3 recognizes a broad range of pathogens, including *S. aureus*,[33] NLRP3 activation was thought to be critical for the pathogenesis of *S. aureus* pneumonia. However, *Nlrp3*$^{-/-}$ mice do not have a remarkable phenotype in *S. aureus* pneumonia, suggesting involvement of an NLRP3-independent host defense mechanism in the response to this pathogen.[13] We showed both myeloid and resident cells express NLRC4 during *S. aureus* pneumonia. Our bone marrow chimeras reveal that NLRC4 expression in both compartments is detrimental with regard to

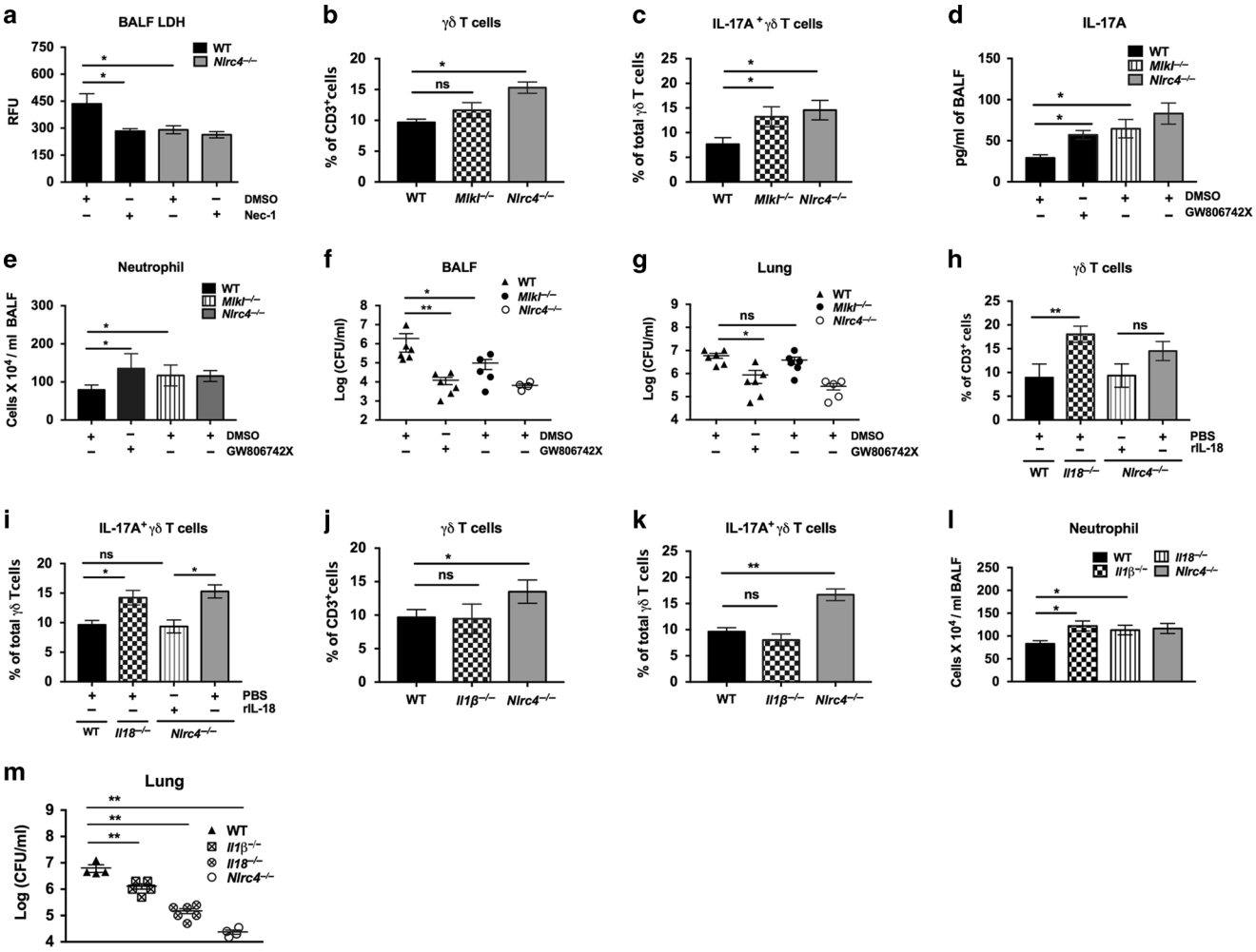

Fig. 6 NLRC4-driven necroptosis and IL-18 suppress 17A-dependent neutrophil recruitment by limiting γδ T cell expansion. (**a**) WT and *Nlrc4*$^{-/-}$ mice were treated with Necrostatin-1 or DMSO intraperitoneally 12 h prior to infection with *S. aureus* (5×10^7 CFU/mouse). RFU of LDH release in BALF was measured at 24 hpi ($n = 5$ mice /group). (**b–g**) WT, *Mlkl*$^{-/-}$, and *Nlrc4*$^{-/-}$ mice were infected with *S. aureus* (5×10^7 CFU/mouse) intratracheally with administration of GW806742X (MLKL inhibitor) or DMSO intraperitoneally 1 h prior. BALF and lungs were harvested at 24 hpi. (**b**) The percentage of total γδ T cells and (**c**) IL-17A-producing γδ T cells in the lungs were determined by flow cytometry. (**d**) IL-17A level, (**e**) number of neutrophils, and (**f**) bacterial burden in BALF, and (**g**) lungs were quantitated. Each symbol represents a single mouse ($n = 4$–6 mice/group). (**h–m**) WT, *Il-18*$^{-/-}$, *Il-1β*$^{-/-}$, and *Nlrc4*$^{-/-}$ mice were infected with *S. aureus* (5×10^7 CFU/mouse) intratracheally. At 24 hpi, the lungs were harvested and processed for flow cytometric analysis. (**h**) The percentage of total γδ T cells and (**i**) IL-17A producing γδ T cells in the lungs of WT, *Il-18*$^{-/-}$, and *Nlrc4*$^{-/-}$ mice receiving recombinant murine IL-18 (1 μg/mouse) or PBS 1 hpi were enumerated. (**j**) The percentage of total γδ T cells and (**k**) IL-17A-producing γδ T cells in the lungs of WT, *Il-1β*$^{-/-}$, and *Nlrc4*$^{-/-}$ mice. (**l**) The number of neutrophils in BALF and (**m**) bacterial burden in the lungs at 24 hpi. Each symbol represents a single mouse ($n = 4$–6/group). Data from a representative experiment are shown. All experiments were performed three times. Statistical significance was determined by one-way ANOVA (followed by Bonferroni's post hoc comparisons) in all experiments. *$p < 0.05$; **$p < 0.01$

bacterial control. It is important to note that the WT → *Nlrc4*$^{-/-}$ group (hematopoietic) had defective bacterial control in the lungs compared to other chimeric groups. Since neutrophil are a major player of *S. aureus* clearance and *Nlrc4*$^{-/-}$ neutrophils are more efficient in controlling bacterial burden, it is possible that NLRC4 expression in myeloid cells of the WT → *Nlrc4*$^{-/-}$ (only hematopoietic expression) group may have dampened phagocytic function of these cells.

Based on data from experiments in THP-1 cells and BMDMs, the NLRC4 was important for caspase-1 activity and IL-1β production in response to *S. aureus* infection. Our findings also indicate that α-hemolysin induces phosphorylation of the NLRC4, which is a PKC-δ-dependent process like in *S. typhimurium*-induced NLRC4 activation in macrophages.[24] However, α-hemolysin expression in *S. aureus* was dispensable for caspase-1 processing. In addition, pulmonary infection with *K. pneumoniae* or *P. aeruginosa* (non-flagellated strains) has been shown to activate the NLRC4 in vivo,[21,22] indicating that virulence factors other than flagellin can activate NLRC4. In this regard, recent reports related to the involvement of NLRC4 activation in obesity-associated tumors, in progression of melanoma tumors, and in diabetic nephropathy suggest that endogenous host proteins may serve as signals for NLRC4 activation.[34–36] Therefore, it is possible that unidentified bacterial components of *S. aureus* or host-derived proteins may act as homologs to known NLRC4-activating bacterial signals.

A number of *S. aureus* toxins are known to activate necroptosis, a highly pro-inflammatory mode of cell death. *S. aureus*-derived pore-forming toxins like Hla, Leukocidin AB, and PSMs leading to cytotoxicity has been well characterized.[7,8,37] Further, necroptosis results in irreparable loss of alveolar macrophages and

anti-inflammatory signals, impeding clearance of *S. aureus*.[17] In addition to reduced phosphorylation of MLKL in the lungs of *Nlrc4*$^{-/-}$ mice, we also found that NLRC4 interacts with RIP3, suggesting a potential role of NLRC4 in necrosome formation. However, it remains unknown if NLRC4 activation also alters the K^+ efflux or contributes to membrane destabilization leading to cell lysis. Unlike *Nlrp3*$^{-/-}$, *Nod2*$^{-/-}$, or *Rip3*$^{-/-}$ mice infected with *S. aureus*,[12,13,17] the central feature of protection in *Nlrc4*$^{-/-}$ mice is their ability to recruit sufficient numbers of neutrophils to the lungs, which is impaired in WT mice. In *Nlrc4*$^{-/-}$ mice, this occurs through the increased frequency of γδ T cells, which provide the IL-17A signal to drive neutrophil influx. Previous studies have shown that IL-17A stimulates granulopoesis in bone marrow and regulates expression of CXC chemokines in lungs to recruit neutrophils.[38,39] In our model, it appears that IL-17A may have contributed to neutrophil recruitment via increased CXCL5 and G-CSF production in *Nlrc4*$^{-/-}$ mice. While numerous immune cells can produce IL-17A at different stages of infection, many studies have highlighted the importance of early IL-17A production by γδ T cells during infection with *S. aureus*[26,40,41] and other extracellular bacteria.[39,42] In addition to γδ T cells, the increased frequency of IL-17A + neutrophils at 24 h post infection may explain that neutrophils are also involved in the recruitment through an autocrine loop. Interestingly, MLKL deficiency or blockade of necroptosis with Nec-1 or MLKL inhibitors improves bacterial clearance, which is also associated with an increased frequency of IL-17A$^+$ γδ T cells and neutrophil recruitment. In addition to necroptosis, NLRC4-induced IL-18 production was found to suppress the γδ T cell–IL-17A axis, as IL-18 deficiency resulted in improved neutrophil recruitment and clearance of *S. aureus*. Similar to our results, IL-18 has also been shown to impede host defense in pneumonia and other disease models.[23,28,43] However, IL-1β, another NLRC4-dependent cytokine, was surprisingly found to be dispensable for this pathway. Thus, these two seemingly disparate NLRC4-dependent mechanisms, the necroptosis and IL-18, work synergistically to impede neutrophil recruitment in *S. aureus* pneumonia. However, future studies will be required to determine how deficiency of necroptosis and IL-18 leads to the increased frequency of IL-17A-producing γδ T cells as early as 6 hpi.

The precise mechanism through which PKC-δ-dependent NLRC4 phosphorylation occurs following Gram-positive (*S. aureus*) infection remains unclear. It should be noted that a wide range of pathogens or host-derived factors, along with environmental pollutants, can activate NLRP3.[33] Along the same lines, it is possible that NLRC4 may recognize host-derived ligands during bacteria-induced inflammation in addition to recognizing pathogens and/or their components. For example, since *S. aureus* -derived pore-forming toxins destabilize the membrane of cells, it is possible that endogenous inflammatory ligands are released and subsequently activates NLRC4. However, elucidation of the specific ligands and mechanisms responsible for NLRC4 activation during Gram-positive *S. aureus* infection of the lung will require future studies.

METHODS

Animals

Nlrc4$^{-/-}$mice were generated as described.[44] The generation of *Il-17a*$^{-/-}$, *Il-17f*$^{-/-}$, *Il-17ra*$^{-/-}$, *Casp1*$^{-/-}$/*11*$^{-/-}$, *Il-1β*$^{-/-}$, *Il-18*$^{-/-}$, and *Mlkl*$^{-/-}$ were described previously.[22,27,45–48] All mouse strains were backcrossed onto the C57BL/6J genetic background at least 10 times. Age- and gender-matched WT controls were used. The Institutional Animal Care and Use Committee of the Louisiana State University approved all animal experiments.

Pneumonia model

The *S. aureus* USA300 strain, its isogenic α-hemolysin (*hla*)-deficient, and MSSA Newman strain were used to induce pneumonia, as described previously.[21,49] Bacterial preparation is detailed in Supplementary Methods. After anesthesia with xylazine/ketamine mix, mice were i.t. inoculated with *S. aureus* at a concentration of 5×10^7 CFUs/50 µl/mouse for pneumonia or 2×10^8 CFUs/50 µl/mouse for survival. Neutrophil depletion was achieved with intraperitoneal injection of 500 µg of anti-Ly6G ab (IA8) (Biolegend) 24 h and 2 h prior to infection.[50] In some experiments, mice were injected intraperitoneally with 300 µg of necrostatin-1 (RIP1 inhibitor) (Calbiochem), 150 µg of Ac-yvad-cmk (Caspase-1 inhibitor) (Caymen Chemical), 200 µg of murine anti-IL-17A antibody (eBiosccience), 100 µg of anti-γδ-TCR antibody (Biolegend), or 200 µg of Rottlerin (PKC-δ inhibitor) (Calbiochem) 12 h prior to infection as described with or without slight modifications.[17,51,52] In other experiments, 100 µl of 10 µM of GW806742X (MLKL inhibitor) (Synkinase) was administered intraperitoneally 1 h prior to infection.[18] In additional experiments, mice were treated i.t. with 1 µg recombinant IL-17A or IL-18 (R&D Systems) 1 hpi.[21,26] Control mice received phosphate-buffered saline (PBS) or DMSO, or control rat IgG2a, as appropriate.

BALF collection, cell counts, and bacterial burden

BALF and organs were collected as described earlier.[21] In brief, mice were sacrificed, the trachea was exposed and cannulated with 20-gauge catheter, and the lungs were flushed four times with 0.8 ml of PBS containing heparin/dextrose. Total and differential cell counts were performed on BALF using light microscopy utilizing Quik-Dip Stain. Lungs/spleen were excised, homogenized in PBS, and plated in serial dilutions on TSA plates to quantitate the bacterial burden in organs after overnight incubation at 37 °C.

Immunofluorescence microscopy

Immunofluorescence staining for NLRC4 expression on tissue slides was performed as described previously.[39] Paraffin-embedded lung sections from healthy or diseased humans (with clinical diagnosis of unspecified bacterial pneumonia) were obtained from Biochain Institute Inc. USA. Following primary antibodies were used: anti-NLRC4 Ab (Cell Signaling), anti-lipocalin-2 Ab for PMNs (R&D systems), anti-CD68 Ab for macrophages (Biolgend), and anti-CD326 Ab for epithelial cells (Biolegend). For mouse slides, we used anti-NLRC4 Ab (EMD Millipore), anti-Ly6G$^+$ Ab for PMNs (Biolegend), anti-CD326 Ab for epithelial cells (Biolegend), and the anti-F4/80 Ab for myeloid cells (Biolegend). Similarly, anti-RIP3 Ab (Cell Signaling) and anti-MLKL (phospho S345) Ab (Abcam) were used to study necroptosis in mouse BMDMs. Alexa-conjugated secondary antibodies (Invitrogen) were used.

Flow cytometry

For flow cytometric analysis, lungs were excised, minced, digested for 90 min at 37 °C in collagenase (2 mg/ml) (Worthington) and DNase I (20 U/ml) (Roche) to obtain single cell suspensions. The following surface marker antibodies were purchased from eBioscience: anti-CD11b (clone M1/70), Ly6G (1A8), F-4/80 (BM8), CD3 (17A2), CD4 (GK1.5), CD8α (53-6.7), γδ-TCR (GL3), and NK1.1 (PK136). For intracellular staining, cells were incubated for 4–5 h with 3 mg/ml of brefeldin A (eBioscience). The BD Cytofix/Cytoperm Kit was used to permeabilize and fix cells before staining with IL-17A (17B7, eBioscience). Isotype controls were used for compensation. Cells were acquired on a FACs caliber (BD Biosciences). FlowJo 10 (Treestar) was used to analyze data.

Western blotting and immunoprecipitation

Western blotting for lung homogenates and cell lysates was performed as described in previous publications.[21] Antibodies to NLRC4 (EMD Millipore), NLRC4 (phospho S533) (ECM Biosciences), Caspase-1 (Adipogen), RIP3 (Cell Signaling), MLKL (phospho S345) (Abcam), and GAPDH (Cell Signaling) were used. The

immunoprecipitation Kit, Dynabeads™ Protein G (Invitrogen), was used to immunoprecipitate RIP3 and NLRC4 according to the manufacturer's protocol.

Bone marrow chimeras

Bone marrow transplantation was performed to create *Nlrc4*$^{-/-}$ chimera mice wherein the *Nlrc4* deficiency was confined to either the hematopoietic cells (*Nlrc4*$^{-/-}$ → WT) or non-hematopoietic tissue (WT → *Nlrc4*$^{-/-}$), as described in our previous publications.[21,39] In brief, recipient mice were lethally irradiated with a single dose of 1000 rad. Each recipient mouse received a tail vein injection of marrow cells (4×10^6/mouse) and was kept on 0.2% neomycin-sulfate drinking water for 2 weeks. Pneumonia was induced at 2 months after the reconstitution. Four groups were generated (WT → WT, WT → *Nlrc4*$^{-/-}$, *Nlrc4*$^{-/-}$ → WT, and *Nlrc4*$^{-/-}$ → *Nlrc4*$^{-/-}$). Our routine procedure results in at least 75–85% reconstitution of blood leukocytes from donor mice, as confirmed by flow cytometry on donor cells expressing GFP.

Statistical analysis

Data are expressed as means ± standard error of mean (s.e.m.) unless otherwise stated. Prism 7.0a software (GraphPad Software Inc.) was used for statistical analyses. The unpaired *t*-test, Mann–Whitney *U* test (non-parametric), or one-way ANOVA (followed by Bonferroni's post hoc comparisons) were used to analyze differences between groups, as appropriate. Survival curves (Kaplan–Meier plot) were compared using log-rank tests. $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ were considered significant.

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AUTHOR CONTRIBUTIONS

Conceived and designed experiments: S.P. and S.J. Performed experiments: S.P., L.G., L.J., P.B. and S.C. Analyzed the data: S.P. and S.J. Wrote the paper: S.P. and S.J.

ADDITIONAL INFORMATION

The online version of this article (https://doi.org/10.1038/s41385-018-0088-2) contains supplementary material, which is available to authorized users.

Competing interests: The authors declare no competing interests.

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