SUPPLEMENTAL METHODS

Bacteria Preparation. Bacterial strains were grown overnight at 37°C in tryptic soy broth, diluted to 1:100 in tryptic soy broth next day, and subculture for 3-4 hours to the mid-logarithmic phase. *S. aureus* strains were harvested by centrifuging the culture at 9000 rpm for 2 minutes, washed twice, and re-suspended in phosphate buffered saline (PBS) to achieve an optical density of 0.5.

THP-1 cells and FAM-FLICA caspase-1 assay. THP-1 (#thp-null) and THP-1 cells overexpressing *Nlrc4* (#thp-nlrc4) were obtained from Invivogen. Culture and maintenance of cells was performed as described in their protocols. The FAM-FLICA caspase-1 assay was performed in THP-1 cells to detect active caspase-1 according to kit's protocol (ImmunoChemistry Technologies)

Cytokine/Chemokines, LDH, and ROS. Human (IL-1β, IL-18 and IL-17A) and Mouse (IL-1β, IL-1α, IL-18, TNF-α, IL-6, MCP-1, IL-17A, and IL-17F) were measured by ELISA as described in their respective protocols (eBioscience/Invitrogen). CXCL1, CXCL2, CXCL5, G-CSF (R&D systems) and HMGB-1 (Chondrex) were quantified according to the manufacturers' protocols. LDH release and % cytotoxicity assays were performed as per manufacturer's guidelines (Promega). The percentage cytotoxicity was calculated as 100x (Experimental-Spontaneous)/ (Maximum-Spontaneous). Total ROS activity was determined using a commercial kit (AAT Bioquest). In some experiments, cells were infected in the presence or absence of murine recombinant IL-17A (R&D Systems).

BMDM/BMDN, bacterial killing assay, and phagocytosis. Bone marrow-derived cells were flushed from tibia and femurs of mice, RBCs lysed, cells were then washed and resuspended in the DMEM containing 10% FBS and Penicillin/Streptomycin (100 U/ml).

Bone marrow cells were then differentiated to macrophages in culture dishes by supplementing MCSF (50 ng/ml) on days 0, 2, 4, 6 for a week. The Mouse Neutrophil Enrichment Kit (Stem Cell) was used to isolate and purify neutrophils through negative selection as described in the manufacturer's protocol. The intracellular killing abilities of BMDM/Ns were evaluated using a gentamicin protection assay with slight modifications [1]. Briefly, BMDMs were infected with multiplicity of infection (MOI 10) of *S. aureus* for 1 hour, washed, incubated with a medium containing gentamicin (250 µg/ml) for 2 hours, washed, and thereafter incubated with a medium containing gentamicin (50 µg/ml) to kill extracellular bacteria. For BMDNs, a medium containing gentamicin (250 µg/ml) was used for 30 minutes at indicated times. The cells were then washed, lysed, and plated by serial dilution in TSA plates to enumerate the CFU of intracellular bacteria. Net phagocytosis of neutrophils was performed utilizing pHrodo Red *S. aureus* BioParticles (Life Technologies). Net phagocytosis was calculated by deducting the average fluorescence intensity of the no-cell negative-control wells from all positive control and experimental wells.

***In vitro* stimulation of purified γδ T cells.** Naïve γδ T cells were purified from spleens of uninfected WT and *Nlrc4*$^{-/-}$ mice using pan-T-cell isolation (MAC Miltenyi Biotec) followed by FACS sorting for CD3^{+} γδ-TCR^{+} cells, as described elsewhere [2]. Purified γδ T cells (1×10^5 cells/well) were then stimulated with *S. aureus* (MOI of 10) for 18 hours in presence or absence of IL-23 (40 ng/ml) (R&D systems). Cell supernatants were collected and IL-17A was quantitated by ELISA. The purity of γδ T cells was found to be ≥87% as determined by flow cytometry.

BrdU staining. The BrdU Flow Kit (BD Biosciences, #559619) was used. In brief, WT

and *Nlrc4*$^{-/-}$ mice received a single dose of 1 mg BrdU solution intraperitoneally 1 hour before infection. Lungs were harvested and processed for flow cytometric staining according to the manufacturer's protocol.

Human Neutrophils. Purified human neutrophils (Astarte Biologics, #1025-3841MA18) were thawed and stimulated with *S. aureus* (MOI 10 or 20), and supernatants were collected for IL-17A measurement.

SUPPLEMENTAL REFERENCES

1. Batra S, Cai S, Balamayooran G, Jeyaseelan S. Intrapulmonary administration of leukotriene B(4) augments neutrophil accumulation and responses in the lung to Klebsiella infection in CXCL1 knockout mice. *J Immunol* 2012; **188**(7)**:** 3458-3468.

2. Maher BM, Mulcahy ME, Murphy AG, Wilk M, O'Keeffe KM, Geoghegan JA *et al.* Nlrp-3-driven interleukin 17 production by gammadeltaT cells controls infection outcomes during Staphylococcus aureus surgical site infection. *Infect Immun* 2013; **81**(12)**:** 4478-4489.

SUPPLEMENTAL FIGURE LEGENDS

Supplemental Figure 1. *S. aureus* activates NLRC4 through PKC-δ to induce IL-1β, IL-18, and necroptosis. (a) Immunoblot analysis of NLRC4 in lungs lysates from healthy and pneumonic patients or from THP-1 and HL-60 cells infected with *S. aureus* (MOI 10) or PBS for 8 hours. (**b**) % of FLICA+ THP-1 and THP1-NLRC4 cells infected with *S. aureus* (MOI 50) or PBS for 6 hours. (**c**) Immunoblot analysis of NLRC4 in lysates of WT and *Nlrc4*$^{-/-}$ -BMDMs infected with S. aureus (MOI 10) for 6 hours. **(d)** Level of IL-1β in the supernatant of WT and *Nlrc4*$^{-/-}$ -BMDMs incubated with *S. aureus* (MOI 20 or 50) or purified α-hemolysin (25 μg/ml) for 6 hours. **(e)** Level of IL-1β in supernatant of WT-BMDM infected with *S. aureus* for 12 hours with or without 1 hour pre-treatment with 10 uM Rottlerin. **(f)** Cytotoxicity in BMDMs, stimulated with *S. aureus* (MOI 100) for 2 hours with or without 1 hour pre-treatment with 100 uM of Necrostatin-1. **(g)** Representative immunofluorescence images and **(h)** percentages showing co-expression of RIP3 (green) and phospho-MLKL (red) in BMDMs stimulated with *S. aureus* (MOI 50) or PBS for 12 hours. Nuclei were stained with DAPI (blue). Original magnification, 40x. **(i-k)** WT and *Nlrc4*$^{-/-}$ mice were inoculated with *S. aureus* (5 X 10^7 CFU/mouse) and BALF was collected at 12 and 24 hpi. **(i)** Relative fluorescence units (RFUs) representing LDH release, **(j)** high mobility group box-1 (HMGB-1), **(k)** IL-1α are measured in BALF. (n= 4-6 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 in all experiment except in cell death assay **(f),** by one-way ANOVA (followed by Bonferroni's

post hoc comparisons). *p<0.05; **p<0.01. *Hla:* α-hemolysin, MOI: multiplicity of infection, DMSO: dimethyl sulfoxide, LDH: Lactate Dehydrogenase.

Supplemental Figure 2. Cytokine/Chemokine profiles of *Nlrc4*$^{-/-}$ mice in *S. aureus* pneumonia. (a-g) WT and *Nlrc4*$^{-/-}$ mice were inoculated intratracheally with 5 x 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. (n= 4-6/group for pneumonia and n=3/group for PBS control). Data are representative of three independent experiments. *p<0.05, ***p<0.001by unpaired t-test.

Supplemental Figure 3. NLRC4 deficiency does not alter phagocytosis or bacterial killing of BMDMs in response to *S. aureus*. (a) BMDMs were isolated from wild type and *Nlrc4*$^{-/-}$ mice and infected with *S. aureus* (MOI 10). The intracellular killing ability of macrophages was assessed at the indicated time points by determining the intracellular bacterial burden. Data are representative of three independent experiments with five technical replicates per group.

Supplemental Figure 4. Multiple immune cell types produce IL-17A in the lungs during *S. aureus* pneumonia. (a-d) Flow cytometric analysis of lungs from WT and *Nlrc4*$^{-/-}$ mice either untreated or intratracheally infected with 5 x 10^7 CFU of *S. aureus*. At 24 hpi, lungs were harvested and processed for flow cytometric analysis. **(a)** Dot plots representation of neutrophils (Ly6G$^+$ CD11b$^+$ cells) and IL-17A$^+$ neutrophils, **(b)** CD4$^+$ CD3$^+$ cells (CD4 T cells) and IL-17A$^+$ CD4 T cells, **(c)** CD8α$^+$ CD3$^+$ cells (CD8 T cells) and IL-17A$^+$ CD8 T cells, and **(d)** NK1.1$^+$ CD3$^-$ cells (NK cells) and IL-17A$^+$ NK cells. (e) Purified human neutrophils were stimulated with *S. aureus* (MOI 10 or 20) or PBS for 12

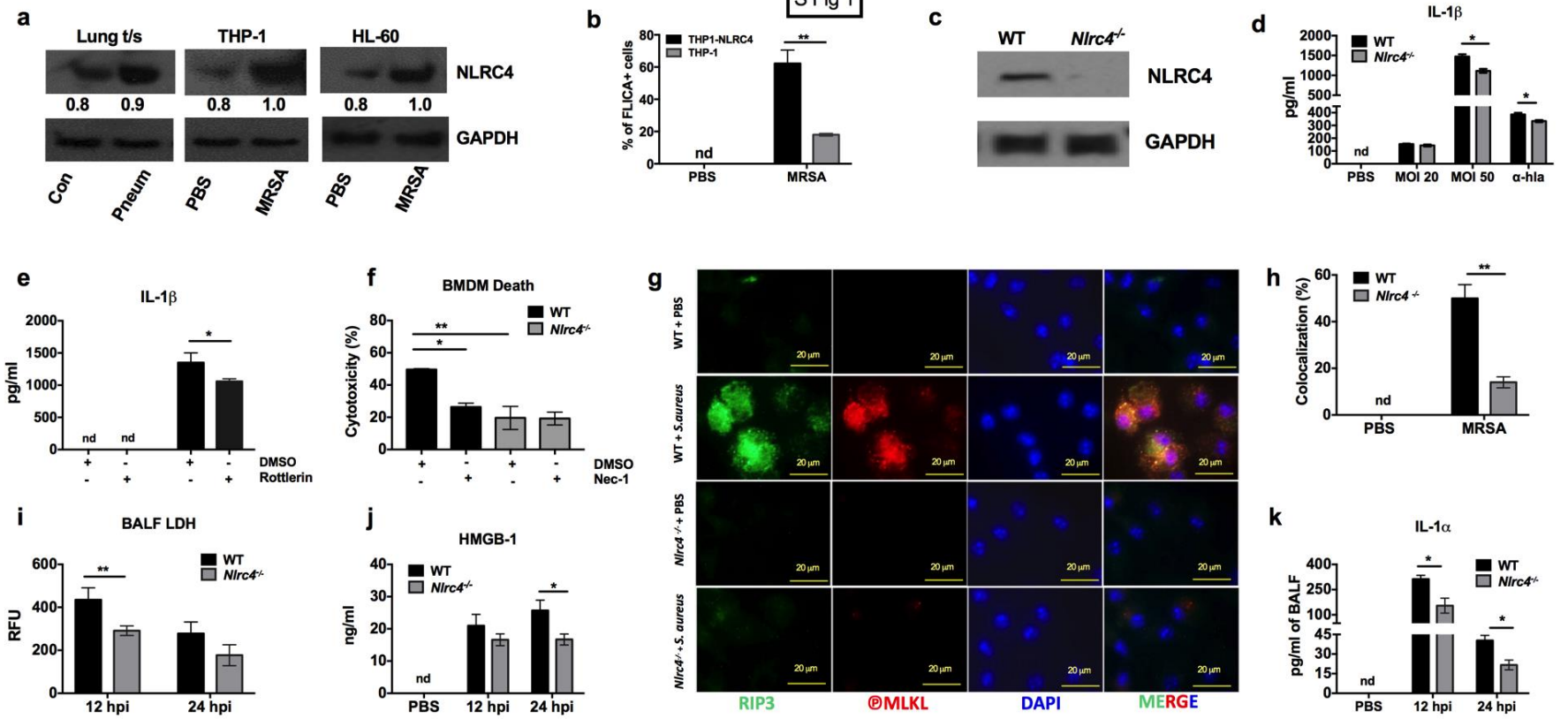
hours and IL-17A level in supernatant were measured. (n= 5-6 mice/ *S. aureus* group, 3 mice/PBS group). Data are representative of three independent experiments.

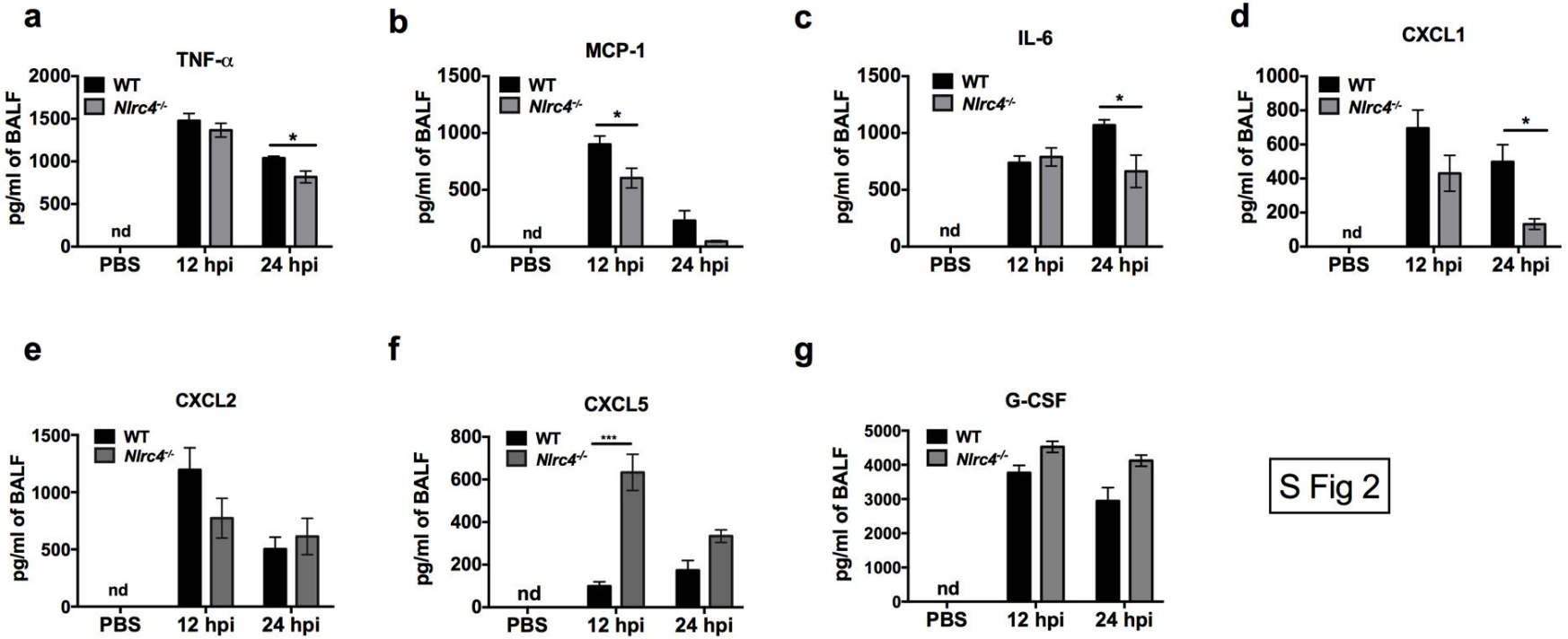
Supplemental Figure 5. Deficiency of MLKL or IL-18, but not of IL-1β, augments the numbers of γδ T cells and IL-17A producing γδ T cells. (a-c) WT, *Mlkl*$^{-/-}$, *Il-18*$^{-/-}$, *Il-1β*$^{-/-}$, and *Nlrc4*$^{-/-}$ mice were infected with *S. aureus* (5 x10^7 CFU/mouse) intratracheally. At 24 hpi, lungs were harvested and processed for flow cytometric analysis. **(a)** Total γδ T cells and IL-17A producing γδ T cells in the lungs of WT, *Mlkl*$^{-/-}$, and *Nlrc4*$^{-/-}$ mice. **(b)** Total γδ T cells and 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 hour post-infection. (**c)** Total γδ T cells and IL-17A producing γδ T cells in the lungs of WT, *Il-1β*$^{-/-}$, and *Nlrc4*$^{-/-}$ mice. (n= 5-6 *S. aureus* mice/group, 3 mice/PBS group). Data are representative of three independent experiments.

Supplemental Figure 6. Blockade of caspase-1 and RIP1 improve the outcome of *S. aureus* pneumonia. (a-c) WT, *Casp1/11*$^{-/-}$ and *Nlrc4*$^{-/-}$ mice were treated with Ac-yvad-cmk (150 μg/mouse) or DMSO intraperitoneally 12 hours prior the infection with *S. aureus* (5 x 10^7 CFU or 2 x10^8 CFU/mouse) intratracheally. **(a)** Bacterial burden in the lungs, **(b)** BALF at 24 hpi, and **(c)** survival over 100 hours were determined. **(d-f)** WT and *Nlrc4*$^{-/-}$ mice were treated with Necrostatin-1 (300 μg/mouse) or DMSO intraperitoneally 12 hours prior to infection with *S. aureus* (5 x10^7 CFU/mouse for pneumonia or 2 x 10^8 CFU/mouse) intratracheally. (**d)** Number of neutrophils, **(e)** bacterial burden in BALF at 24 hpi, and **(f)** survival over 100 hours were determined. **(g-i)** WT mice were treated with Rottlerin (PKC-δ inhibitor) (200 μg/mouse) or DMSO intraperitoneally 12 hours prior to infection with *S. aureus* (5 x10^7 CFU/mouse) intratracheally. (**g)** Level of IL-1β, (**h**) number of neutrophils,

(i) bacterial burden in lungs and BALF at 24 hpi were determined. Data are representative of three independent experiments. Kaplan–Meier plots were used to show survival of mice from each group. Statistical significance was determined by one-way ANOVA (followed by Bonferroni's *post hoc* comparisons **(a, b, d, e)**, log-rank test **(c,f)**, unpaired t-test (**g, h**), and Man-Whittney (**i**). *$p<0.05$; **$p<0.01$; ***$p<0.001$. (n= 4-6/group for pneumonia and n= 10/group for survival).

S Fig 1


a
Lung t/s
THP-1
HL-60
NLRC4
0.8 0.9 0.8 1.0 0.8 1.0
GAPDH
Con
Pneum
PBS
MRSA
PBS
MRSA
b
THP1-NLRC4
THP-1
% of FLICA+ cells
nd
PBS
MRSA
c
WT
Nlrc4-/-
NLRC4
GAPDH
d
IL-1β
WT
Nlrc4-/-
pg/ml
nd
PBS
MOI 20
MOI 50
α-hla
e
IL-1β
pg/ml
nd nd
DMSO
Rottlerin
f
BMDM Death
WT
Nlrc4-/-
Cytotoxicity (%)
DMSO
Nec-1
g
WT + PBS
WT + S.aureus
Nlrc4-/- + PBS
Nlrc4-/- + S. aureus
20 μm
RIP3
PMLKL
DAPI
MERGE
h
WT
Nlrc4-/-
Colocalization (%)
nd
PBS
MRSA
i
BALF LDH
WT
Nlrc4-/-
RFU
12 hpi
24 hpi
j
HMGB-1
WT
Nlrc4-/-
ng/ml
nd
PBS
12 hpi
24 hpi
k
IL-1α
WT
Nlrc4-/-
pg/ml of BALF
nd
PBS
12 hpi
24 hpi


a
TNF-α
pg/ml of BALF
2000
1500
1000
500
0
WT
Nlrc4-/-
nd
*
PBS
12 hpi
24 hpi
b
MCP-1
pg/ml of BALF
1500
1000
500
0
WT
Nlrc4-/-
nd
*
PBS
12 hpi
24 hpi
c
IL-6
pg/ml of BALF
1500
1000
500
0
WT
Nlrc4-/-
nd
*
PBS
12 hpi
24 hpi
d
CXCL1
pg/ml of BALF
1000
800
600
400
200
0
WT
Nlrc4-/-
nd
*
PBS
12 hpi
24 hpi
e
CXCL2
pg/ml of BALF
1500
1000
500
0
WT
Nlrc4-/-
nd
PBS
12 hpi
24 hpi
f
CXCL5
pg/ml of BALF
800
600
400
200
0
WT
Nlrc4-/-
***
nd
PBS
12 hpi
24 hpi
g
G-CSF
pg/ml of BALF
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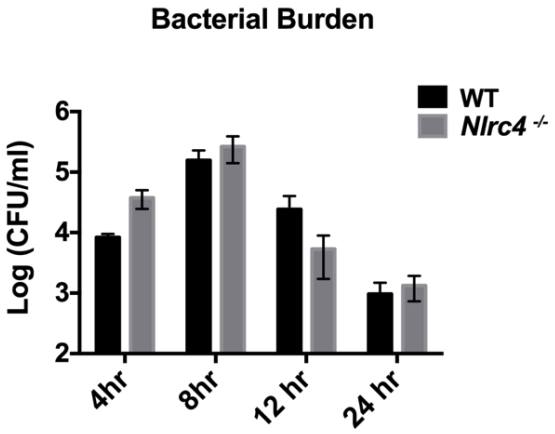
4000
3000
2000
1000
0
WT
Nlrc4-/-
nd
PBS
12 hpi
24 hpi

S Fig 2

S Fig 3



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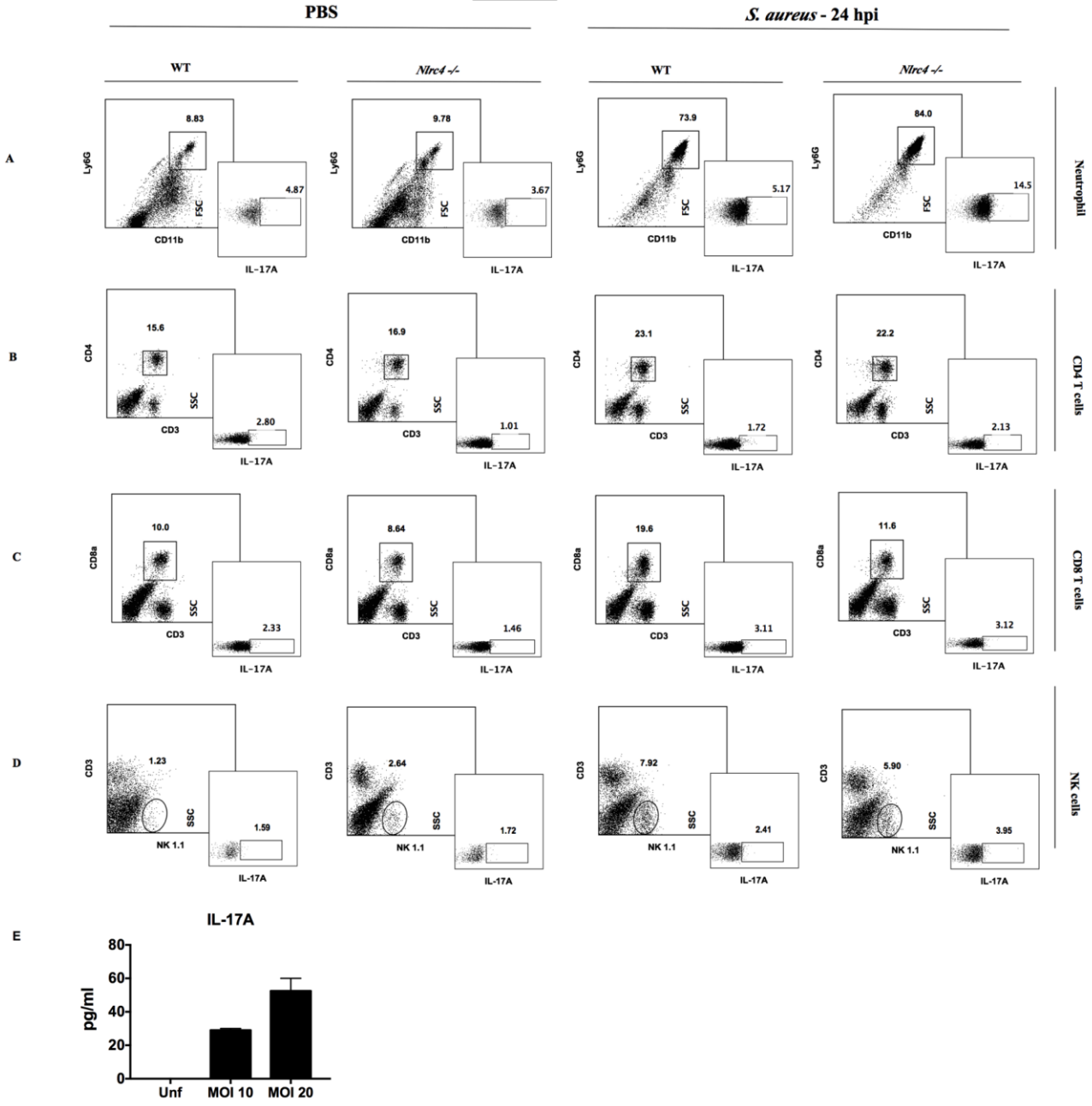
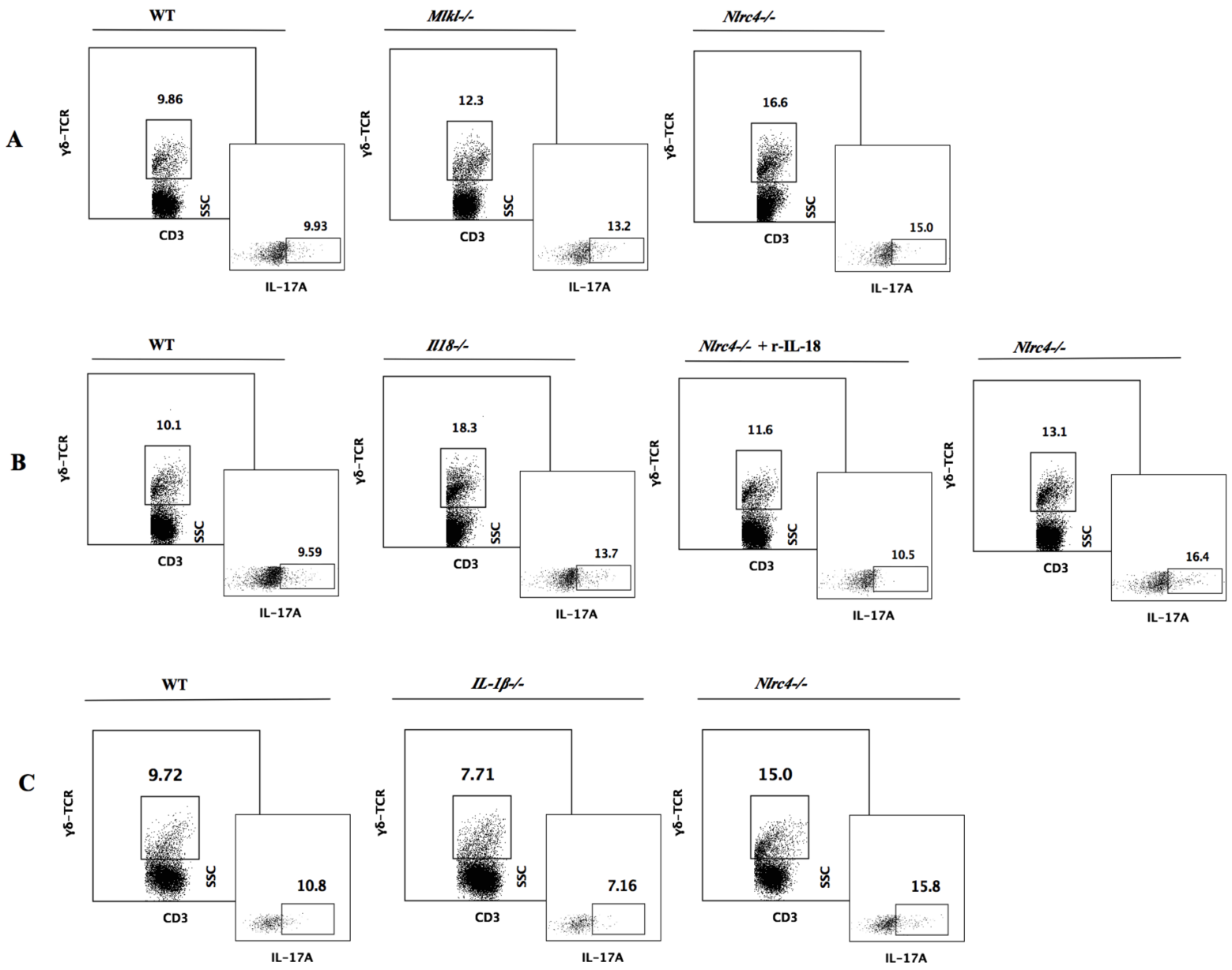
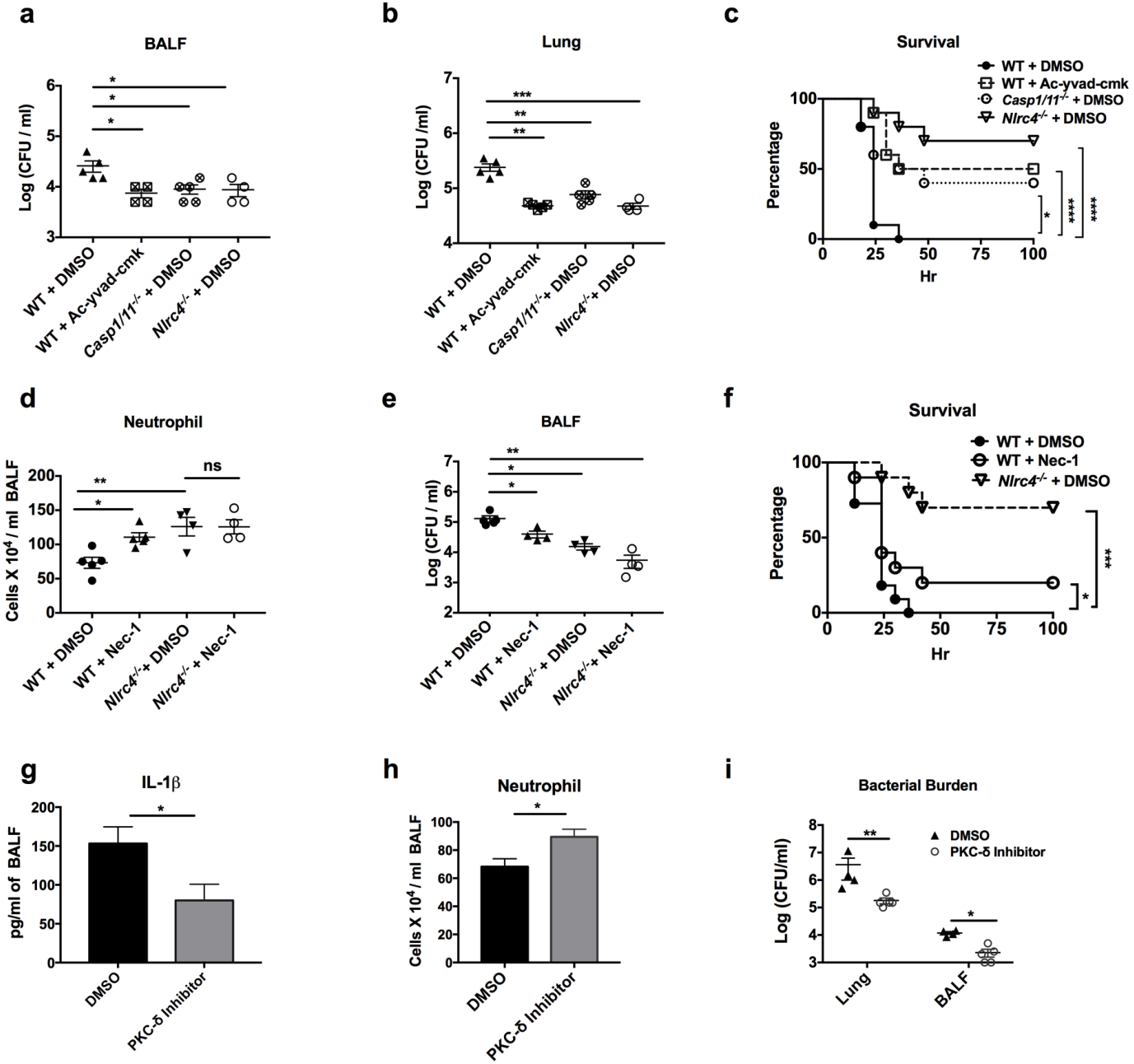

PBS
S. aureus - 24 hpi
WT
Nlrc4 -/-
WT
Nlrc4 -/-
A
Ly6G
CD11b
FSC
IL-17A
8.83
4.87
9.78
3.67
73.9
5.17
84.0
14.5
Neutrophil
B
CD4
CD3
SSC
IL-17A
15.6
2.80
16.9
1.01
23.1
1.72
22.2
2.13
CD4 T cells
C
CD8a
CD3
SSC
IL-17A
10.0
2.33
8.64
1.46
19.6
3.11
11.6
3.12
CD8 T cells
D
CD3
NK 1.1
SSC
IL-17A
1.23
1.59
2.64
1.72
7.92
2.41
5.90
3.95
NK cells
E
IL-17A
pg/ml
80
60
40
20
0
Unf
MOI 10
MOI 20

S Fig 5


A
WT
9.86
9.93
Mlkl-/-
12.3
13.2
Nlrc4-/-
16.6
15.0
γδ-TCR
SSC
CD3
IL-17A
B
WT
10.1
9.59
Il18-/-
18.3
13.7
Nlrc4-/- + r-IL-18
11.6
10.5
Nlrc4-/-
13.1
16.4
C
WT
9.72
10.8
Il-1β-/-
7.71
7.16
Nlrc4-/-
15.0
15.8

S Fig 6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BALF
Log (CFU / ml)
WT + DMSO
WT + Ac-yvad-cmk
Casp1/11-/- + DMSO
Nlrc4-/- + DMSO
b
Lung
Log (CFU /ml)
c
Survival
Percentage
Hr
WT + DMSO
WT + Ac-yvad-cmk
Casp1/11-/- + DMSO
Nlrc4-/- + DMSO
d
Neutrophil
Cells X 10^4 / ml BALF
ns
WT + Nec-1
Nlrc4-/- + DMSO
Nlrc4-/- + Nec-1
e
BALF
f
Survival
WT + Nec-1
g
IL-1β
pg/ml of BALF
DMSO
PKC-δ Inhibitor
h
Neutrophil
i
Bacterial Burden
Log (CFU/ml)
Lung
BALF