

Additional materials

Materials and Methods

Ethics statement

The study was approved by the Clinical Research Ethics Committee of The First Affiliated Hospital of Chongqing Medical University, and all subjects provided informed consent, and written informed consent was obtained from all subjects prior to participation according to the Declaration of Helsinki. All animal experiments were discussed with and approved by the Animal Care and Use Committee of the Chongqing Medical University and carried out according to the recommendations in the guide for the care and use of laboratory animals conformed to animal protection laws of China and applicable guidelines.

Study Population

Patients were randomly selected from The First Affiliated Hospital of Chongqing Medical University intensive care unit between 2010 and 2013. The diagnosis of sepsis was based on the criteria recommended by the American College of Chest Physicians and Society of Critical Care Medicine Consensus Conference.¹ On the time point admission to ICU, the following items for each patient were recorded: age, sex, Acute Physiology and Chronic Health Evaluation II (APACHE II) score, Sequential Organ Failure Assessment (SOFA) score, and blood samples were taken from all septic patients for laboratory examinations, such as the counts of white blood cells (WBC) and the levels of C-reaction proteins (CRP). Patients with HIV infection, with organ transplantation, or receiving chemotherapy or corticosteroids in the past 8

weeks were excluded from the study. Control samples were obtained from healthy donors with no medical problems in the medical examination center of The First Affiliated Hospital of Chongqing Medical University.

Mice

C57BL/6 mice aged 6–8 weeks were obtained from and raised at Chongqing Medical University. IL-27R^{-/-} (WSX-1-deficient) mice raised on C57BL/6 background were from The Jackson Laboratory.

Animal models

CLP was used as a model of systemic sepsis syndrome as previously described.² Briefly, mice were anesthetized intraperitoneally (i.p.) with a mixture of xylazine (4.5mg/kg) and ketamine (90mg/kg), and the cecum was exposed, ligatured at its external third, and punctured through and through with a 26-gauge needle. The cecum was then placed back in the peritoneal cavity, and the incision was closed with surgical staples. Sham-operated (control) animals underwent identical laparotomy, the cecum was exposed but not ligated or punctured and was then replaced in the peritoneal cavity. All mice were given preoperative and postoperative analgesia (ibuprofen, 200 µg/ml in drinking water), starting 24 h before until 48 h after surgery.

Induction of secondary pneumonia

To create a relevant model of secondary pneumonia, mice were anesthetized with an i.p. ketamine (4.5mg/kg) and xylazine (90mg/kg) mixture, and then the trachea was exposed, and 30 µL of 1×10^5 *P. aeruginosa* (strain UI-18) was administered intratracheally (i.t.) to CLP or sham animals 24 h post CLP or sham surgery. Sample

harvesting and processing were then performed.

Lung leukocyte preparation and pulmonary histopathology

At designated time points, the mice were euthanized by CO₂ inhalation. Lungs were then removed from euthanized animals and leukocytes prepared as previously described.³ Briefly, lung slurries were enzymatically digested for 30 minutes at 37°C. The total lung cell suspension was pelleted, resuspended, and spun through a 40% Percoll gradient to enrich for leukocytes. Cell counts and viability were determined using Trypan blue exclusion counting on a hemacytometer. Cytospin slides were prepared and stained with a Wright-Giemsa stain. For histologic examination, the lungs were inflated with 0.5% agarose under 25 cm water pressure, fixed in 10% buffered formalin for 24 h. Next, formalin-fixed, paraffin-embedded 6-mm sections of lungs were used for immunohistochemistry by Haematoxylin and Eosin (H&E) staining.

Pathology score (PA) assessment

Histology of lung sections from each mouse was examined after staining with hematoxylin and eosin. The lung inflammation score were evaluated according to the semiquantitative scoring system including necrosis or formation of abscess, interstitial inflammation, endothelialitis, bronchitis, edema, thrombi, pleuritis, and percentage of the lung surface demonstrating confluent (diffuse) inflammatory infiltrate by a blinded pathologist.

Determination of lung and plasma *P. aeruginosa* CFU

At the designated time points, plasma was collected, and the right ventricle was

perfused with 1 ml PBS, then lungs were removed aseptically and placed in 1 ml sterile saline. The tissues were then homogenized with a tissue homogenizer under a vented hood. Serial 1:5 dilutions of both lung homogenates and plasma were made. Ten microliters of each dilution was plated on soy base blood agar plates to determine lung CFU.

Determination of splenic immune cell counts

Spleens were harvested at the time of sacrifice from all groups of mice. Splenocytes were dissociated by gently pressing through a 70 μ m filter and then washed. The total number of splenocytes was determined using a Beckman-Coulter (Fullerton, CA, USA) cell counter. Splenocytes were also stained with fluorochrome-conjugated antibodies (BD PharMingen) for specific cell population counts (CD4 and CD8 for T cells, and B220 for B cells). Flow cytometric analysis was performed on a FACSCalibur flow cytometer (BD Biosciences).

Determination of Delayed-Type Hypersensitivity (DTH)

Mice underwent CLP or sham surgery as described above. At day 4 post-surgery, mice were immunized with 100 μ l of 10 mM 2,4,6-trinitrobenzene-sulfonic acid (TNBS) subcutaneously. At day 8, mice had antigenic challenge with 30 μ L of 10mM TNBS in the right footpad. PBS was injected in the left footpad as a control. Measurements (μ M) of footpad swelling were taken 24 h later and represent the difference between the right and left footpad.

Alveolar macrophages (AMs) and neutrophils harvesting and culture

Neutrophils were purified from the bone marrow by discontinuous Percoll gradient

centrifugation as previously reported⁴. AMs were isolated by adherence of BAL fluid for 1 h in Dulbecco's minimum essential medium at 37 °C, 5% CO₂, and nonadherent cells were removed by replacement of culture medium with antibiotic-free medium.

Quantitative real-time RT-PCR

Total cellular RNA was extracted from cells and organs with RNeasy columns (QIAGEN), including DNase I digestion. Quantitative real-time PCR analysis for IL-27 EBI3 and p28, WSX-1, and GAPDH was performed using specific primers Quantitect Primer/Probe assays (QIAGEN). An average value of gene expression after GAPDH normalization was used as a calibrator to determine the relative levels of target gene. The relative expression of target genes was calculated using the $2^{-\Delta C(t)}$ method.

Phagocytosis and bacterial killing assays

Neutrophils (1×10^6 cells) were infected with *P. aeruginosa* (multiplicity of infection, 100) at 37 °C for 30 min. Cells were washed with buffer containing tobramycin (100 µg/ml) to remove extracellular bacteria and were lysed. Live intracellular bacteria were quantified by culture of lysates for determination of bacterial uptake ($t = 0$) and intracellular killing ($t = 1$ h). Killing was calculated from the percentage of colonies present at $t = 1$ h as compared to $t = 0$, as follows: $100 - [\text{number of colony-forming units (CFUs)}_{t = 1 \text{ h}} / \text{number of CFUs}_{t = 0 \text{ h}}]$.

AMs were infected with *P. aeruginosa* at an MOI ratio of 1:10 at 37 °C for 1 h, and cells were then lysed in PBS containing 0.1% Triton 100 for assessment of phagocytosis ($t = 0$), and additional samples were incubated for 1 additional hour ($t =$

2 h) to assess bacterial killing as described above. In some experiments, AMs and neutrophils were treated with recombinant IL-27 (50 ng/ml, R&D systems) in the presence or absence of anti-IL-10 neutralizing antibodies (2 µg/ml, R&D systems).

Measurement of cytokine/chemokine

The concentrations of IFN- γ , TNF- α , IL-1 β , CXCL1, CXCL2, CXCL10, IL-6, IL-12 and IL-17 were determined with commercially available ELISA kits from Biolegend, while IL-27 levels were determined by ELISA kits from R&D Systems according to the manufacturer's instructions.

Flow cytometric analysis

AMs or neutrophils were incubated with FITC/PE-labelled anti-WSX1 (R&D Systems), anti-CD40, anti-CD80, anti-CD86, anti-Mac-1, or anti-LFA-1 (BD Pharmingen) antibodies and their corresponding mouse IgG isotype (R&D Systems). After final washing, cells were resuspended in 1% paraformaldehyde in PBS. Expression of surface molecules on 2000 viable cells was then quantitatively analyzed by flow cytometry (FACSCalibur flow cytometer; BD Biosciences) in terms of mean fluorescence intensity (MFI).

Depletion of AMs

Dichloromethylenebisphosphonate (clodronate) was from Roche Diagnostics. Phosphatidylcholine was obtained from Lipoid, and cholesterol was purchased from Sigma-Aldrich. The clodronate-encapsulated liposomes and PBS-encapsulated liposomes were prepared as described.⁵ Clodronate-encapsulated liposomes (100 µL) were delivered intranasally (i.n.) to deplete AMs at 48 h before bacterial infection.

PBS-encapsulated liposomes were delivered in a similar fashion as a control.
Microscopic examination of bronchoalveolar lavage fluid (BALF) indicated >90%
depletion at the time of infection.

Depletion of neutrophils

Neutrophil depletion was performed as previously described.⁶ Briefly, mice were
injected intravenously (i.v.) with 0.1 mg of RB6-8C5 monoclonal antibodies (mAb) to
mouse Ly6G (anti-Gr-1, BD Biosciences) with rat IgG as a control at 24 h before
bacterial infection.

Antibody-mediated neutralizations *in vivo*

IL-27 neutralization was performed by intravenously (i.v.) administration of 100 µg of anti-IL-27 antibodies (R&D systems) on day 0 (same day as CLP), followed by booster doses of 50 µg on day 1, and then mice were i.t. challenged with *P. aeruginosa*. Sample harvesting and processing were then performed at indicated times.

IL-27 *in vivo* treatment

We treated mice i.p. under light isoflurane anesthesia with 2 µg of recombinant mouse
IL-27 (R&D systems) in 100 µl of phosphate buffered saline (PBS) at 24 h before
bacterial infection. In parallel, mice were injected solely with saline as control.

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Supplementary Figure legend

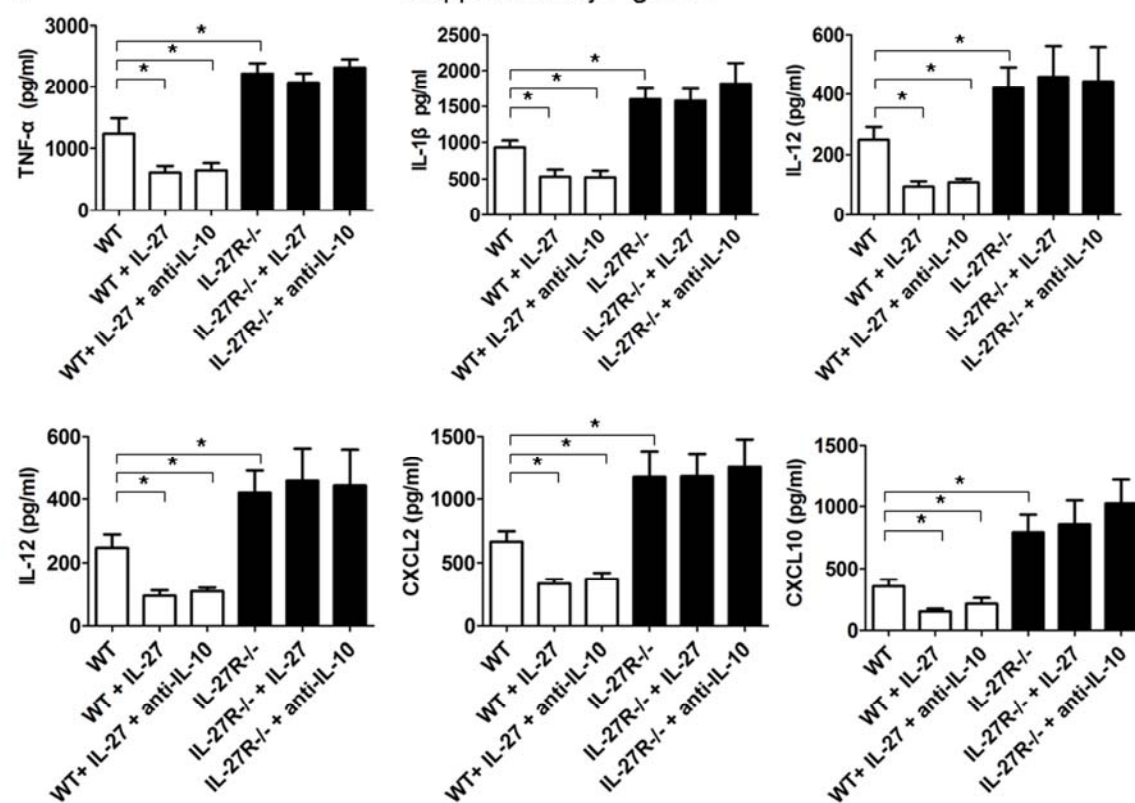
Supplementary Figure 1. IL-27 modulated immune responses of AMs. (A) AMs (1×10^4) were stimulated *ex vivo* with heat-killed (HK) *P. aeruginosa* (equivalent of 1×10^6 CFUs per milliliter) in the presence or absence of recombinant IL-27 (50 ng/ml) and anti-IL-10 neutralizing antibodies (2 μ g/ml) for 24 h, after which cytokine/chemokine were determined in supernatants by ELISA. (B) Cell-surface expression of CD40, CD80 and CD86 by AMs after stimulation with HK *P. aeruginosa* in the presence or absence of recombinant IL-27 (50 ng/ml) and

anti-IL-10 neutralizing antibodies (2 µg/ml) for 24 h determined by flow cytometry. Data were expressed as mean ± SEM and analyzed using one-way ANOVA, * $P < 0.05$ when compared between groups denoted by horizontal lines.

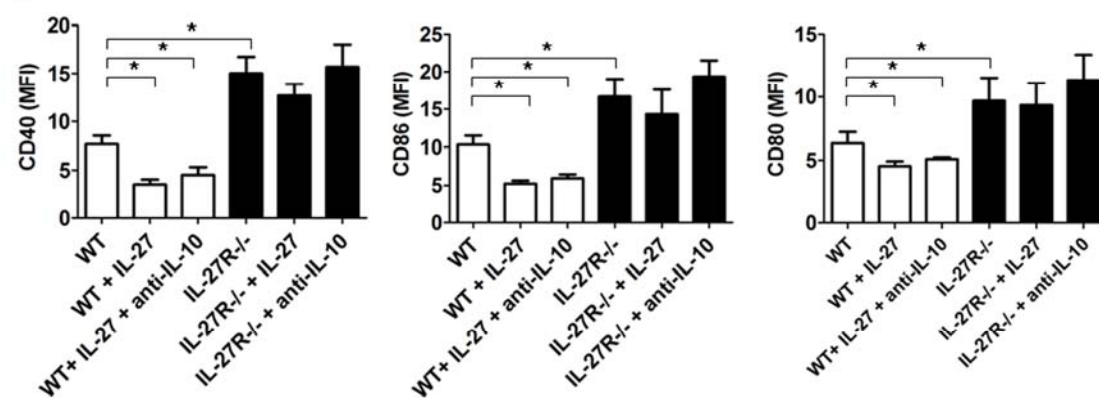
Supplementary Figure 2. IL-27 modulated immune responses of neutrophils. (A) Neutrophils (1×10^5) were stimulated *ex vivo* with HK *P. aeruginosa* (equivalent of 1×10^6 CFUs per milliliter) in the presence or absence of recombinant IL-27 (50 ng/ml) and anti-IL-10 neutralizing antibodies (2 µg/ml) for 24 h, after which TNF- α and IL-1 β were determined in supernatants by ELISA. (B) Cell-surface expression of Mac-1 and LFA-1 by neutrophils after stimulation with HK *P. aeruginosa* in the presence or absence of recombinant IL-27 (50 ng/ml) and anti-IL-10 neutralizing antibodies (2 µg/ml) for 24 h determined by flow cytometry. Data were expressed as mean ± SEM and analyzed using one-way ANOVA, * $P < 0.05$ when compared between groups denoted by horizontal lines.

Supplementary Figure 1

A

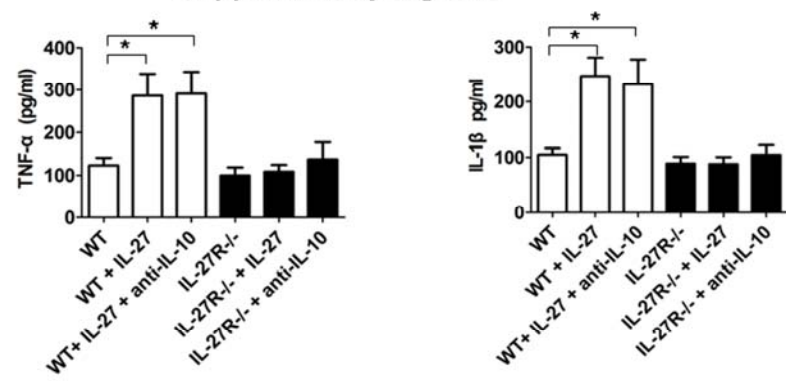


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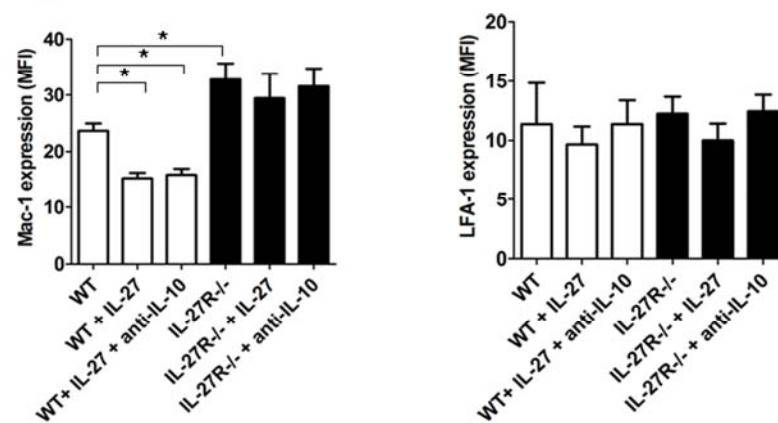


Supplementary Figure 2

A



B



Supplementary Table 1: Patient and healthy donor characteristics

Characteristic	Sepsis (n=25)	Healthy control (n=21)
Age (y)	66 ± 11	61 ± 8
Male/female gender, No.	15/10	12/9
Smoker	15 (60%)	11 (52.4%)
Major surgery	2 (8 %)	—
Multiple trauma	5 (20%)	—
Peritonitis	7 (28%)	—
Pancreatitis	11 (44%)	—
APACHE II Score	17.6 ± 7.5	—
SOFA score	7.9 ± 3.1	—
WBC	9.8 ± 5.1	5.6 ± 3.6
CRP	108.7 ± 95.6	—
Length of ICU stay	7.5 ± 6.5	—
Length of hospital stay	21.5 ± 12.5	—

Data are presented as mean ± SD, or number (%) where indicated. APACHE II: Acute Physiology and Chronic Health Evaluation II; SOFA=Sepsis-related Organ Failure Assessment; WBC=white blood cells; CRP=C-reaction protein.