CONTRIBUTION OF IL-38 IN LUNG IMMUNITY DURING PSEUDOMONAS AERUGINOSA-INDUCED PNEUMONIA

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ABSTRACT—Objective: Interleukin-38 (IL-38), a new type of cytokine, is involved in processes such as tissue repair, inflammatory response, and immune response. However, its function in pneumonia caused by Pseudomonas aeruginosa (P. aeruginosa) is still unclear. Methods: In this study, we detected circulating IL-38 and cytokines such as IL-1β, IL-6, IL-17A, TNF-α, IL-8, and IL-10 in adults affected by early stage pneumonia caused by P. aeruginosa. Collected clinical data of these patients, such as the APACHE II score, levels of PCT, and oxygenation index when they entering the ICU. Using P. aeruginosa-induced pneumonia WT murine model to evaluate the effect of IL-38 on Treg differentiation, cell apoptosis, survival, tissue damage, inflammation, and bacterial removal. Results: In clinical research, although IL-38 is significantly increased during the early stages of clinical P. aeruginosa pneumonia, the concentration of IL-38 in the serum of patients who died with P. aeruginosa pneumonia was relatively lower than that of surviving patients. It reveals IL-38 may insufficiently secreted in patients who died with P. aeruginosa pneumonia. Besides, the serum IL-38 level of patients with P. aeruginosa pneumonia on the day of admission to the ICU showed significantly positive correlations with IL-10 and the PaO2/FiO2 ratio but negative correlations with IL-1β, IL-6, IL-8, IL-17, TNF-α, APACHE II score, and PCT In summary, IL-38 might be a molecule for adjuvant therapy in P. aeruginosa pneumonia. In experimental animal models, first recombinant IL-38 improved survival, whereas anti-IL-38 antibody reduced survival in the experimental pneumonia murine model. Secondly, IL-38 exposure reduced the inflammatory response, as suggested by the lung injury, and reduced cytokine levels (IL-1β, IL-6, IL-17A, TNF-α, and IL-8, but not IL-10). It also increased bacterial clearance and reduced cell apoptosis in the lungs. Furthermore, IL-38 was shown to reduce TBK1 expression in vitro when naive CD4+ T lymphocytes were differentiated to Tregs and played a protective role in P. aeruginosa pneumonia. Conclusions: To summarize, the above findings provide additional insights into the mechanism of IL-38 in the treatment of P. aeruginosa pneumonia.

KEYWORDS—Apoptosis, cytokines, IL-38, P. aeruginosa pneumonia, Tregs

INTRODUCTION

In recent years, broad-spectrum antibacterial drugs, or immunosuppressants, have been used widely in clinical practice and invasive treatments, such as various operations and punctures, which breach the natural barriers of the human body (1-4). The incidence of Pseudomonas aeruginosa (MDR-PA) infection in clinics is increasing. P. aeruginosa is a conditional pathogen, which often causes bacteremia, pneumonia, urinary tract infections, infections after burns, and secondary infections in cystic fibrosis (5-7). Due to the continuous formation of drug-resistant strains and constant improvements in drug resistance, P. aeruginosa has become one of the most serious conditional pathogens of acquired infections in hospitals (8, 9). Particularly, in the intensive care unit (ICU), the increasing number of multi-drug resistant P. aeruginosa causing pneumonia has challenged the application of antibiotics (10). Pulmonary immunity plays a vital role in fighting against pulmonary respiratory pathogens; simultaneously, diverse inflammatory regulators (like growth factors, cytokines, and chemokines) regulate the responses to an injury or infection (11). Therefore, it is important to better understand the cellular and molecular immune responses and pulmonary immunity regarding microbial infections to identify new opportunities for combating bacterial pneumonia.

Interleukin-38 (IL-38) represents a novel cytokine belonging to the IL-1 family. It shares 41% homology with IL-1Ra and

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Our manuscript confirming the study is reported in accordance with ARRIVE guidelines.

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Ethics approval: Human subjects: This study was carried out in accordance with the recommendations of the Ethics Committee of the First Affiliated Hospital of Chongqing Medical University with written informed consent from all subjects. All subjects gave written informed consent in accordance with the Declaration of Helsinki. The protocol was approved by the Ethics Committee of the First Affiliated Hospital of Chongqing Medical University. The number is 2020-847. Animal subjects: This study was carried out in accordance with the recommendations of The Institutional Animal Care and Use Committee at Chongqing Medical University. All experimental protocols were approved by the Institutional Animal Care and Use Committee at Chongqing Medical University.

Consent for publication: We confirm that the manuscript has been read and approved by all named authors and that there are no other persons who satisfied the criteria for authorship but are not listed. We further confirm that the order of authors listed in the manuscript has been approved by all of us.

The authors report no conflicts of interest.

Authors’ contributions: Conception hypothesis and design: CJ-W. Data acquisition and analysis: QW. Manuscript preparation: XC. Revised manuscript: CJ-W. Searched and collected bibliography: XC and ZZ-Y.

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43% homology with IL-36Ra; thus, it has been identified as the IL-1 family receptor antagonist (12). Recent studies have found that IL-38 can competitively bind to IL-1R1, preventing IL-1R accessory protein (IL-1RAcP) recruitment, and causing anti-inflammation in inflammatory bowel disease (IBD) (13), osteoarthritis (OS) (14), sepsis (15), and other inflammatory diseases (16). Previous research by our group showed that IL-38 protects the cecal ligation and punctures the (CLP)-or lipopolysaccharide (LPS)-mediated acute respiratory distress syndrome (ARDS) model by reducing Th17 differentiation (17). Therefore, the present work focused on characterizing the effect of IL-38 on lung immunity to resist P. aeruginosa-induced pneumonia (P.A. pneumonia). For this, we examined IL-38 production among patients admitted to the ICU with P.A. pneumonia. Then, the host immune responses to the administration of IL-38 neutralizing antibody and recombinant IL-38 protein were determined by adopting the P.A. pneumonia model constructed through intranasal injection.

**MATERIALS AND METHODS**

**Human subjects**

The blood of patients diagnosed with pneumonia on day 0, 1, 3, 5, and 7 after entering the ICU of the First Affiliated Hospital of Chongqing Medical University were collected. According to the diagnostic criteria for P. aeruginosa (P.A.) pneumonia (pneumonia caused by P. aeruginosa) was diagnosed from X-ray films by the presence of continuous infiltration, accompanied by at least one of the symptoms of hypothermia (<35.0°C) or fever (≥38.0°C), pleuritic chest pain, cough, altered breathing sounds upon auscultation, and dyspnea (18). Definite causative pathogens were confirmed for isolates of P. aeruginosa obtained either from sputum culture, or good-quality sputum containing <10 epithelial cells and >25 polymorphonuclear cells in each particle field (100× magnification), and the dominant growth in sputum culture containing ≥10^7 colony-forming units (CFUs)/ml (19). Patients with pneumonia combined with P.A. were included in the P.A. pneumonia group (n = 27), and without P.A. infection were included in patients control subjects (22 patients randomly selected). We obtained patient data, such as the Acute Physiology and Chronic Health Evaluation II (APACHE II) score, levels of procalcitonin (PCT), and oxygenation index (PaO2/FiO2) when they entering the ICU. This study randomly selected. We obtained patient data, such as the Acute Physiology and Chronic Health Evaluation II score. The APACHE II score system includes: (1) acute physiology score, (2) agepoints, (3) chronichealthpoints (1). (d) The patient’s white blood cell (WBC), procalcitonin (PCT), C-reactive protein (CRP), PaO2/FiO2 ratio and APACHE II score were collected within 24 h of entering our ICU unit. (e) The survival indicates the patient’s survival on the 28th day.

**Human serum cytokine measurements**

Blood samples were collected through venipuncture from patients infected by P. aeruginosa, other ICU cases, or healthy people. The blood samples were centrifuged for 10 min at 1,000 g and 4°C immediately after collection. Serum samples were then divided evenly into the Eppendorf (EP) tubes and preserved at −80°C for subsequent experiments. The ELISA test kit (R&D Systems, California, # DY9110–05) was used for measuring IL-38 levels according to a previous method (17). Serum levels of IL-6, IL-8, IL-10, IL-1β, and TNF-α were detected at the Clinical Testing Center of The First Affiliated Hospital of Chongqing Medical University.

**Animals**

We obtained 8- to 12-week-old male C57BL/6 mice weighing 20 to 24 g from the Laboratory Animal Center of the Chongqing Medical University (Chongqing, China) under the license number SYXK (Chongqing, China) 2018–0003. All animals were housed under specific pathogen-free (SPF) conditions (relative humidity (RH) between 50% and 60%, temperature at 24°C, and light/dark cycle of 12/12 h). The mice had free access to food and water and were healthy (without infection) during the experiment. We randomly divided 35 mice into seven groups (normal control group, P.A. pneumonia model [24 h] group, P.A. pneumonia model (72 h) group, sham-operated group, P.A. pneumonia + IgG group, P.A. pneumonia + rIL-38 group, and P.A. pneumonia + anti-IL-38 group), each group with five mice.

Each animal was treated according to the guidelines for the care and use of laboratory animals in China. Our experimental protocols were approved by the Institutional Animal Care and Use Committee of the Chongqing Medical University.

**P. aeruginosa-induced pneumonia mouse model**

To perform experiments in vivo, 5 mg/kg xylazine and 100 mg/kg ketamine were used to anesthetize the animals, followed by the intranasal infection of P. aeruginosa (5 × 10^7 contained in 50 μL PBS) to simulate the natural pattern of human pneumonia infection (20). Each experiment was performed following the guidelines of the Institutional Animal Care and Use Committee of the Chongqing Medical University.

**Survival study**

The effect of IL-38 antibody: Thirty mice (n = 10/group) were randomly divided into a Sham-operated group, a P. aeruginosa + IgG group, and a P. aeruginosa + anti-IL-38. All mice had free access to food and water and were monitored every 24 h for 10 days to analyze the long-term effects of anti-IL-38.

The effect of recombinant IL-38: Thirty mice (n = 10/group) were randomly divided into a Sham-operated group, a P. aeruginosa + rIL-38 group, and a P. aeruginosa + rIL-38. All mice had free access to food and water and were monitored every 24 h for 10 days to analyze the long-term effects of rIL-38.

**In vivo administration of recombinant murine IL-38**

Recombinant murine IL-38 protein (1 μg, Adipogen, #AG-40B-0101) (15) was administered by intratracheal injection (i.t.) 2 h after bacterial infection. The mouse IgG was similarly injected as a control vehicle.

**Table 1. Characteristics of the study population**

| Characteristic | Pseudomonas aeruginosa pneumonia patient (n = 27) | Patient controls (n = 22) | Healthy controls (n = 17) |
|---------------|-----------------------------------------------|--------------------------|--------------------------|
| Age (years)   | 61 ± 8                                        | 57 ± 10                  | 59 ± 6                   |
| Biological sex (proportion of male) | 16/11 (59.2%) | 14/8 (63.6%) | 10/7 (58.8%) |
| WBC, 10/L     | 13.18 ± 3.93                                 | 12.79 ± 4.63             | 6.11 ± 3.28              |
| PCT, ng/mL    | 20.45 ± 9.41                                 | 9.62 ± 6.50              | 0.02 ± 0.01              |
| CRP, mg/mL    | 195.43 ± 93.60                               | 214.68 ± 82.80           | 73.86 ± 22.08            |
| P. aeruginosa, no. of patients | 27 | NA | NA |
| PaO2/FiO2 ratio | 232 ± 37                                     | 241 ± 46                 | NA                       |
| APACHE II score | 14 ± 4                                       | 16 ± 5                   | NA                       |
| Ventilator free days | 12.86 ± 5.68 | 8.73 ± 4.17 | NA |
| ICU free days  | 16 ± 6                                       | 10 ± 5                   | NA                       |
| Survival      | 20/27                                        | 17/22                    | NA                       |

(a) Data as a percentage of patients or median ± interquartile range. (b) ARDS acute respiratory distress syndrome. (c) APACHE II score: Acute Physiology and Chronic Health Evaluation II score. The APACHE II score system includes: (1) acutephysiology score, (2) agepoints, (3) chronichealthpoints (1). (d) The patient’s white blood cell (WBC), procalcitonin (PCT), C-reactive protein (CRP), PaO2/FiO2 ratio and APACHE II score were collected within 24 h of entering our ICU unit. (e) The survival indicates the patient’s survival on the 28th day.
IL-38 suppression in vivo

For blocking IL-38 in vivo, intranasal injection of 50 μg rat anti-mouse IL-38 antibody (R&D Systems, California, #MAB7774) (15) dissolved in 100 μL PBS was performed 2 h postinfection. The controls were injected with a mouse IgG.

Bronchoalveolar lavage fluid (BALF) and lung tissue collection

After 24 or 72 h of P. aeruginosa i.t., mice were sacrificed after being anesthetized and the BALF and lung tissues were harvested immediately (21). Then, the chest was tapped, the right bronchus was bundled, and the left lung was rinsed. The right lung was resected, then the upper right lobe was harvested for counting bacterial quantity, and the remaining tissue samples were immediately stored at −70 °C until further analysis.

Determination of lung bacterial burden

The right upper lung lobe was collected in an aseptic environment and homogenized using sterile saline (1 mL) by a tissue homogenizer in a vented hood. The lung homogenate was obtained by gradient dilutions, where 10 mL samples were added on the dry tryptic soy-base blood agar plates in solutions of different dilutions and incubated at 37 °C overnight. Then, we calculated the number of CFUs as the total CFUs/lung.

Measurement of inflammatory mediators

IL-38 concentration was measured by a murine IL-38 ELISA kit (R&D Systems, California, #DY2427–05) or the human IL-38 quantification ELISA kit (R&D Systems, California, #DY9110–05), as appropriate. We also measured inflammatory mediators, including TNF-α, IL-1β, IL-6, IL-17A, and IL-10, in the BALF or lung homogenate, using the Mice Cytokine Magnetic Bead Panel Kit (eBioscience, USA) according to specific instructions. IL-8 concentration in the BALF or lung homogenate was measured by a mouse IL-8 ELISA kit (Mybiosource, USA, #MBS7606860).

Histology

After inflation by 0.5% agarose, 10% formalin was used to fix lung tissues for 24 h, followed by formalin fixation, paraffin embedding, and slicing into 6 μm sections for immunohistochemical (IHC) staining. A pathologist assessed the pathology score of the lung surface according to previous descriptions. Typically, the pathologist was blinded to the information regarding edema, bronchitis, intra-alveolar inflammation, interstitial inflammation, endothelitis, pleuritis, and the proportion of the lung surface showing syncretic inflammatory infiltrate.

TUNEL assay

The TUNEL assay was performed to measure cell apoptosis by using the In Situ Cell Apoptosis Detection Kit I, POD (Roche, Switzerland) according to the manufacturer’s protocol. Briefly, 4-μm sections were deparaffinized with xylene and rehydrated using gradient ethanol. The activity of endogenous peroxidase was blocked using 3% hydrogen peroxide (H2O2) for 10 min, and the sections were incubated in a stop/wash buffer thrice, with 2 min of rinsing each time. Then, each section was incubated with the anti-digoxin antibody diluted at 1:20 in a reaction buffer (digoxigenin-labeled nucleotides) at 37 °C for 2 h. The sections were stained with a stop/wash buffer thrice, with 2 min of rinsing each time. Then, each section was incubated with the anti-digoxin antibody diluted at 1:100 for 30 min at 37 °C, and later with ABC at 37 °C for another 30 min. The 3’,3’-Diaminobenzidine chromogen was used to incubate sections for approximately 20 min, and the sections were counterstained with hematoxylin to measure apoptosis. Subsequently, we randomized five fields of view (FOVs) in each section (400× magnification). Finally, we measured the proportion of TUNEL-positive cells in each of the five FOVs at 400× magnification.

Western blot (WB) analysis

Using the protein extraction kit (Beyotime, China), total protein was extracted from lung tissues or T lymphocytes. The BCA protein detection kit (Pierce, USA) was used to measure the protein content. After separating the proteins...
using 10% SDS-PAGE, they were transferred onto the nitrocellulose membranes. Next, 5% (w/v) skimmed milk in Tris-buffered saline containing 0.05% Tween-20 was used to block the membranes, followed by overnight membrane incubation using the β-actin antibody (#3700) or anti-TBK1/NAK (E9H5S) antibody (both obtained from the Cell Signaling Technology, USA, #51872) at 4°C. An imaging system (BIO-RAD) was used for band densitometry.

Isolation cells from lung tissue

After the mice were sacrificed, 5 mL of PBS/0.6 mM EDTA was injected into the right ventricular cannula for lung perfusion. The lung was then isolated from the surrounding tissue and added to medium containing digestive enzymes (RPMI 1640, 10%FCS, 175U/mLcollagenase, 75U/mLDNase 1, 0.2U/mLpancreatic elastase, 100 U/mL penicillin, and 100 mg/mL streptomycin) for incubation for 45 min at 37°C. The resulting suspension was passed three times through a 19- gauge needle to break up clumps and then through a 40-mm filter to remove debris. The leukocytes were enriched by discontinuous Percoll gradient centrifugation and recovered at the interface between 40% Percoll and 70% Percoll layers. Then, cells obtained from lungs for flow cytometric analysis.

Flow cytometry

To separate the cells from the supernatant, we centrifuged the BALF at 400 g for 10 min and washed the separated cells with PBS before converting them into pellets, followed by flow cytometric analysis. Monoclonal antibodies Foxp3 and CD4 were used, which were stained using the anti-Foxp3-APC, anti-CD4-FITC, and Fixation/Permeabilization kits (eBioscience, USA) following specific protocols. Cells (10⁶) were harvested by the FACScan flow cytometer (Becton Dickinson, USA) and analyzed by the Flow Jo software 7.6.

Cell purification and culture

Peripheral blood mononuclear cells (PBMCs) were isolated from the mouse spleen by the Lymphocyte Separation Medium (GE Healthcare, USA). Then, naive CD4⁺ T cells were isolated from the collected PBMCs by magnetically activated cell sorting (MACS, Miltenyi Biotec, Bergisch Gladbach, Germany) with the use of the Naïve CD4⁺ T Cell Isolation Kit II (STEMCELL Technologies, Canada) according to specific protocols. The purity of naive CD4⁺ T cells (>90%) was determined by flow cytometry. Cells were cultured using the RPMI 1640 growth medium (Gibco, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS) at 5% CO₂ and 37°C.

Treg cell subset generation

Treg cell subsets were produced by culturing Treg cells in L-glutamine (2 mM), β-mercaptoethanol (50 mM), anti-CD3 (5 μg/mL), anti-CD28 (2 μg/mL), IL-2 (50 U/mL), and TGF-β (2.5 ng/mL) for 3 days. To determine the role...
of IL-38 in induction, recombinant murine IL-38 protein was added to the culture medium. The levels of surface markers and intracellular staining were determined by flow cytometry.

Statistical analysis

Data were statistically analyzed in SPSS19.0 (IBM, Armonk, NY) and are shown as mean ± SD or median (with interquartile ranges). Mann-Whitney U tests were performed to assess the differences between two specific groups, and one-way ANOVA was performed for comparing multiple groups. Survival curves were assessed by the log-rank (Mantel-Cox) test. Differences between and among groups were considered to be statistically significant at P < 0.05.

RESULTS

IL-38 levels in the serum of adult patients with P.A. pneumonia

For the 27 adults affected by P.A. pneumonia, the serum IL-38 levels on the day of ICU admission markedly increased compared to that in the normal controls and ICU controls (Fig. 1A). High circulating levels of IL-38 were detected between the onset of P.A. pneumonia and 3 days after the onset of the disease. The serum IL-38 levels showed a decreasing trend but markedly increased on day 7 relative to the levels in the normal controls (Fig. 1B). The serum IL-38 level of the surviving patients on the day of admission to the ICU was also significantly higher than that of patients with P.A. pneumonia (Fig. 1C). Besides, the serum IL-38 level of patients with P.A. pneumonia on the day of admission to the ICU showed significantly positive correlations with IL-10 and the PaO2/ FiO2 ratio (oxygenation index) but showed negative correlations with IL-1β, IL-6, IL-8, IL-17, TNF-α, APACHE II score, and PCT (Fig. 1D).

P. aeruginosa impaired the host pulmonary immunity in mice

As we have observed that IL-38 is highly expressed in patients with P.A. pneumonia in the early stage, and then gradually decreases. Next, whether this expression trend is also the same in our experimental model. Subsequently, we adopted the extensively used mouse model of bacterial pneumonia induced through the intranasal injection of

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**Fig. 3.** IL-38 blockade aggravated Pseudomonas aeruginosa-induced pneumonia. C57BL/6 mice were administered with anti-IL-38 antibodies (50 μg, R&D Systems) at 2 h after modeling. Mouse IgG served as the control group. (A) Pneumonia mouse survival (n = 10 mice/group) after IL-38 suppression following modeling by anti-IL-38 treatment, with mouse IgG used as the control. Kaplan-Meier analysis was performed to compare between the two groups through logrank tests; # P < 0.01 relative to isotypical IgG-treated septic mice. (B) Representative H&E stained lung tissues after 24 h of infection with P. aeruginosa and treatment with IgG or anti-IL-38 antibodies. Histological scores of the P. aeruginosa-induced pneumonia after exposure to recombinant anti-IL-38 or IgG (n = 5 mice/group). (C) Cell apoptosis was determined by TUNEL assay, and TUNEL-positive cells showed dark-brown nuclei. (D) Bacterial number in the mouse BALF (n = 5 mice/group) following anti-IL-38 or IgG treatment, at 24 h following modeling. The comparison between the two groups was performed by one-way ANOVA followed by the LSD multiple comparisons test.
**P. aeruginosa.** At 24 h postinfection, IL-38 levels markedly increased in the lung homogenates and BALF in *P. aeruginosa*-infected C57BL/6 mice (*P* < 0.01). After 72 h, its level decreased gradually; the decrease was significant (Fig. 2A). The lung histopathological and CFU examination showed that when the infection was prolonged, the number of CFUs in the lungs and the lung injury score gradually increased (Fig. 2B and C). Furthermore, the results of the flow cytometry analysis also showed that the expression of Treg cells in the BALF and lungs had a similar trend as IL-38 (Fig. 2D). When the P.A. pneumonia lasted for a longer time in the mouse model, the BALF or serum levels of cytokines, such as TNF-α, IL-1β, IL-6, IL-17A, and IL-8, showed an increasing trend, whereas that of IL-10 showed a decreasing trend (Fig. 2E and F). Collectively, the above results suggested that the mouse model of P.A. pneumonia had similar IL-38 expression to human P.A. pneumonia. Additionally, IL-38 was possibly associated with Treg cells.

**IL-38 blockade aggravated P.A. pneumonia**

After observing the levels of IL-38 in experimental and clinical sepsis, we used IL-38 antibodies for determining the effect of IL-38 on P.A. pneumonia. After P.A. pneumonia was induced in C57BL/6 mice, IL-38 antibodies were used to treat mice 2 h postoperatively. After treatment, the P.A. pneumonia mouse survival rate remarkably decreased relative to the survival of the mice treated with the control IgG (Fig. 3A). The H&E- stained lung section analysis revealed a higher lung injury score in mice from the P.A. pneumonia group compared to those in the IgG group following IL-38 antibody exposure (Fig. 3B). Additionally, the proportion of TUNEL-positive cells were elevated after treatment with IL-38 antibodies in the P.A. pneumonia group of mice (Fig. 3C). Furthermore, P.A. pneumonia mice exposed to IL-38 antibody treatment showed a higher mortality rate, which was related to an increased bacterial load, as verified through the comparison of CFUs in the BALF (Fig. 3D).

**Table 2. Cytokine Levels in Mice Lung Homogenate or BALF during *P. aeruginosa*-induced Pneumonia after treatment with anti-IL-38**

| Cytokine       | Sham-operated | *P. aeruginosa*+IgG | *P. aeruginosa*+anti-IL-38 |
|----------------|---------------|---------------------|---------------------------|
| **Lung homogenate** |               |                     |                           |
| TNF-α          | 105.97± 18.59*** | 1255.32± 317.76     | 1824.67± 404.13*          |
| IL-6           | 1350.26± 156.29**** | 15160.37± 4430.59    | 21043.11± 3028.03*         |
| IL-1β          | 320.21± 26.38**  | 951.32± 254.73      | 1636.45± 468.54*           |
| IL-8           | 55.32± 13.11***  | 313.75± 73.81       | 478.09± 76.98**            |
| IL-17A         | 44.59± 10.03**** | 256.54± 52.48       | 400.87± 91.13*             |
| IL-10          | 808.85± 189.48*** | 2386.21± 851.60     | 1292.86± 274.91*           |
| **BALF**       |               |                     |                           |
| TNF-α          | 102.13± 33.89**** | 422.12± 137.91     | 866.40± 288.04*            |
| IL-6           | 1087.07± 209.59**** | 15012.05± 2679.06   | 20653.54± 3649.02*         |
| IL-1β          | 136.25± 48.52*** | 623.99± 216.47      | 1197.54± 273.81**          |
| IL-8           | 80.43± 31.95**** | 336.29± 72.93       | 553.30± 80.19***           |
| IL-17A         | 24.71± 8.52****  | 123.14± 19.43       | 238.12± 41.92***           |
| IL-10          | 1300.69± 321.35**** | 4006.26± 390.18    | 2534.03± 461.71***         |

Data are expressed as the median (interquartile range) of n = 5 mice per group. Measurements are expressed as picograms per milliliter. *p*<0.05, **p<0.01, ***p<0.001, ****p<0.0001, by one-way ANOVA followed by LSD multiple comparisons test compared with *P. aeruginosa*+IgG group.

**Fig. 4.** (A and B) IL-38 inhibition increased the release of pro-inflammatory factors but decreased the release of anti-inflammatory factors in the P.A. pneumonia mouse model. After recombinant IL-38 or IgG treatment, cytokine levels in the BALF and lung homogenates in five mice were measured using the mouse cytokine magnetic bead panel kit at 24 h following modeling. Increased levels of IL-6, IL-1β, IL-8, IL-17A, and TNF-α, and a decreased level of IL-10 in the BALF and lung were observed following recombinant IL-38 stimulation. (C) T cells were isolated from the mouse BALF and lungs. Flow cytometry was performed to determine the proportion of CD4+ Foxp3+ Tregs, and the data from five mice were averaged for the final result. The comparison between the two groups was performed by one-way ANOVA followed by the LSD multiple comparisons test.

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Administration of anti-IL-38 increased the production of cytokines and ablated Treg cells during P.A. pneumonia

The effect of anti-IL-38 on *P. aeruginosa*-induced cytokines in the BALF and lungs was evaluated 24 h after modeling. For the P.A. pneumonia model, anti-IL-38 exposure enhanced cytokine response, leading to a significant increase in the levels of TNF-α, IL-1β, IL-6, IL-17, and IL-8, but not IL-10 (Fig. 4A and B). Moreover, as suggested by the results of the flow cytometry assay, the Treg cells in the BALF and lungs remarkably decreased after anti-IL-38 exposure for 24 h (Fig. 4C).

IL-38 administration protected against P.A. pneumonia

After anti-IL-38 treatment, bacterial pneumonia was aggravated in mice. We conducted a rescue assay to determine the impact of recombinant IL-38 protein exposure. After 2 h from the onset of P.A. pneumonia, recombinant IL-38 protein treatment enhanced the survival of the treated mice compared to the control mice exposed to vehicle treatment (Fig. 5A). This highlighted the therapeutic effect of IL-38 on P.A. pneumonia. The recombinant IL-38 protein-treated mice showed a decreased bacterial load in the BALF 2 h postmodeling (Fig. 5B). Mice with therapeutic rIL-38 administration also exhibited a lower lung injury score and pulmonary TUNEL-positive cells (Fig. 5C and D).

IL-38 regulated inflammatory responses in the P.A. pneumonia mouse model

For assessing whether IL-38 protected the mice from P.A. pneumonia, the mice were treated with recombinant IL-38 protein or IgG. The rIL-38 treated group exhibited a significant decrease in cytokines and chemokines (e.g., TNF-α, IL-1β, IL-6, IL-17A, and IL-8) in the lung homogenates and BALF, relative to the IgG group, whereas, IL-10 levels were markedly elevated (Fig. 6A and B). Notably, treatment with recombinant IL-38 protein significantly enhanced Treg cells in the BALF and lungs of IL-38-treated mice compared to that in the IgG-treated mice (Fig. 6C). The above results elucidated the IL-38 related immune mechanism in the regulation of P.A. pneumonia in mice.

IL-38 promoted the differentiation of regulatory T lymphocytes through downregulation of the TBK1 signaling pathway

Treg lymphocytes in the mouse BALF have been previously suggested to markedly aggravate the effects of P.A. pneumonia.
in mice treated with recombinant IL-38 protein. To better understand, the role of IL-38 in the differentiation of Treg lymphocytes, we isolated the splenic naïve CD4+ T lymphocytes for in vitro culture. After rIL-38 treatment for 3 days, naïve CD4+ T lymphocytes were found to differentiate into Treg cells (Fig. 7A). The TBK1 signaling pathway is important in the differentiation of naïve CD4+ T lymphocytes to Treg cells. To elucidate the effect of IL-38 on the TBK1 signal transduction pathway, a WB assay was performed for detecting TBK1 expression in T lymphocytes. The TBK1 expression showed a gradually decreasing trend in T lymphocytes with the increase in the level of recombinant IL-38 (Fig. 7B).

**DISCUSSION**

*Pseudomonas aeruginosa*-induced pneumonia is a common and refractory disease in the intensive care unit (ICU). With the progress of the disease, patients are prone to complications such as ARDS and multiple organ dysfunction syndrome (MODS), which endanger their lives (19, 22, 23). As *P. aeruginosa* can easily develop drug resistance, traditional treatment methods like antibacterial drugs, mechanical ventilation, fluid support, etc. are not sufficient (24). In recent years, studies have revealed that the inflammatory responses mediated by inflammatory factors play a vital role in the outcome of P.A. pneumonia, and the imbalance between pro-inflammatory and anti-inflammatory factors can worsen the disease (25, 26). As an important inflammatory mediator, interleukin is a vital part of the disease occurrence and prognosis (27–30). Some researchers performed detailed studies on IL-6, IL-8, and TNF-α in elderly patients with severe pneumonia, and found that the above cytokines were significantly higher in those patients (31). These cytokines can cause artery spasm, platelet aggregation, abnormal blood viscosity, and microvascular thrombosis, causing multiple organ failure (32–35). Therefore, targeted therapy specific to immune activity is one promising approach. IL-38 is a new type of anti-inflammatory cytokine, which has an antiinflammatory effect in inflammatory bowel disease (13), osteoarthritis (14), sepsis (15), and other inflammatory diseases (16). Our previous studies found that in the model of cecal

**Table 3. Cytokine Levels in Mice Lung Homogenate or BALF during *P. aeruginosa*-induced Pneumonia after treat with rIL-38**

|                | Sham-operated | P. aeruginosa+lgG | P. aeruginosa+rIL-38 |
|----------------|---------------|-------------------|----------------------|
| **Lung homogenate** |               |                   |                      |
| TNF-α          | 174.43± 59.36*** | 1228.83± 376.55   | 735.20± 238.45*      |
| IL-6           | 1844.3± 355.41*** | 16064.7± 1831.95 | 9546.31± 1325.11***  |
| IL-1β          | 257.99± 95.59*** | 827.21± 242.15    | 446.59± 188.15*      |
| IL-8           | 75.63± 15.05**** | 375.65± 82.97     | 178.13± 65.25**      |
| IL-17A         | 44.07± 16.05**   | 196.19± 64.92     | 108.30± 33.82*       |
| IL-10          | 1086.38± 239.44*** | 2684.49± 640.83   | 3870.09± 831.76*     |
| **BALF**       |               |                   |                      |
| TNF-α          | 102.79± 22.05*** | 667.94± 219.45    | 381.99± 73.45*       |
| IL-6           | 1078.56± 324.01**** | 12675.99± 1950.04 | 6705.68± 2832.35***  |
| IL-1β          | 104.74± 55.03**** | 696.34± 188.75    | 377.26± 135.65**     |
| IL-8           | 72.65± 23.48**   | 404.71± 114.09    | 155.57± 43.68**      |
| IL-17A         | 36.02± 11.48**   | 149.40± 36.49     | 78.94± 24.99**       |
| IL-10          | 706.69± 197.68** | 3387.75± 1185.29  | 5095.99± 1204.81*    |

Data are expressed as the median (interquartile range) of n = 5 mice per group. Measurements are expressed as picograms per milliliter. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001, by one-way ANOVA followed by LSD multiple comparisons test compared with P. aeruginosa+lgG group.

Fig. 6. (A and B) IL-38 treatment decreased the generation of pro-inflammatory factors in the pneumonia models. After the recombinant IL-38 treatment, cytokine levels in the BALF and lung homogenates in five mice were measured using the mouse cytokine magnetic bead panel kit at 24 h following modeling. Decreased levels of IL-8, IL-1β, IL-6, IL-17A, and TNF-α, and an elevated level of IL-10 in the BALF and lungs were observed following recombinant IL-38 stimulation. (C) We isolated T cells from the BALF or lungs of mice. The frequency of Tregs was subsequently determined by flow cytometry and the results were from five mice per time point. Shown here are representative dot plots. The comparison between the two groups was performed by one-way ANOVA followed by the LSD multiple comparisons test.
ligation and puncture (CLP)-induced ARDS, IL-38 played a protective role by downregulating the differentiation of Th17 (17). In this study, we observed that IL-38 may be insufficiently secreted in the early stage of patients with P.A. pneumonia and cause of death. And, it is negatively correlated with a variety of pro-inflammatory cytokines. This suggested that IL-38 might be a novel molecule for adjuvant therapy in P.A. pneumonia.

The experimental induction of P.A. pneumonia resulted in increased local IL-38 levels in mice, which was associated with the higher IL-38 levels found in clinical studies. Although in our previous study there was no statistical difference in the expression of IL-38 in the lungs after 6 and 24 h in the LPS- induced ARDS model, it showed an increasing trend in the initial stage of infection and then gradually decreased, which was consistent with the results of our present study. This may be related to the dose of LPS and the virulence of *P. aeruginosa*. Therefore, on further investigation, we found that IL-38 administration protected mice from experimental P.A. pneumonia and IL-38 blockade aggravated the experimental model. Lung damage caused by *P. aeruginosa* is associated with the unregulated inflammation resulting from a significant increase in chemokines and cytokines (36).

Consequently, IL-38-mediated protection during the experimental P.A. pneumonia was related to the mitigation of inflammation. IL-38 possibly counteracted the different inflammatory disorders, shown by the reduced systemic and local inflammation (such as chemokines, IL-17A, IL-6, TNF-α, etc.), and improvement of bacterial clearance. Therefore, we also found that the administration of recombinant IL-38 protein can effectively reduce *P. aeruginosa* from the lungs, and the clearance rate of *P. aeruginosa* was reversed after administering the IL-38 antibody.

Endotoxin (lipopolysaccharide LPS) is the main pathogenic substance in *P. aeruginosa*, and its virulence is related to its
quantity (37). The LPS-mediated acute lung injury (ALI) represents one of the inflammatory pulmonary disorders, which is characterized by the overproduction of pro-inflammatory factors, alveolar epithelial cell apoptosis, and inflammatory cell infiltration (38, 39). The control of abnormal inflammation and apoptosis greatly helps to improve the prognosis (40). The aggravated alveolar epithelial cell apoptosis is the main cause of ALI (41), and the overproduction of inflammatory factors like ROS, IL-1β, IL-6, and TNF-α, results in cell apoptosis (42, 43). Therefore, the inhibition of epithelial cell apoptosis provides a target for the treatment of ALI (44). In this study, we found that lung apoptosis markedly aggravated P.A. pneumonia in the mouse model. Nonetheless, recombinant IL-38-based treatment remarkably decreased mouse lung apoptosis. The above results indicated another key IL-38-mediated regulatory mechanism in P.A. pneumonia.

The occurrence, development, and outcome of bacterial pneumonia are largely related to cellular immune dysfunction (45). The body’s immune response against bacterial infection is mainly via cellular immunity (46). T lymphocytes are the core cells that mediate cellular immunity, and they are also the regulators of the immune response (47). When the body is invaded by microorganisms, T lymphocytes differentiate, proliferate, synthesize, and secrete a series of cytokines, and produce specific cellular immunity (48). Treg, an important subgroup of CD4+ T lymphocytes, controls autoimmune reactivity through cell contact and produces inhibitory cytokines (49). It has an immunosuppressive function that can reduce inflammation in the body (50). Treg cells have been shown to reduce lung injury by regulating the immune response (51). Immunoglobulins can further promote the production of Treg cells and immunosuppressive function to improve the progression of P.A. pneumonia (52). According to our results, IL-38 elevated the Treg cell proportion in the BALF of the experimental mice. To better understand, the role of IL-38 in the differentiation of T lymphocytes, we isolated splenic naïve CD4+ T lymphocytes, controls autoimmune reactivity through cell contact and produces inhibitory cytokines (49). It has an immunosuppressive function that can reduce inflammation in the body (50). Treg cells have been shown to reduce lung injury by regulating the immune response (51). Immunoglobulins can further promote the production of Treg cells and immunosuppressive function to improve the progression of P.A. pneumonia (52). According to our results, IL-38 elevated the Treg cell proportion in the BALF of the experimental mice. To better understand, the role of IL-38 in the differentiation of T lymphocytes, we isolated splenic naïve CD4+ T lymphocytes from mice to culture in vitro. According to our results, IL-38 remarkably enhanced the differentiation of naïve CD4+ T lymphocytes into Treg cells. Furthermore, T lymphocyte differentiation requires the activation of signaling pathways. T-cell subset development is mostly modulated via STAT phosphorylation of different cytokine stimuli. However, it has been strongly suggested that other signal transduction pathways are also related to the regulation of the differentiation of T cells (53). TANK Binding Kinase 1 (TBK1) is a serine/threonine methionine kinase at the center of the signal pathway that maintains homeostasis of the immune system (54) and is abundantly expressed in lymphocytes (55). Studies have shown that TBK1 can be used as a signal center and cell-type-specific regulator for virus-induced lung injury. It regulates the recruitment and cytokine expression of inflammatory macrophages in the lungs (56). Moreover, TBK1 deficiency effectively inhibits the phosphorylation at S727 of STAT3, which regulates T cell activation and autophagy (55). Other studies have found that the TBK1 inhibitor Alexino (ALX) effectively reduces TBK1/AKT and TBK1/IRF3 signals and increase Tregs to alleviate the progression of autoimmune encephalomyelitis (EAE) (57). It is speculated that the competitive binding of IL-38 with IL-36R can block the recruitment of the trimeric complex composed of IL-1RAcP and inhibit the strong inflammatory signal in the cell (13). A recent study showed that IL-38 enhanced CD4+ CD25+ Treg cells for immunosuppression, thus facilitating the improvement of host immunity and sepsis prognosis (58). Our in vitro cell experiments confirmed that IL-38 effectively reduced the TNK1 signal expression of initial T lymphocytes, enhanced the differentiation of naïve T lymphocytes to Treg cells.

**CONCLUSION**

Our data indicate that IL-38 plays an important protective role during *P. aeruginosa*-induced pneumonia by regulating the dynamic balance of pro-inflammatory and anti-inflammatory factors, enhancing the elimination of *P. aeruginosa* bacteria and reducing cell apoptosis. Furthermore IL-38 could promote the differentiation of naïve CD4+ T cells into CD4+Foxp3+ Tregs, which help control inflammation in P.A. pneumonia, and these effects of IL-38 were increased through inhibiting TBK1 pathway.

REFERENCES

1. Pappas G, Saplaoura K, Falagas ME: Current treatment of pseudomonal infections in the elderly. *Drugs Aging* 26(5):363–379, 2009.
2. Jara MC, Frediani AV, Zehetmeyer FK, Bruhn FRP, Muller MR, Miller RG, Nascente PDS: Multidrug-resistant hospital bacteria: epidemiological factors and susceptibility profile. *Microbiol Drug Resist* 27(3):433–440, 2021.
3. Omar T, Ziltener P, Chamberlain E, Cheng Z, Johnston B: Mice lacking gammadelta T cells exhibit impaired clearance of pseudomonas aeruginosa lung infection and excessive production of inflammatory cytokines. *Infect Immun* 88(6):111, 2020.
4. Lynch JP 3rd: Hospital-acquired pneumonia: risk factors, microbiology, and treatment. *Chest* 119(2(Suppl)):373S–384S, 2001.
5. Bassetti M, Vena A, Crozatto A, Righi E, Guery B: How to manage Pseudomonas aeruginosa infections. *Drugs Context* 7:212527, 2018.
6. Salerian AJ: Burn wound infections and Pseudomonas aeruginosa. *Burns* 46 (1):257–258, 2020.
7. Warren AE, Boullaine-Larsen CM, Chandler CB, Chiotti K, Kroll E, Miller SR, Taddi F, Seremet-Gaudelus I, Ferromi A, McInnerny K, et al.: Genotypic and phenotypic variation in *Pseudomonas aeruginosa* reveals signatures of secondary infection and mutator activity in certain cystic fibrosis patients with chronic lung infections. *Infect Immun* 79(12):4802–4818, 2011.
8. Ding C, Yang Z, Wang J, Liu X, Cao Y, Pan Y, Han L, Zhan S: Prevalence of *Pseudomonas aeruginosa* and antimicrobial-resistant *Pseudomonas aeruginosa* in patients with pneumonia in mainland China: a systematic review and metaanalysis. *Int J Infect Dis* 49:119–128, 2016.
9. Raman G, Avendano EE, Chan J, Merchant S, Puzniak L: Risk factors for hospitalized patients with resistant or multidrug-resistant *Pseudomonas aeruginosa* infections: a systematic review and metaanalysis. *Int J Infect Dis* 79:13, 2018.
10. Ribeiro A, Crozatti MTL, Silva AAD, Macedo RS, Machado AMO, Silva ATA: *Pseudomonas aeruginosa* in the ICU: prevalence, resistance profile, and antimicrobial susceptibility. *Rev Soc Bras Med Trop* 53:e20180498, 2019.
11. Mizgerd JP: Respiratory infection and the impact of pulmonary immunity on lung health and disease. *Am J Respir Crit Care Med* 186(9):824–829, 2012.
12. van de Veerdonk FL, de Graaf DM, Joosten LA, Dinarello CA: Biology of IL-38 and its role in disease. *Infect Immun* 281(1):191–196, 2018.
13. Bouvet MA, Bart G, Penhoat M, Aimand J, Brunil B, Charrier C, Morel F, Lecron JC, Rolli-Derkinderen M, Bourreille A, et al.: Distinct expression of interleukin (IL)-36alpha, beta and gamma, their antagonist IL-36Ra and IL-38 in psoriasis, rheumatoid arthritis and Crohn’s disease. *Clin Exp Immunol* 184(2):159–173, 2016.
14. Jiang L, Zhou X, Huang C, Bao J, Li J, Xu K, Dong D, Wu L: The elevated expression of IL-38 serves as an anti-inflammatory factor in osteoarthritis and its protective effect in osteoarthritic chondrocytes. *Int Immunopharmacol* 94:107489, 2021.
15. Xu F, Lin S, Yan X, Wang C, Tu H, Yin Y, Cao J: Interleukin 38 protects against lethal sepsis. *J Infect Dis* 218(7):1175–1184, 2018.
27. Shindo Y, Fuchs AG, Davis CG, Eitas T, Unsinger J, Burnham CD, Green JM, Coopersmith CM, Amiot DM 2nd, Stromberg PE, Dunne WM, Davis CG, Weber A, Zimmermann C, Mausberg AK, Dehmel T, Kieseier BC, Hartung HP, Wang T, Hou Y, Wang R: A case report of community-acquired Pseudomonas aeruginosa pneumonia during sepsis associated immunity and increased clot lysis time due to IL-1beta, IL-6 and IL-8.

19. von Baum H, Welte T, Marre R, Suttorp N: Ewig S, group Cs. Community-acquired pneumonia through Enterobacteriaceae and Pseudomonas aeruginosa: criteria.

17. Chai YS, Lin SH, Zhang M, Deng L, Chen Y, Xie K, Wang CJ, Xu F: IL-38 is a novel cytokine that improves host immunity and survival in a two-hit model of Pseudomonas aeruginosa pneumonia.

34. Bester J, Matshailwe C, Pretorius E: Simultaneous presence of hypercoagulation and increased clot lysis time due to IL-1beta, IL-6 and IL-8. Cytokine 110:237242, 2018.

16. Xu WD, Huang AF: Role of interleukin-38 in chronic inflammatory diseases: a comprehensive review. Front Immunol 9:1462, 2018.

18. Xia Y, Zou Q, Xie J, Liu Y, Wang R: A case report of community-acquired Pseudomonas aeruginosa pneumonia complicated with MODS in a previously healthy patient and related literature review. BMC Infect Dis 19(1):130, 2019.

25. Liu ZF, Zheng D, Fan GC, Peng T, Su L: Heat stress prevents lipopolysaccharide-induced apoptosis in pulmonary microvascular endothelial cells by blocking calpain/p38 MAPK signalling. Apoptosis 21(8):896–904, 2016.

10. Wang T, Hou Y, Wang R: A case report of community-acquired Pseudomonas aeruginosa pneumonia complicated with MODS in a previously healthy patient and related literature review. BMC Infect Dis 19(1):130, 2019.

22. Wang T, Hou Y, Wang R: A case report of community-acquired Pseudomonas aeruginosa pneumonia complicated with MODS in a previously healthy patient and related literature review. BMC Infect Dis 19(1):130, 2019.

16. Xu WD, Huang AF: Role of interleukin-38 in chronic inflammatory diseases: a comprehensive review. Front Immunol 9:1462, 2018.

18. Xia Y, Zou Q, Xie J, Liu Y, Wang R: A case report of community-acquired Pseudomonas aeruginosa pneumonia complicated with MODS in a previously healthy patient and related literature review. BMC Infect Dis 19(1):130, 2019.