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ORIGINAL ARTICLE

Partial Depletion of Regulatory T Cells Enhances Host Inflammatory Response Against Acute *Pseudomonas aeruginosa* Infection After Sepsis

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Abstract— Immune dysfunction contributes to secondary infection and worse outcomes in sepsis. Regulatory T cells (Tregs) have been implicated in sepsis-induced immunosuppression. Nevertheless, the role of Tregs in secondary infection after sepsis remains to be determined. In the present study, a two-hit model which mimics clinical conditions was used and the potential role of Tregs in secondary *Pseudomonas aeruginosa* infection post-sepsis was investigated. Results showed that mice were susceptible to secondary *P. aeruginosa* infection 3 days, but not 7 days, post-cecal ligation and puncture (CLP). The levels of IL-17A, IL-1β, and IL-6 remained low in CLP mice after *P. aeruginosa* infection, while the levels of IL-10 increased significantly. Additionally, increased number of Tregs in both lung and spleen was observed in "two-hit" mice. Injection with PC61 (anti-CD25) mAb reduced the number of Tregs by 50% in spleen and 60% in lung of septic mice. This partial depletion of Tregs elevated IL-17A, IL-1β, and IL-6 production and decreased IL-10 levels in septic mice with *P. aeruginosa* infection, leading to lower bacterial load, attenuation of lung injury, and improvement of survival. The present findings demonstrate that Tregs play a crucial role in secondary *P. aeruginosa* infection after sepsis by modulating the inflammatory response.

KEY WORDS: sepsis; secondary infection; nosocomial infection; sepsis; regulatory T cells; CD25.

INTRODUCTION

Sepsis is a life-threatening organ dysfunction caused by a dysregulated host response to microbial infection [1]. Due to the advances in early goal-directed therapy, new antibiotics, and adjunct strategies, more and more septic patients survive the phases of acute circulation failure and organ dysfunction. It has been found that about 60–70% of septic deaths occur in the late phase (≥ 3 days) of the disease and that most late deaths were related to ICU-acquired complications, including nosocomial infections [2]. Recently, a prospective study found that ICU-acquired infections contributed to overall mortality in septic patients [3]. Similarly, our previous study demonstrated that the risk of late death for septic shock patients with

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secondary infection was about 5.8 times higher than that without secondary infection [4].

It is well-known that, after a transient hyper-inflammatory phase, patients suffering from sepsis rapidly progress to a prolonged immunosuppressive state which is characterized by the defects of both innate and adaptive immune responses [5–8]. The unique immunosuppressive status of sepsis patients contributes to their susceptibility to secondary infection. $CD4^+CD25^+$ regulatory T cells (Tregs) have been shown to be crucial in regulating the immune responses in tumor immunity, transplantation tolerance, and infectious diseases [9–11]. Forkhead/winged helix transcription factor p3 (Foxp3) is principally found within the $CD4^+CD25^+$Treg cell population and plays an important role in the development and functionality of these cells [12]. Tregs control the immune response by inhibiting the activation of effector T lymphocytes and suppressing the maturation and function of antigen presenting cells [13]. The enhanced suppressive function of $CD4^+CD25^+$Tregs has been shown to be associated with fatal outcomes in burn patients [14]. Similar results were also observed in patients with septic shock. Additionally, various clinical studies illustrated that the expansion of $CD4^+CD25^+$Tregs and elevated Foxp3 levels were associated with a higher risk of nosocomial infection in critically ill patients including sepsis [15–17]. So, we hypothesized that depletion of Tregs might be an effective strategy to prevent secondary infection in sepsis.

It should be noted that, at different phases of sepsis, the same immune cells may have different functions. It has been reported that $Foxp3^+$Tregs are crucial for minimizing host tissue damage when the initial cytokine storm has significantly decreased in septic animals and that these cells are required for recovery from severe sepsis [7]. Moreover, the complete loss of $Foxp3^+$Tregs has been proven to increase the number of deaths from sepsis in animal models [18]. As about 80% of $CD25^+$lymphocytes and 90% of $CD4^+CD25^+$T cells express Foxp3, the monoclonal rat anti-mouse CD25 clone PC61 was widely used to deplete Tregs in experimental studies, and a 30–60% reduction in $Foxp3^+$cells in the spleen was observed after PC61 mAb treatment [19–21]. So, PC61 mAb was used to deplete Tregs in the present study. We found that Tregs contributed to immunosuppression and secondary infection in sepsis. Depletion of Tregs by PC61 mAb treatment rescues septic mice with secondary *Pseudomonas aeruginosa* infection. Thus, Tregs may be a potential therapeutic target for limiting secondary infection in sepsis.

Fig. 1. Time course analysis of susceptibility to *P. aeruginosa* infection after sepsis. Survival curves of septic mice with secondary *P. aeruginosa* infection 3 days (**a**) and 7 days (**b**) post-CLP. Septic mice and sham controls were infected with *P. aeruginosa* 3 or 7 days after surgery. Results of three independent studies were combined. $n = 10$ per group. **c** Pathological score of lung tissue in each group 24 h after secondary infection. Pathological scores of lung tissues in six groups were shown ($n = 8$). **d** Lung colony-forming units (CFUs). BAL fluids of each groups were collected for culturing and the number of bacterial colonies was counted 24 h later ($n = 8$). PA, *P. aeruginosa*; Sal, saline; CLP, cecal ligation and puncture. $^{***}p < 0.001$.

MATERIALS AND METHODS

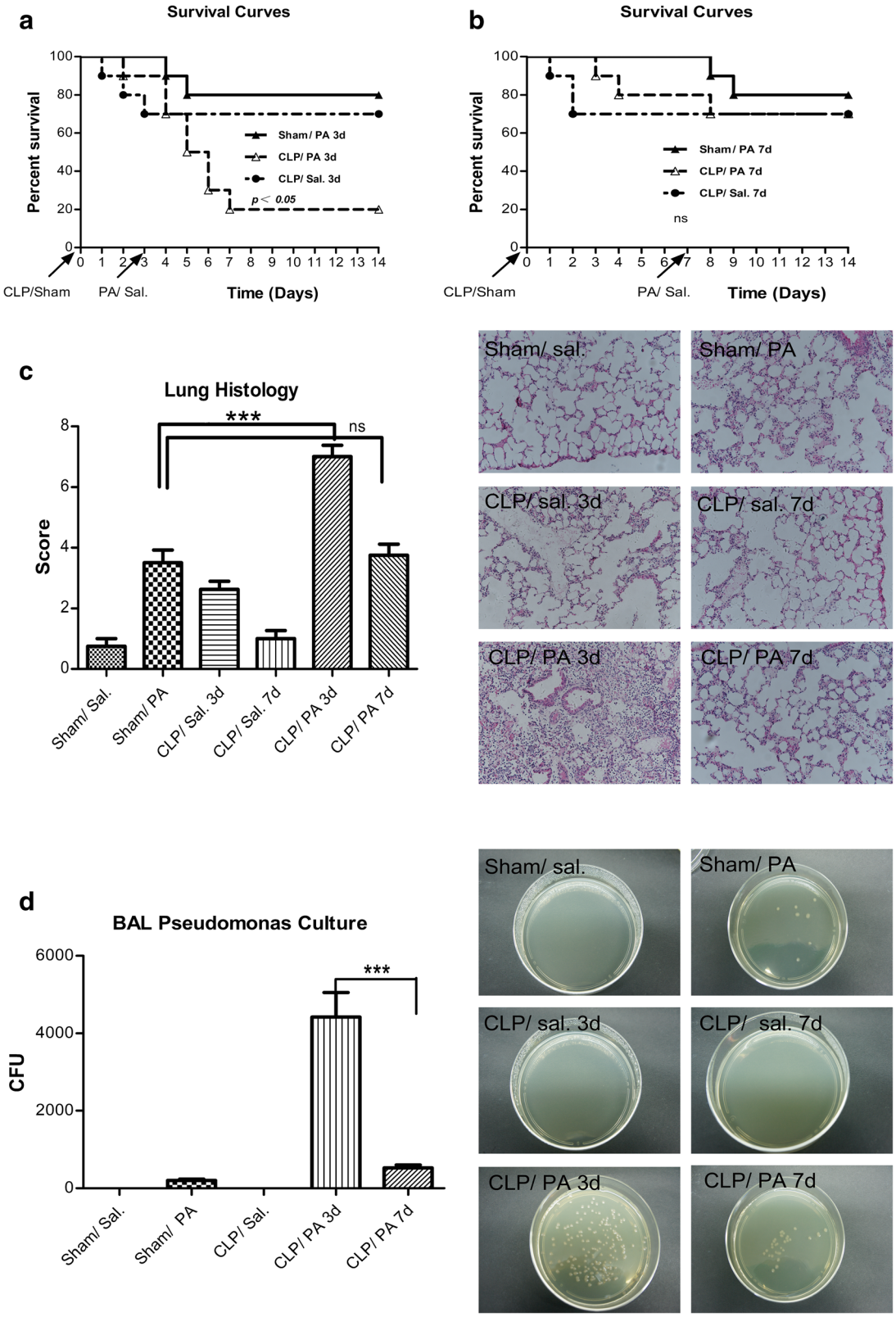
Mice

C57BL/6 female mice, 6–8 weeks old, were purchased from Shanghai Slack Laboratory Animal Co. Ltd. and raised at the Wenzhou Medical University. All mice experiments were conducted in accordance with the guidelines proposed by the Wenzhou Medical University Institutional Animal Care and Use Committee.

"Two-Hit" Mice Model

Cecal ligation and puncture (CLP) was performed as the first hit and intratracheal injection of *P. aeruginosa* was the second hit. In brief, mice were anesthetized with halothane (5% induction, 2% maintenance) and the abdomen was prepped. Then, a 1-cm midline incision was made and the cecum was exposed. The cecum was punctured through and through using a 27-gauge needle with 50% cecal ligation. Sham animals underwent the same procedure without cecum ligation and puncture. The mice were resuscitated with a subcutaneous injection of 0.5 ml sterile saline. Imipenem-cilastatin (Tienam, 25 mg/kg in 0.5 ml of saline) was given starting 6 hours (h) after surgery and continuing every 12 h for 2 days (d). Over 7 days, the mortalities of the CLP animals with and without antibiotic treatment were approximately 10–20 and 40%, respectively.

P. aeruginosa (ATCC27316) were prepared as previously described [22]. In brief, *P. aeruginosa* was grown overnight at 37 °C until reaching a stationary phase. Cells were resuspended in sterile saline and the bacterial concentration was calculated with the DENSIMAT method. At 3 and 7 days post-CLP, surviving mice were anesthetized again with mixture of ketamine and xylazine. Mice were held in a "head-up" position and their tracheas were exposed. Then, 20 μl of *P. aeruginosa* suspension (4×10^5 colony-forming unit [CFU]) was slowly injected *via* the


a
Survival Curves
Percent survival
Sham/ PA 3d
CLP/ PA 3d
CLP/ Sal. 3d
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CLP/Sham
PA/ Sal.
Time (Days)
b
Survival Curves
Percent survival
Sham/ PA 7d
CLP/ PA 7d
CLP/ Sal. 7d
ns
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trachea. Mice receiving an equal volume of sterile saline were used as controls.

Depletion of Tregs

Evidence illustrated that 200 μg/mouse PC61 mAb was enough to maintain a persistent low number of Tregs in septic mice [23]. In the present study, mice were i.p. injected with 200 μg of PC61 (BioLegend, San Diego, CA) or rat IgG1 (BioLegend, San Diego, CA) in 200 μl PBS 24 h before *P. aeruginosa* infection. Then, the changes in proportion of Tregs in spleen and the number of $Foxp3^+$ cells in lung were analyzed.

Lung Tissue and Bronchoalveolar Lavage Fluid Collection

The mice were sacrificed by overdose anesthesia 24 h after intratracheal injection of *P. aeruginosa* or saline. Lung tissues and bronchoalveolar lavage fluids (BALFs) were collected immediately. The whole lung tissues were perfused with 4% paraformaldehyde and saved in paraformaldehyde for an additional 36 h for histologic examination. BALFs were collected by lavaging the lung tissue with 2 × 0.5 ml saline. In brief, the trachea was exposed again and 0.5 ml sterile saline was slowly injected into the lungs and retrieved carefully. The bronchoalveolar lavage operation was conducted twice for each mouse.

Cell Preparation and Analysis of Spleen Tregs

Lymphocytes were isolated from single cell suspensions of spleens from sham and septic mice using lymphoprep according to the manufacturer's instructions. In order to analyze the proportion of $CD4^+CD25^+Foxp3^+$Treg cells, splenic lymphocytes (1×10^6) were stained with anti-mouse CD4-FITC antibody and CD25-APC antibody (eBioscience, San Diego, CA) or isotype control antibodies for 30 min on ice. For intracellular Foxp3 staining, cells were stained with anti-mouse Foxp3-PE (eBioscience, San Diego, CA) antibody or isotype antibody after permeabilization treatment. Cells were analyzed by flow cytometry using a FACScan (BD Bioscience, Mountain View, CA).

Lung Immunohistochemical Analysis of $Foxp3^+$ Cells

Lung immunohistochemical examination was carried out on formalin-fixed paraffin-embedded tissue with anti-Foxp3 mAb (eBioscience, San Diego, CA) according to the manufacturer's instructions. The number of $Foxp3^+$ cells was calculated by a pathologist blinded to the study protocol.

Bacteria Culture

In order to evaluate the lung bacterial clearance ability, BALF samples were collected 24 h after *P. aeruginosa* administration and bacterial culture tests were performed. The number of lung bacterial colonies was calculated by the gradient dilution and plate paint isolation methods. The lung BALFs were collected immediately after the mice were sacrificed and BALFs (100 μl) were spread evenly on the *P. aeruginosa* selective medium for bacterial culturing. The number of bacterial colonies was observed and calculated 24 h later.

Histopathology of Lung Tissues

Mice lung tissues were fixed in 4% paraformaldehyde for 36 h. Routine histologic techniques were performed to lung specimens. Lungs were embedded in paraffin and 3-μm sections of whole lung were stained with H&E for examination. Qualitative and semi-quantitative histopathological evaluations were carried out by a pathologist blinded to the study protocol.

Cytokine Quantification

IL-1β, IL-6, and IL-17A play crucial roles in host defense against *P. aeruginosa* infection [24–26]. IL-10 has been reported to be an important cytokine mediator of sepsis-induced immunosuppression [27]. Lung tissues were harvested and lung homogenates were prepared for cytokine analysis. IL-1β, IL-6, IL-10, and IL-17A concentrations were analyzed in duplicate using ELISA kits from R&D Systems in accordance with the manufacturer's guidance.

Statistical Analysis

Data were represented as the mean ± SEM. Differences between two groups were assessed using Student's *t* test, and comparisons between more than two groups were done with the Kruskall-Wallis test or one-way analysis of variance (ANOVA). Survival was analyzed by log-rank test. All calculations were made by the Prism 5.0 statistical program (GraphPad software, San Diego, CA). $P < 0.05$ were accepted as statistically significant.

RESULTS

Time Course Analysis of Susceptibility to *P. aeruginosa* Infection After Sepsis

As clinical data illustrated that most secondary infections in septic patients were observed 3 days after

admission, the susceptibility of mice to *P. aeruginosa* infection was evaluated 3 and 7 days post-CLP. The mortality of mice was examined every 24 h up to 14 days post-CLP. As shown in Fig. 1a, the mortality of mice infected with *P. aeruginosa* 3 days post-CLP was higher than that of animals undergoing CLP or *P. aeruginosa* infection alone. Histological changes were assessed in lung. As shown in Fig. 1c, significantly higher numbers of inflammatory cells, more fluid accumulation, and increased lung histopathology semi-quantitative scores were observed in the mice undergoing CLP followed by *P. aeruginosa* pneumonia 3 days after surgery compared to the sham mice infected with *P. aeruginosa* ($p < 0.0001$). The number of bacterial colonies was calculated 24 h after culturing the BALFs. As expected, no bacterial colonies were observed in BALFs from both septic mice and sham controls. As shown in Fig. 1d, the number of bacterial colonies in septic mice infected with *P. aeruginosa* 3 days post-CLP was much higher than that of sham mice with *P. aeruginosa* infection (4420 ± 1273 CFU *versus* 200 ± 72 CFU) ($p < 0.0001$). Nevertheless, when challenged with *P. aeruginosa* 7 days after surgery, there were no

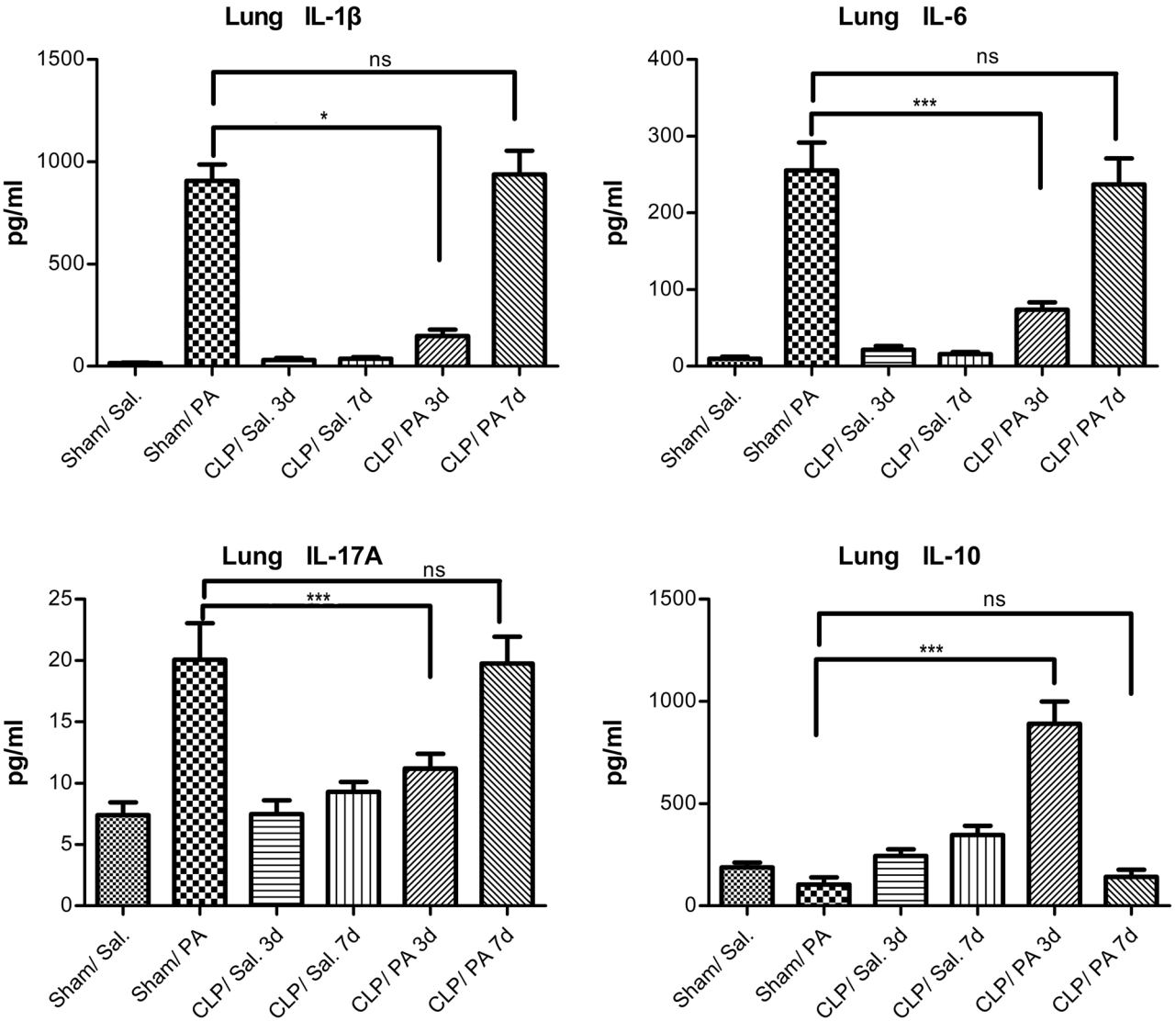

Fig. 2. Lung cytokine levels in septic mice with *P. aeruginosa* infection. CLP mice and sham controls were received *P. aeruginosa via* the trachea as a second hit 3 or 7 days after surgery. 24 h later, the lungs of each group were collected. The protein levels of IL-17A, IL-1β, IL-6, and IL-10 were measured in tissue lysates by ELISA test ($n = 8$). PA, *P. aeruginosa*; Sal, saline; CLP, cecal ligation and puncture. $^{*}p < 0.05$, $^{***}p < 0.001$.

statistically significant differences in the histopathological alterations, mortalities, and the number of bacterial colonies between septic mice and sham controls (Fig. 1b–d).

Lung Cytokine Levels in Septic Mice with *P. aeruginosa* Infection

As shown in Fig. 2, there were increased levels of pro-inflammatory cytokines, including IL-1β ($p < 0.0001$), IL-6 ($p < 0.0001$), and IL-17A ($p < 0.05$), in the lungs of septic mice infected with *P. aeruginosa* 3 days post-CLP when compared to sham mice with *P. aeruginosa* infection. Meanwhile, mice infected with *P. aeruginosa* 3 days post-CLP had increased levels of anti-inflammatory cytokine IL-10 ($p < 0.0001$) (Fig. 2). However, results showed that there were no statistically significant differences in cytokine levels between CLP mice infected with *P. aeruginosa* and sham controls with *P. aeruginosa* infection 7 days after surgery (Fig. 2).

Increased Tregs in Post-Septic Mice After *P. aeruginosa* Challenge

The number of Tregs in the spleen and lungs was evaluated 24 h after secondary *P. aeruginosa* infection. The results showed that there was an almost twofold increase in the number of $Foxp3^+$ cells in lung tissues of septic mice after secondary *P. aeruginosa* infection 3 days post-CLP (11.9 ± 3.843 *versus* 6.1 ± 3.855, $p < 0.0001$) (Fig. 3A). The proportion of regulatory T cells in the spleens of CLP mice infected with *P. aeruginosa* 3 days after surgery was about 1.6 times higher than in CLP mice (8.47 ± 1.259 *versus* $5.3 \pm 1.035\%$, $p < 0.05$) (Fig. 3B). Similar results were observed 7 days after surgery (Fig. 3B).

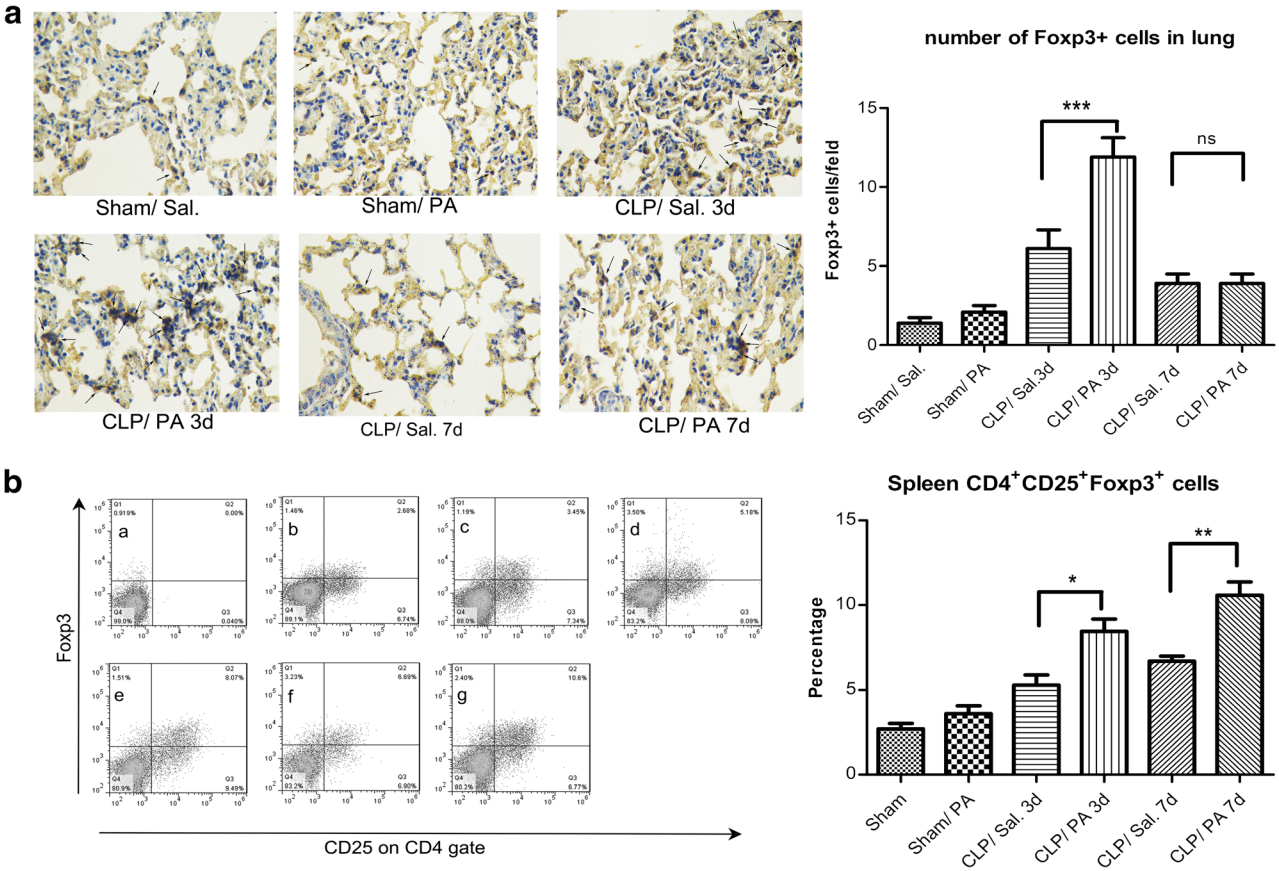

Fig. 3. Increased Tregs in post-septic mice after *P. aeruginosa* challenge. Mice underwent CLP or sham surgery and then received *P. aeruginosa via* the trachea as a second hit 3 or 7 days after surgery. (**A**) Immunohistochemical analysis of $Foxp3^+$ cells in lung tissues (image: × 400). (**B**) Flow cytometric analysis of $CD4^+CD25^+Foxp3^+$ cells in spleen ($n = 8$). a, isotype; b, sham + sal.; c, sham + PA; d, CLP + sal. 3d; e, CLP + PA 3d; f, CLP + sal. 7d; g, CLP + PA 7d. PA, *P. aeruginosa*; Sal, saline; CLP, cecal ligation and puncture. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

Depletion of Tregs Improved Survival of Septic Mice After Secondary Infection

Given the above results that the increased number of Tregs was associated with secondary *P. aeruginosa* infection in sepsis, PC61 mAb (200 μg/mouse, i.p.) was used to deplete the Tregs in septic mice. Results showed that injection with PC61 mAb reduced the number of Tregs by about 50% in spleen (7.96 ± 1.418 *versus* 3.992 ± 1.114%) and 60% in lung (15.1 ± 6.935 *versus* 5.9 ± 2.331%) of septic mice (Fig. 4B) ($p < 0.001$). Additionally, depletion Tregs with PC61 mAb significantly improved survival of septic mice with *P. aeruginosa* infection ($p < 0.05$) (Fig. 5a).

Decreased Susceptibility of Septic Mice to *P. aeruginosa* Infection After Treg Depletion

As shown in Fig. 5b, the number of bacterial colonies in lung was decreased in mice treated with PC61 mAb compared with IgG1-treated mice (2237 ± 1170 CFU *versus* 4775 ± 1026 CFU) ($p < 0.01$). After Treg depletion, the morphology of the lung tissue was observed by histological examination. As shown in Fig. 5c, decreased numbers of inflammatory cells, less fluid accumulation, and lower lung histopathology semi-quantitative scores were observed in mice treated with PC61 mAb compared to IgG1-treated mice (7 ± 1.07 *versus* 2.5 ± 0.53, $p < 0.0001$).

Lung Cytokine Levels in Mice After Treg Depletion

The protein levels of IL-17A, IL-1β, IL-6, and IL-10 were measured in lung tissue by using ELISA kits. As indicated in Fig. 6, the production of IL-1β, IL-6, and IL-17A was significantly increased after Treg depletion in septic mice that underwent *P. aeruginosa* pneumonia ($p < 0.05$). Additionally, the protein levels of IL-10 in lung were decreased after Treg depletion ($p < 0.001$) (Fig. 6).

DISCUSSION

Sepsis is one of the leading causes of death in critically ill patients. Due to the improved treatment strategies, more and more septic patients survive the initial hyper-inflammatory phase and enter a sustained immunosuppressive phase. The immune dysfunction in sepsis lead to the host cannot effectively control the primary infection. More importantly, septic patients who survive the early phase of the disease are at high risk for secondary nosocomial

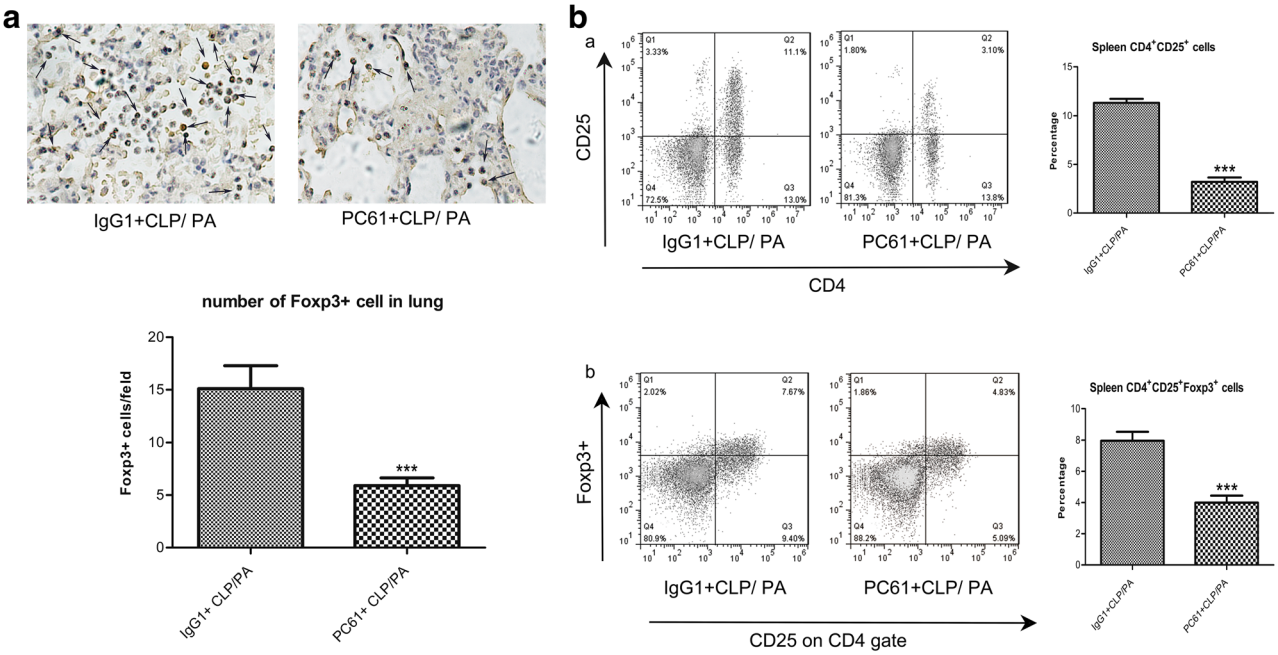

Fig. 4. The number of Foxp3$^+$ cells in lung and regulatory T cells in spleen of mice treated with PC61 mAb or IgG1 mAb. Mice that underwent CLP were infected with *P. aeruginosa* 3 days after surgery. PC61 mAb or IgG1 mAb was administrated 24 h before *P. aeruginosa* infection. (**A**) Immunohistochemical analysis of Foxp3$^+$ cells in lung tissues (image: × 400). (**B**) Flow cytometric analysis of CD4$^+$CD25$^+$ cells (a) and CD4$^+$CD25$^+$Foxp3$^+$ cells (b) in spleen. PA, *P. aeruginosa*; Sal, saline; CLP, cecal ligation and puncture. ***$p < 0.001$, compared with lgG1 mAb group.

infection [28]. Evidence illustrated that nosocomial infections occurred in 13.5% of sepsis ICU admissions, and the incidence of secondary infection reached 39% in patients with septic shock [29, 30]. Recently, secondary infection has been proved to be the major cause of death among patients with sepsis [4], and effective prevention of secondary infection has become a potential measure to further reduce the mortality of the disease.

There are several factors associated with the incidence of secondary infection in septic patients, including advanced age, the severity of the disease, and the length of ICU stay. Nevertheless, these factors are relatively objective causes of secondary infection and it is hard to control them in clinical work. As mentioned above, immune dysfunction in sepsis is associated with increased incidence of secondary infection. Previous studies have shown that the production of IL-10 contributed to the secondary infection in sepsis [27]. In the present study, the levels of IL-10 in mice infected with *P. aeruginosa* 3 days post-CLP increased significantly. It is reported that IL-17 exerts a protective effect in the airway by favoring the clearance of pathogens during acute *P. aeruginosa* infection [31]. Our results demonstrated that the levels of IL-17A in lung increased significantly after acute

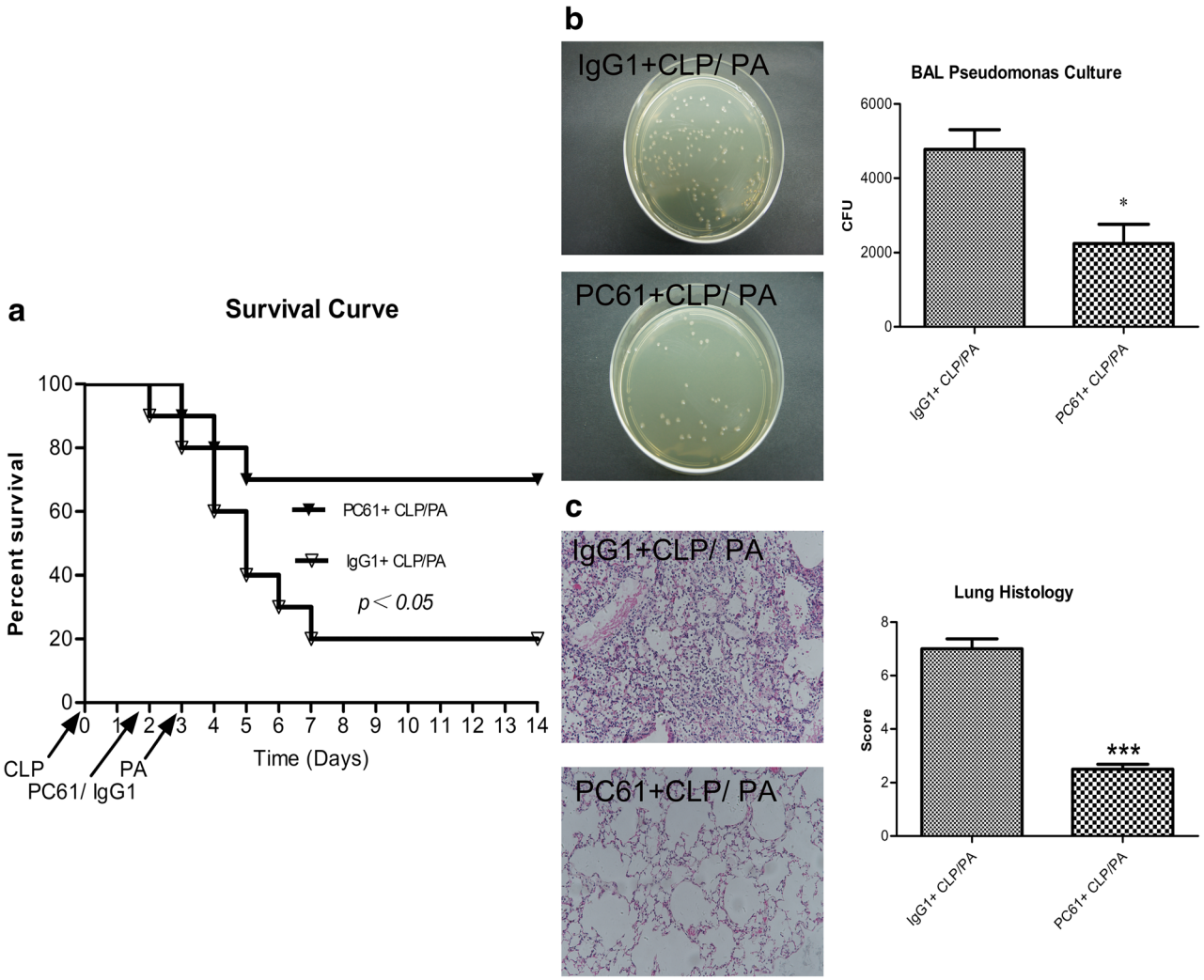

Fig. 5. Decreased susceptibility of septic mice to *P. aeruginosa* infection after Treg depletion. Mice undergoing CLP were infected with *P. aeruginosa* 3 days after surgery. PC61 mAb or IgG1 mAb was administrated 24 h before *P. aeruginosa* infection **a** survival curves of mice treated with PC61 mAb or IgG1 mAb ($n = 10$). **b** Lung colony-forming units (CFUs). BAL fluids of mice treated with PC61 mAb and IgG1 mAb were collected for culturing. The number of bacterial colonies was counted 24 h later ($n = 8$). **c** Pathological score of lung tissue in each group 24 h after secondary infection. Pathological scores of lung tissues in two groups were shown ($n = 8$). PA, *P. aeruginosa*; Sal, saline. $^*p < 0.05$, $^{***}p < 0.001$, compared with lgG1 mAb group.

P. aeruginosa infection in sham-operated mice. However, secondary *P. aeruginosa* infection 3 days post-CLP did not cause an increase in IL-17A levels. Similarly, at the same time point, the levels of IL-1β and IL-6, which are critical for the host defense against pneumonia due to *P. aeruginosa*, remained low in mice which underwent CLP followed by *P. aeruginosa* infection. Additionally, significant increases in the number of bacterial colonies were observed in post-CLP mice after *P. aeruginosa* infection while the inflammatory cell infiltration in lung was increased, as consistently observed in previous studies. These seemingly contradictory results may be explained by the fact that the neutrophils and macrophages which are recruited to the lung in septic patients with secondary infection appear to have impaired abilities to secrete pro-inflammatory cytokines and clear pathogens [27, 32].

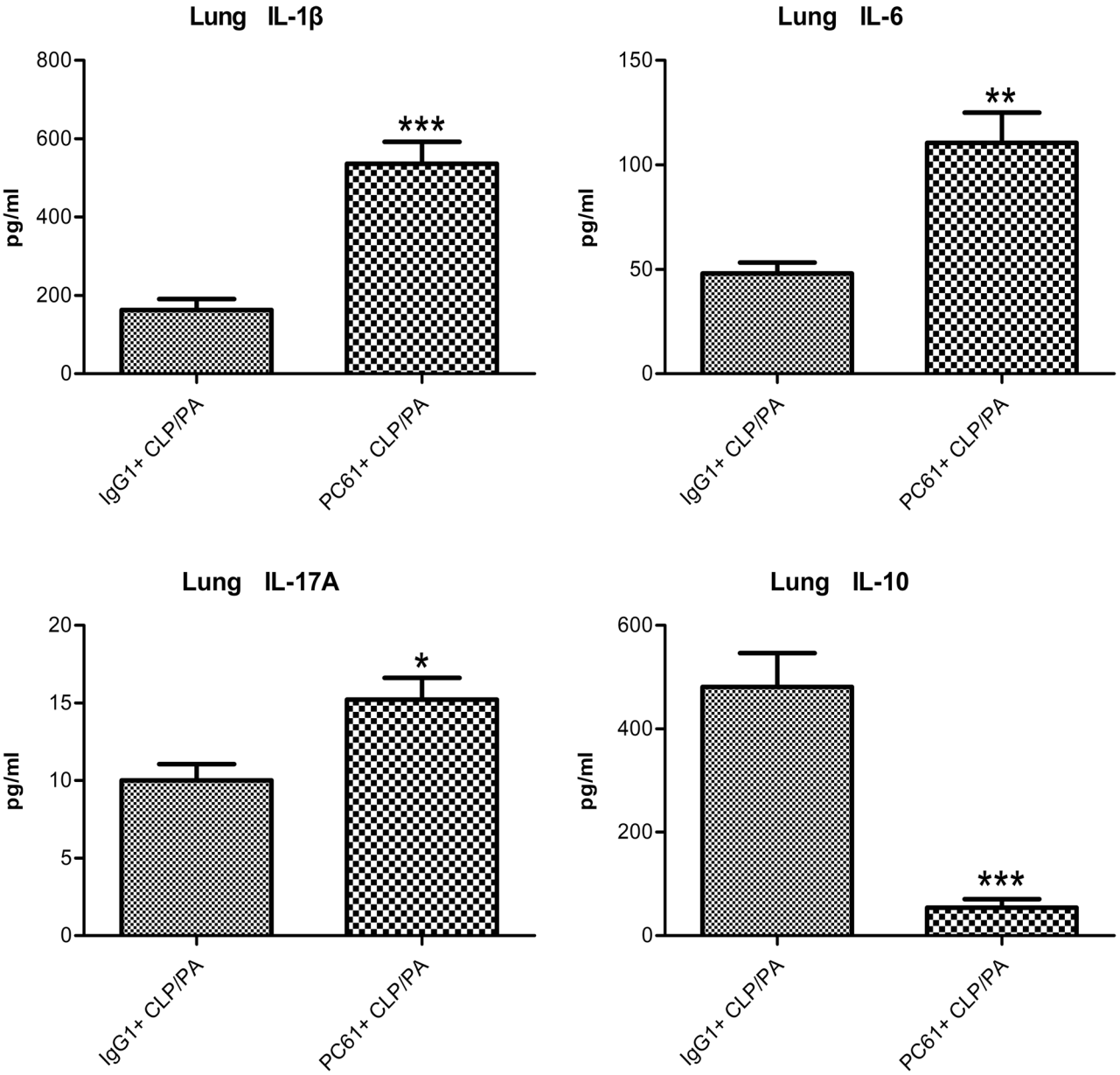

Fig. 6. Lung cytokine levels in mice after Treg depletion. Septic mice were infected with *P. aeruginosa* 3 days post-CLP. PC61 mAb or IgG1 mAb was given 24 h before *P. aeruginosa* infection. The protein levels of IL-17A, IL-1β, IL-6, and IL-10 were measured in tissue lysates by ELISA test ($n = 8$). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, compared with lgG1 mAb group.

It has been demonstrated that Tregs were associated with the defects in immune cell responses in sepsis. Increased levels of pro-inflammatory cytokines and enhanced immune cell responses after the depletion of Tregs were observed in many studies [33–35]. In the present study, the levels of $CD4^+CD25^+Foxp3^+$Tregs in spleen and $Foxp3^+$ cells in lung were increased in post-septic mice after secondary infection, indicating that the expansion of these cells may contribute to secondary infection in sepsis. Accordingly, Tregs were depleted using the monoclonal rat anti-mouse CD25 clone PC61. Although not all $Foxp3^+$T cells express CD25, PC61 mAb treatment can cause a 30–60% reduction in $Foxp3^+$Tregs and enhance the host immune response against tumors and pathogens [19–21]. After PC61 mAb treatment, the levels of IL-1β, IL-6, and IL-17A were increased and the production of IL-10 was decreased significantly in the "two-hit" model. Due to the improvement of immune response after Treg depletion in sepsis, the lung bacterial clearance ability was enhanced. As a result, the inflammatory cell recruitment to lung and the mortality of the animals were decreased.

Sepsis is a highly complex and non-linear process. If immunotherapy is applied during the wrong phase of the sepsis, it may worsen outcomes by causing excessive inflammation or by impairing the immune response. Unfortunately, the markers used to guide immunotherapy treatment are still lacking, although HLA-DR, PD-L1, and others are currently under consideration [36–38] . While a single biomarker does not reflect the overall degree of immunosuppression, the shift in host susceptibility to pathogens may be a useful method to evaluate the immune status of sepsis in experimental studies. In the present study, increased susceptibility to *P. aeruginosa* infection 3 days post-CLP and elevated levels of Tregs were observed. So, Tregs were depleted before secondary infection and improved host response to *P. aeruginosa* in septic mice was observed. Additionally, an antibiotic was used to help CLP mice survive the early phase of sepsis to mimic clinical conditions in our study.

CONCLUSION

In conclusion, our current findings demonstrate that Tregs contribute to secondary infection in sepsis by modulating the inflammatory response. While more research is needed, our work suggests that Tregs may be an effective target for the development of drugs that prevent secondary infection and reduce mortality in sepsis.

COMPLIANCE WITH ETHICAL STANDARDS

All mice experiments were conducted in accordance with the guidelines proposed by the Wenzhou Medical University Institutional Animal Care and Use Committee.

Conflict of Interest. The authors declare that they have no conflict of interest.

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