

Original Article

Inflammatory responses and histopathological changes in a mouse model of *Staphylococcus aureus*-induced bloodstream infectionsDan Wu^{1,*}, Shusheng Zhou^{2,*}, Shijing Hu², Bao Liu²¹ Department of Emergency Medicine, Nanjing First Hospital, Nanjing Medical University, Nanjing, China² Department of Critical Care Medicine, Affiliated Provincial Hospital of Anhui Medical University, Hefei, China

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Abstract

Introduction: *Staphylococcus aureus*-induced bloodstream infections (BSIs) remain a prevalent clinical challenge and the underlying pathogenesis is still poorly understood. The aim of this study was to investigate the inflammatory responses and histopathological changes in BSIs in mice.

Methodology: Male C57BL/6 mice were inoculated with *S. aureus* intravenously to induce BSIs. The survival rate, weight loss, and murine sepsis scores (MSS) were monitored in BSI and phosphate-buffered saline (PBS) control mice. Blood samples and tissue homogenates were plated on agar plates to determine the bacterial burden. Inflammatory proteins and cytokines were determined by enzyme-linked immunosorbent assay (ELISA) kits. Histopathologic changes were assessed by pathological inflammation score (PIS) and macroscopic and microscopic examinations.

Results: BSI mice induced by 4.5×10^8 CFU/mL *S. aureus* showed ~70% survival rate, higher sepsis scores, significantly decreased body weight, elevated levels of white blood cell (WBC) counts, C-reactive protein (CRP), procalcitonin (PCT), interleukin (IL)-1 β , IL-6, and tumor necrosis factor (TNF)- α . Prominent correlations were found between elevated CRP and PCT levels as well as among IL-1 β , IL-6, and TNF- α . Pathological changes and higher PIS were also observed in BSI mice.

Conclusions: Our results demonstrate that inflammatory proteins (PCT and CRP) and cytokines (IL-6, IL-1 β and TNF- α) play an important role in the inflammatory responses and histopathological changes in *S. aureus*-induced BSIs.

Key words: *Staphylococcus aureus*; bloodstream infections; inflammation; cytokines; Histopathology.*J Infect Dev Ctries* 2017; 11(4):294-305. doi:10.3855/jidc.7800

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Copyright © 2017 Wu *et al.* This is an open-access article distributed under the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.**Introduction**

Severe infections can be life threatening, and the high prevalence rates of intensive care unit (ICU)-acquired infections raise patient safety concerns worldwide [1]. Infections are caused by infectious agents such as viruses, fungi, and bacteria. Recent studies show that there is a high incidence of serious bacterial infections caused by *Staphylococcus aureus* (*S. aureus*), which may cause pulmonary infection, digestive tract infection, urinary infection, skin and soft tissue infection, and bloodstream infections (BSIs) [2]. The incidence of *S. aureus*-induced bloodstream infections has significantly increased in critically ill patients and accounts for 20%–30% mortality rate worldwide [3,2]. *S. aureus*-induced bloodstream infections can cause sepsis or septic shock, and the incidence of sepsis and sepsis-related deaths is rising [4]. A better understanding of the pathogenesis of *S. aureus*-induced BSI is essential for the development of

therapeutic methods and outcome improvement. To date, several experimental animal models of sepsis have been developed, including exogenous administration of toxins or live bacteria, cecal ligation and puncture (CLP), and abdominal sepsis, which mimic pathophysiological changes typically seen in septic patients [5,6]. In our study, we used a simple but valuable animal model of BSIs by injecting *S. aureus* to C57BL/6 mice via tail vein to investigate the inflammatory responses and pathological changes in BSIs [4].

Methodology*Ethics statement*

The animal experiment was conducted in strict compliance with the Animal Welfare Act and the laboratory's animal regulations. All animal procedures were approved by the ethics committee of Affiliated Provincial Hospital of Anhui Medical University,

which has been accredited by the National Laboratory Animal Management Regulations. Animal care, bacterial inoculation, and animal sacrifice were supervised by the professional staff of the Animal Experiment Center of the Affiliated Provincial Hospital of Anhui Medical University.

Strain culture

S. aureus strain ATCC29213 used in this study was methicillin resistant, coagulase positive and Panton-Valentine Leukocidin (PVL) positive [7]. The strain was obtained from the Clinical Microbiology Laboratory of Affiliated Provincial Hospital of Anhui Medical University. *S. aureus* strains were cultured in Luria-Bertani (LB) broth (Merck Millipore, Boston, USA) at 37°C with shaking (170 × g). After overnight growth, bacteria were diluted 1:100 (v/v) with fresh LB broth and grew for another 4–6 hours to reach the mid-log phase at optical density (OD)₆₀₀ 0.5. The bacteria were harvested by centrifugation (3,000 × g, 5 minutes, at room temperature), washed three times, and re-suspended in sterile phosphate-buffered saline (PBS) (BBI, Shanghai, China) at OD₆₀₀ 1, while bacterial colony forming units (CFUs) were calculated as 4.5 × 10⁸/mL [8].

Animals

Male C57BL/6 mice (20–25 grams, 6–8 weeks of age) were purchased from the Experimental Animal Center of Anhui province (EACA, Hefei, China). Mice were maintained in a pathogen-free environment with an ambient temperature of 21–23°C and a 12-hour dark-light cycle. Mice were housed in sterile cages with sterile feed and water in the Animal Experiment Center of the Affiliated Provincial Hospital of Anhui Medical University.

Animal model of S. aureus-induced BSIs

Mice were inoculated with different concentrations (4.5 × 10⁹, 4.5 × 10⁸, 4.5 × 10⁷, 4.5 × 10⁶, 4.5 × 10⁵ and 4.5 × 10⁴ CFU/mL) of *S. aureus* via the tail vein (IV) (n = 10/group). All operations followed the aseptic principle and the bacterial dose was 0.1 mL/10 g body weight. Clinical signs, body weight, and survival rate were monitored every day for 7 days post-infection. The median lethal dose (LD₅₀) and 95% confidence interval measured by Karber method were chosen as the best concentration of infection in this study. Animals were anesthetized with 10% chloral hydrate (0.004 mL/g) intraperitoneally and euthanized by cardiac puncture to avoid pain and suffering.

The detection of white blood cell, C-reactive protein, and procalcitonin

Blood samples from the mice of the most suitable infection concentration group were collected by cardiac puncture at each time point (1, 3, 6, 12, 24, 48, 72, 96, 120 and 168 hours post-infection). One set of blood samples (500 uL) were mixed with 15% ethylene diamine tetraacetic acid (EDTA) solution (200 uL) in endotoxin-free tubes and were used for white blood cell (WBC) detection. Another set of blood samples were collected in 1.5-mL microcentrifuge tubes, coagulated for 1 hour at room temperature, centrifuged (4000 × g, 4°C, 15 minutes), and then the serum C-reactive protein (CRP) (expressed as ug/mL) and procalcitonin (PCT) (expressed as ng/mL) levels were measured using a commercially available diagnostic kit (Life Diagnostics, West Chester, USA) according to the manufacturer's instructions.

Measurement of serum and tissue cytokines levels

The levels of cytokines, IL-1β, IL-6, and TNF-α, in serum and tissue homogenates were measured by enzyme-linked immunosorbent assay (ELISA) kits (Neo Bioscience, Shenzhen, China) according to the manufacturer's instructions. Blood was coagulated for 1 hour at room temperature and centrifuged (4000 × g, 4°C, 15 minutes) in a refrigerated centrifuge, and then the supernatant was carefully aspirated to clean tubes and stored at -80°C until further analysis.

Organs (lungs, liver, and kidneys) were aseptically removed from animals and washed in ice-cold saline. Tissues were fully homogenized in PBS (10 mg tissues per 50 uL PBS) with sterile glass tissue mill and centrifuged at 12,000 × g, 4°C for 30 minutes in the refrigerated centrifuge. The supernatant was collected and stored at -80°C until use. The samples were measured by Ascent Software Version 2.6 (Thermo, Boston, USA) using a multifunction protein array reader Multiskan MK3 (Thermo, Boston, USA) and data were analyzed with SPSS version 16.0 (IBM, Armonk, USA).

Bacterial burden in blood and tissue homogenates

To determine bacterial dissemination to organs during bacteremia, blood samples (10 uL) or tissue homogenates (liver, lungs, and kidneys) (10 uL) were diluted tenfold in PBS (90 uL), and then plated in duplicates on mannitol salt agar plates (BD, New York, USA). Bacterial burden was enumerated by colony-forming units (CFUs) after a 24–48-hour incubation period in an aerobic chamber at 37°C.

Histology

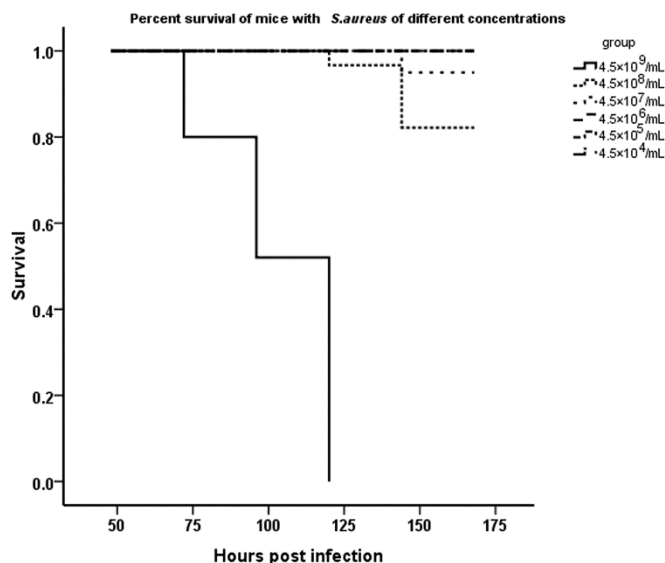
To detect the inflammation in organs, lungs, liver, and kidneys, tissues were collected at each time point after infection. Tissues were fixed in 10% neutral-buffered formalin (BBI, Shanghai, China), dehydrated using a series of ethanol (BBI, Shanghai, China), infiltrated, and embedded in paraffin (BBI, Shanghai, China). Paraffin-embedded samples were sectioned to a 2- μm thickness, deparaffinized, rehydrated, and stained with hematoxylin and eosin (H&E) (BBI, Shanghai, China). All H&E stained sections were examined using an optical microscope (Olympus Optical BX41, Tokyo, Japan). Images were captured and analyzed with the JD801 pathological image analysis system (JEDA, Beijing, China) under 400 × magnification.

To evaluate the pathological changes in tissues during acute inflammation, pathological inflammation scoring (PIS) system was used as previously described [9]: 0 = no or occasional cells; 1 = few loosely arranged cells; 2 = many cells in the peripheral parts of the perivascular space; and 3 = numerous cells in the perivascular space. The total score for each mouse was estimated as the sum of scores from each category for that individual. All sections were examined and scored by an experienced pathologist (Professor Chen, from the department of pathology, Affiliated Provincial Hospital of Anhui Medical University, China), who was blinded to the treatment conditions of mice.

Statistics

Statistical analysis was performed using SPSS 16.0 (IBM, Armonk, USA). Data were calculated as mean ± standard deviation (SD). The differences between two groups were analyzed using an unpaired two-tailed Student’s t-test, followed the Kolmogorov-Smirnov (K-S) test. One-way analyses of variance (ANOVA) followed by the Tukey test for multiple comparisons were used to compare multiple groups. Linear

Figure 1. Survival rate of mice induced with different concentrations *S. aureus* during the 7-day observation period.



Mice were IV infected with 4.5×10^9 CFU/mL, 4.5×10^8 CFU/mL, 4.5×10^7 CFU/mL, 4.5×10^6 CFU/mL, 4.5×10^5 CFU/mL, and 4.5×10^4 CFU/mL *S. aureus* (n = 10/group).

correlation analysis was used to measure the correlation of the CRP, PCT, cytokine levels, and the pathological inflammation scores. Significance was placed at $p < 0.05$.

Results

Different concentrations of S. aureus and survival rate

A total of 60 mice (n = 10/group) were infected (i.v.) with 4.5×10^9 , 4.5×10^8 , 4.5×10^7 , 4.5×10^6 , 4.5×10^5 , or 4.5×10^4 CFU/mL *S. aureus* (0.1 mL/10 g body weight). The survival rate was monitored for 7 days consecutively post-infection. In BSI mice, the survival rate was 0% in the 4.5×10^9 CFU/mL group, 70% in the 4.5×10^8 CFU/mL group, 90% in the 4.5×10^7 CFU/mL group, 100% in the 4.5×10^6 CFU/mL 4.5

Table 1. The body weight and MSS of mice.

| Time | N | Body weight (g) | | Mean MSS | |
|-------|----|-----------------|--------------|----------------|-----|
| | | BSI | PBS | BSI | PBS |
| 1 h | 20 | 20.52 ± 1.63 | 20.60 ± 1.35 | 0.25 ± 0.08 | 0 |
| 3 h | 20 | 20.53 ± 1.70 | 20.70 ± 1.34 | 1.07 ± 0.12** | 0 |
| 6 h | 20 | 20.43 ± 1.55 | 21.00 ± 1.54 | 1.69 ± 0.10** | 0 |
| 12 h | 20 | 20.43 ± 1.19 | 21.20 ± 1.03 | 2.24 ± 0.28** | 0 |
| 24 h | 20 | 20.30 ± 1.09 | 21.60 ± 1.26 | 7.73 ± 0.12** | 0 |
| 48 h | 20 | 19.88 ± 1.05* | 22.20 ± 1.34 | 8.99 ± 0.67** | 0 |
| 72 h | 20 | 19.00 ± 0.92* | 23.40 ± 0.97 | 9.73 ± 0.27** | 0 |
| 96 h | 20 | 18.07 ± 1.10* | 24.10 ± 0.88 | 13.51 ± 2.15** | 0 |
| 120 h | 20 | 17.10 ± 1.45* | 25.10 ± 0.88 | 16.15 ± 0.12** | 0 |
| 168 h | 20 | 15.00 ± 0.71* | 26.20 ± 0.92 | 20.04 ± 0.12** | 0 |

MSS: murine sepsis score; BSI: bloodstream infection; PBS: phosphate buffered saline; *Significant at $p < 0.001$ when compared with the PBS group in the terms of body weight; **Significant at $p < 0.001$ when compared with the PBS group in the terms of MSS.

$\times 10^5$ CFU/mL, and 4.5×10^4 CFU/mL groups (Figure 1). In the PBS control group (n = 10), the survival rate was 100% throughout the experiment (data not shown). Therefore, 4.5×10^8 CFU/mL was chosen as the best concentration, based on the survival rate and the median lethal dose.

Clinical symptoms in BSI mice

Weight loss

After inoculation with 4.5×10^8 CFU/mL *S. aureus*, the body weights of surviving mice were monitored for 7 days and recorded at each time point (n = 10/each time point) (Table 1). Mice in the PBS control group (n = 10) showed an increase in body weight during the 7-day period without mortality. In contrast, mice in the BSI group showed a decrease in body weight during the 7-day period. There was no significant difference in body weight between the two groups before infection (p > 0.05). The body weight loss started at 24 hours post-infection (p < 0.001 versus the PBS group) and lasted until the mice died.

Murine sepsis score

A murine sepsis score (MSS) was used to evaluate the clinical conditions of mice with sepsis [10]. The MSS is based on simple observations. It might be easily implemented in any mouse model of sepsis and is fully compatible with any investigative timeline because of its non-invasive nature. MSS includes seven criteria: appearance, level of consciousness, activity, response to stimulus, eyes, respiration rate, and respiration quality. Each of these variables is given a score between 0 and 4, which results in an aggregate score. The MSSs for BSI mice at each time point are shown in Table 1. Compared to the mice in the PBS control group (a mean score of 0), the sepsis scores in BSIs group were significantly higher (p < 0.001). Moreover, MSS increased gradually over time. The results obtained

were based on the survival outcomes shown in Figure 1. For the mice that were inoculated with 4.5×10^8 CFU/mL *S. aureus*, the mortality rate was 12% (12/100), and the MSS score was 10 at 72 hours post-infection; the mortality rate was 22% (22/100), and the MSS score was 15 at 5 days post-injection.

WBC, CRP, and PCT levels

The WBC counts in BSI mice induced by 4.5×10^8 CFU/mL *S. aureus* were significantly elevated, compared to the control group (p < 0.05) (Table 2). WBC counts began to increase at 3 hours post-infection, peaked at 72–96 hours, and declined at 120 hours in BSI mice. CRP levels were significantly elevated at 12–96 hours post-infection and PCT levels were significantly elevated at 12–120 hours post-infection, compared to the PBS control group (p < 0.001) (Table 2). CRP levels in the BSI group slightly increased at 6 hours, peaked at 24–48 hours, declined at 72 hours, and returned to normal levels at 5 days post-infection. PCT levels in BSIs group were first detected at 2–3 hours post-infection, increased rapidly at 12–48 hours post-infection, and then decreased gradually to the normal level.

Bacterial counts in blood samples and tissue homogenates

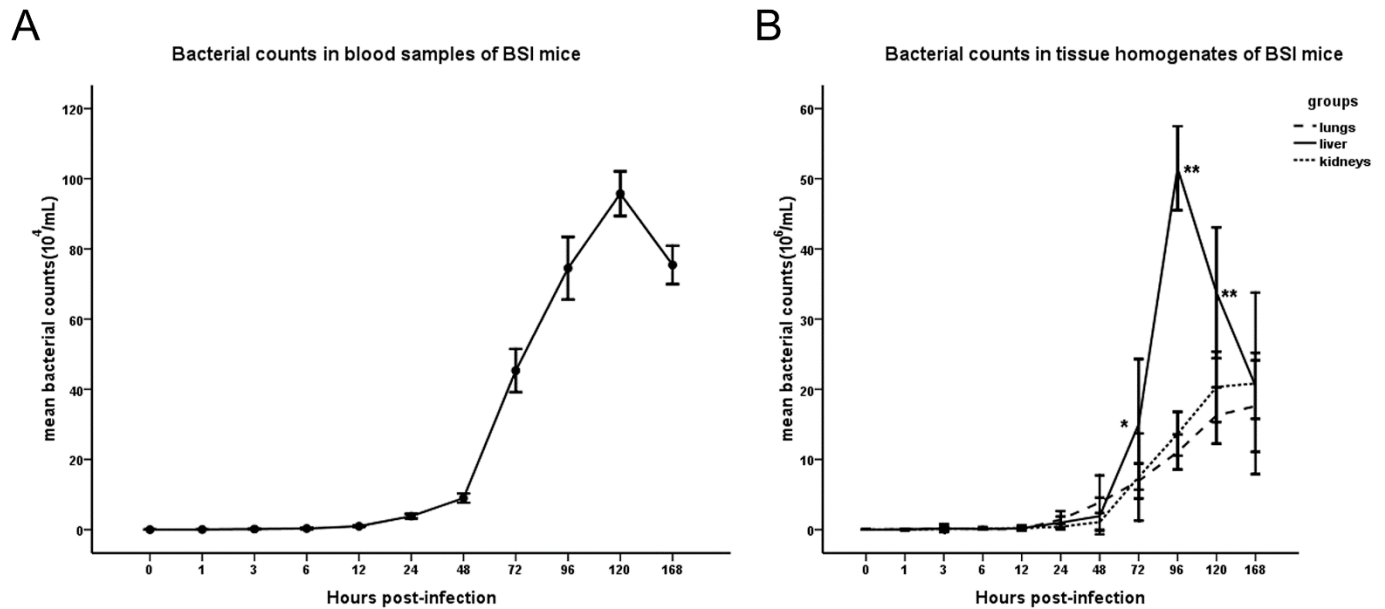
The blood samples and tissue homogenates of BSI mice induced by 4.5×10^8 CFU/mL *S. aureus* were plated on agar plates. The bacterial growth was regarded as the infection in blood and tissues. The typical colonies on the plate were circular protuberances 1.0 ~ 2.0 mm in diameter, yellow or white, thick, luster, and transparent hemolysis ring. The bacterial counts in the blood increased gradually in the BSI group, reached a peak of $(92.34 \pm 2.03) \times 10^4$ CFU/mL at 96–120 hours post-infection, and then declined afterwards (Figure 2A).

Table 2. WBC and serum levels of CRP and PCT in BSI mice.

| Time | N | WBC ($\times 10^9/L$) | | CRP levels (ug/mL) | | PCT levels (ng/mL) | |
|-------|----|-------------------------|-------------|--------------------|-------------|--------------------|--------------|
| | | BSIs | PBS | BSIs | PBS | BSIs | PBS |
| 1 h | 20 | 1.70 ± 1.91 | 1.71 ± 1.14 | 3.28 ± 0.53 | 3.20 ± 0.62 | 0.048 ± 0.04 | 0.022 ± 0.02 |
| 3 h | 20 | 4.44 ± 0.70* | 1.20 ± 0.65 | 3.32 ± 0.53 | 3.22 ± 0.61 | 0.049±0.04 | 0.020 ± 0.02 |
| 6 h | 20 | 5.68 ± 0.72* | 1.74 ± 0.97 | 6.00 ± 0.79 | 3.28 ± 0.61 | 0.202 ± 0.06 | 0.021 ± 0.02 |
| 12 h | 20 | 7.09 ± 0.36* | 1.89 ± 0.59 | 22.90 ± 5.66** | 3.30 ± 0.59 | 2.000 ± 0.38** | 0.020 ± 0.02 |
| 24 h | 20 | 7.34 ± 0.40* | 1.55 ± 0.49 | 38.40 ± 6.51** | 3.34 ± 0.50 | 3.962 ± 0.34** | 0.021 ± 0.02 |
| 48 h | 20 | 9.13 ± 0.45* | 2.20 ± 0.72 | 60.80 ± 5.63** | 3.38 ± 0.51 | 6.796 ± 1.16** | 0.022 ± 0.03 |
| 72 h | 20 | 10.44 ± 0.85* | 2.26 ± 0.49 | 30.96 ± 5.87** | 3.46 ± 0.50 | 4.684 ± 0.66** | 0.019 ± 0.02 |
| 96 h | 20 | 13.71 ± 1.29* | 2.46 ± 1.30 | 15.00 ± 4.06** | 3.50 ± 0.66 | 2.496 ± 0.56** | 0.020 ± 0.02 |
| 120 h | 20 | 11.75 ± 1.67 | 1.85 ± 1.02 | 4.66 ± 0.68 | 3.54 ± 0.61 | 0.806 ± 0.97** | 0.023 ± 0.03 |
| 168 h | 20 | 10.68 ± 1.47 | 2.32 ± 1.19 | 3.52 ± 0.40 | 3.54 ± 0.68 | 0.064 ± 0.01 | 0.021 ± 0.02 |

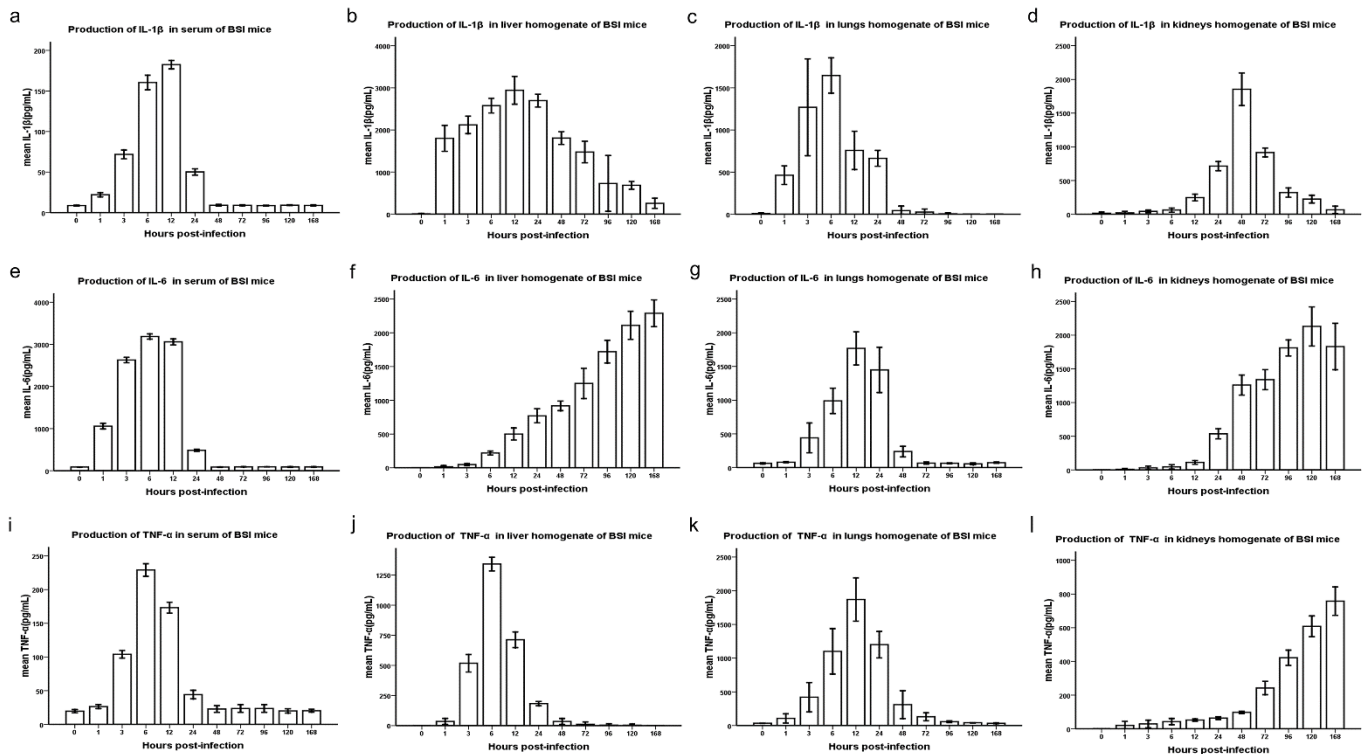
WBC: white blood cell; CRP: C-reactive protein; PCT: procalcitonin; BSI: bloodstream infection; *Significant at p < 0.05 when compared with the PBS group in the terms of WBC; **Significant at p < 0.01 when compared with the PBS group in the terms of CRP and PCT.

Figure 2. Bacterial counts in blood samples and tissue homogenates of BSI mice at each time point.



A: Bacterial counts were performed in blood samples (10⁴CFU/mL); B: Bacterial counts were performed in tissue homogenates (liver, lungs, and kidneys) (10⁶ CFU/organ). The data were expressed as the mean ± standard deviations. *p < 0.05 **p < 0.001.

Figure 3. Inflammatory cytokine levels in BSI and control mice.



Serum and tissue homogenates (liver, lungs, and kidneys) were collected from BSI and PBS control mice at 1, 3, 6, 12, 24, 48, 72, 96, 120, and 168 hours post-infection (n = 10/group). The concentrations of three inflammatory cytokines (IL-1β, IL-6, and TNF-α) were measured in serum (A), liver homogenates (B), lung homogenates (C), and kidney homogenates (D) using enzyme-linked immunosorbent assay kits. Mean values are shown with SEM error bars.

Table 3. The association of CRP, PCT, and PIS in *S. aureus*-induced BSI mice.

| | PCT | | PIS | |
|-----|-------|---------|-------|---------|
| | r | p | r | p |
| CRP | 0.949 | < 0.001 | 0.747 | < 0.001 |
| PCT | - | - | 0.747 | < 0.001 |

CRP: C-reactive protein; PCT: procalcitonin; PIS: pathological inflammation scoring; BSI: bloodstream infection; **p < 0.001.

The bacterial counts significantly elevated at 72–120 hours post-infection in liver homogenates, compared to lung and kidney homogenates (p < 0.05) (Figure 2B). In liver homogenates, the bacterial counts reached a peak of $(51.5 \pm 2.41) \times 10^6$ CFU/organ at 96 hours post-infection, and then declined afterwards. The bacterial counts in lung homogenates began to increase at 12 hours and reached a peak of $(17.6 \pm 2.62) \times 10^6$ CFU/organ at 168 hours post-infection. The bacterial counts in kidney homogenates gradually increased at 48 hours post-infection and reached a peak of $(20.8 \pm 5.20) \times 10^6$ CFU/organ at 7 days post-infection. Bacterial counts were not observed in blood or any tissue homogenates in PBS control group (data not shown).

IL-1β, IL-6, TNF-α levels in serum and tissue homogenates

The IL-1β, IL-6 and TNF-α levels in serum and homogenates (liver, lungs, and kidneys) were evaluated during the 7-day period post-infection (Figure 3). The cytokine levels in serum and lung and liver homogenates peaked at 6–24 hours post-infection. The IL-1β level in kidney homogenates peaked at 48 hours post-infection, while the IL-6 levels in liver and kidney homogenates and TNF-α in kidney homogenates kept increasing during the 7-day period post-infection. In contrast, the cytokine levels in serum and tissue homogenates in the PBS control group were very low.

Histopathology

In BSI mice, there were varies degrees of alveolar hemorrhage, pulmonary edema, and areas of necrosis in the lungs at 48–120 hours post-infection. In the liver of BSI mice, congestion, swelling, and hemorrhage of the surface were observed at 48–120 hours post-infection. In the kidneys of BSI mice, there were expansion, congestion, and multiple focal abscess at renal surface at 48–120 hours post-infection. The liver, lungs, and kidneys in the PBS control mice were normal, without any signs of infection (Figure 4A).

In the PBS control group, the lungs had normal appearance of alveoli, capillaries, bronchioles, and epithelium; the liver had normal hepatic lobule; the kidneys had normal glomeruli and kidney tubules. The lungs in the BSI mice showed diffused pathological

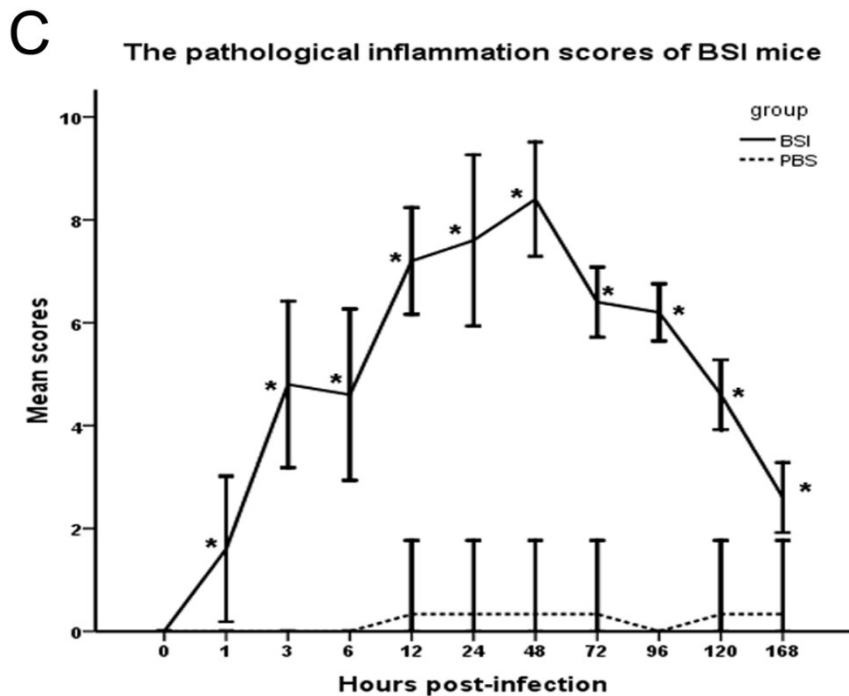
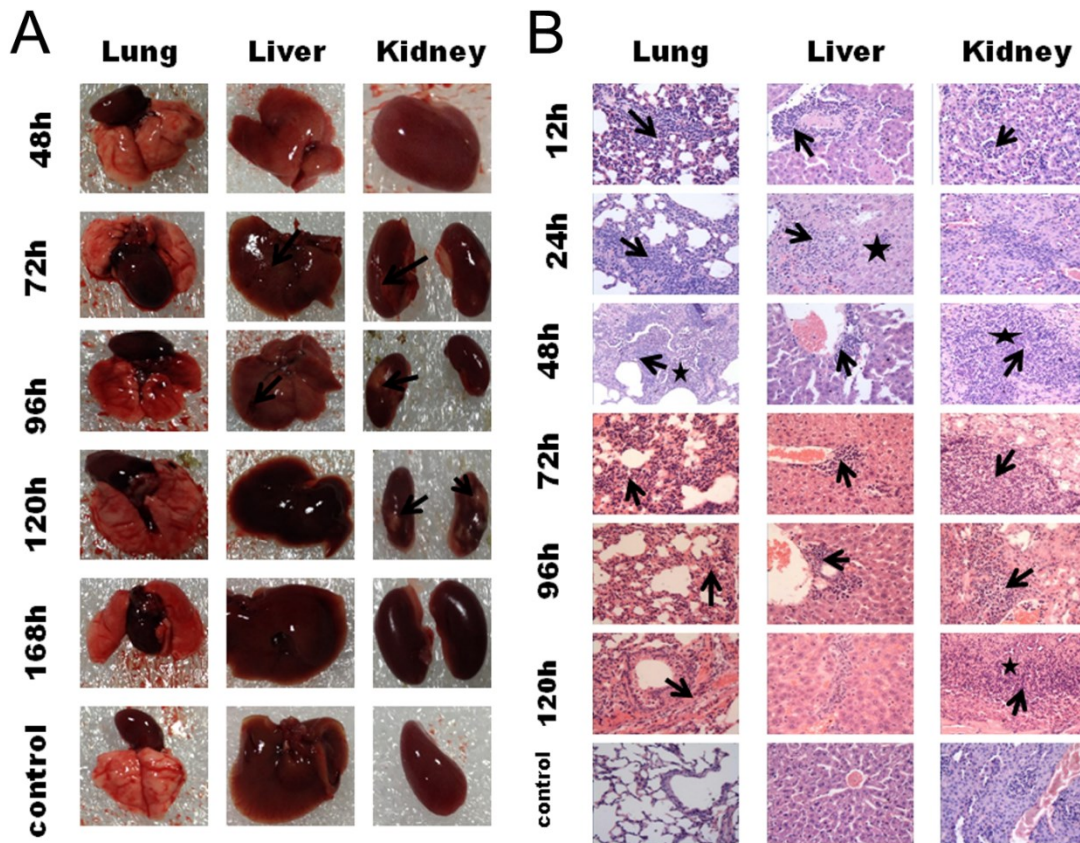
changes characterized by alveolar congestion, inflammatory cell (neutrophils and lymphocytes) infiltration into the airspace or surrounding blood vessels, and abscess or necrosis of the lung tissues. Most striking changes were observed at 12–72 hours post-infection, while mild pathologic changes with few lymphocytes were observed at 96–168 hours post-infection. There was no visible pathologic change at 1–6 hours post –infection (data not shown). In the liver of BSI mice, parenchymal cells showed vary degrees of vacuolization, degeneration, necrosis, and loss of organization and structures. Recruitment of inflammatory cells, mainly neutrophils, at the liver surface and perivascular areas was observed. These pathological changes associated with damage and inflammations were observed at 12–72 hours post-infection in BSI mice. Histopathological analysis of kidneys revealed abscesses formed by neutrophils, as well as necrosis and calcification of kidney tubules at 24–120 hours post-infection (Figure 4B).

The pathological inflammation scores were significantly higher in the BSI group compared with the PBS control group (p < 0.001). The scores peaked at 24–48 hours post-infection and then decreased afterwards (Figure 4C).

Association of the inflammatory proteins, cytokines, and the pathological inflammation scores

The association of the inflammatory proteins (CRP and PCT), cytokines (IL-1β, IL-6 and TNF-α), and PIS in BSI mice was explored. Prominent correlation was found between the elevated CRP and PCT (p < 0.001) (Table 3). There were significant positive correlations of TNF-α and IL-6 levels in serum, liver, lungs, and kidneys (p < 0.001), as well as significant correlations between IL-1β and IL-6 levels in serum, liver, and lungs (p < 0.001) (Table 4). Correlations between IL-1β and CRP or PCT in both serum and tissues (liver, lungs, and kidneys) was also found (p < 0.05). The correlation between IL-6 and CRP was only found in serum and liver. The correlation between IL-6 and PCT was only found in serum. The correlation of TNF-α and PCT was only found in serum and liver (p < 0.05) (Table 5).

Figure 4. Pathological changes in three organs and pathological inflammation scores in BSI and control mice.



A: The gross pathological changes in lungs, liver, and kidneys were evaluated at 48–168 hours post-infection in BSI and PBS control mice. In liver, the arrow indicates the hemorrhage on the surface of liver of BSI mice. In kidneys, the arrows indicate the abscesses in kidneys of BSI mice. B: Representative photomicrographs of HE-stained organs (magnification 400 ×). Sections were from the lungs, liver, and kidneys of BSI and PBS control mice at 12–120 hours post-infection. In lungs, liver, and kidneys, the arrow indicates neutrophils infiltration, and the asterisk indicates tissue necrosis in BSI mice. C: Pathological inflammation scores. Sections from lungs, liver, and kidneys of BSI and PBS control mice at each time point were stained with H&E and evaluated with a previously described scoring system. The scores were calculated as mean ± SEM (*p < 0.001).

Table 4. The association of IL-1β, IL-6, and TNF-α levels in serum and tissue homogenates (liver, lungs, kidneys) in *S. aureus*-induced BSI mice.

| | | IL-6 | | TNF-α | |
|-------|---------|-------|-----------|-------|-----------|
| | | r | p | r | p |
| IL-1β | Serum | 0.928 | < 0.001** | 0.963 | < 0.001** |
| | Liver | 0.989 | < 0.001** | 0.643 | < 0.001** |
| | Lungs | 0.603 | < 0.001** | 0.623 | 0.006* |
| | Kidneys | 0.237 | 0.098 | 0.180 | 0.210 |
| IL-6 | Serum | - | - | 0.932 | < 0.001** |
| | Liver | - | - | 0.642 | < 0.001** |
| | Lungs | - | - | 0.942 | < 0.001** |
| | Kidneys | - | - | 0.857 | < 0.001** |

BSI: bloodstream infection; *p < 0.01 **p < 0.001.

In addition, correlations were also found between PIS and cytokines (IL- 6, IL-1β, and TNF-α) or inflammatory proteins (CRP or PCT) in serum and most of the tissues (p < 0.05) (Table 5).

Discussion

In this study, we created a simple and stable mouse model of *S. aureus*-induced bloodstream infections. We found significant changes in inflammatory proteins and cytokines, histopathological changes and clinical symptoms, as well as prominent correlations among these changes in *S. aureus*-induced BSI mice.

Why were these time points chosen?

Previous studies of bloodstream infections in animal models have used different observation time points, including 2, 24, and 6–16 hours [9,11]. In different animal models, the best observation time in BSIs caused by different types of bacteria is variable. In this study, we investigated the inflammatory responses and pathological changes after *S. aureus*-induced bloodstream infection in mice. We chose different observation time points, including 1, 3, 6, 12, 24, 48, 72, 96, 120 and 168 hours post-injection and

investigated the trend of the changes at these time points. We defined the optimal observation time points based on our results, which will be used in our future study to establish a reliable mouse model of *S. aureus*-induced bloodstream infection.

Compared to CLP model

For more than 20 years, the CLP rodent model has been the most widely used model for experimental sepsis and is currently considered the gold standard in sepsis research [5,6]. Although CLP is considered a clinically relevant model of Gram-negative bacterial or polymicrobial sepsis, we still need to identify the single microorganism in blood culture at the primary stage of sepsis to apply targeted treatment for specific bacteria. Therefore, we established a mouse model of a specific bacterial sepsis and explored the best concentration of infection. The animal model used in our study was established in 1990 [12]. However, most of the previous studies have focused on the Gram-negative bacteria (such as *Escherichia coli*) BSIs. The studies on the Gram-positive bacteria (*S. aureus*) blood infections are rare. In our study, we performed more detailed observations, selected more observation time points,

Table 5. The association of cytokines, CRP, PCT, and PIS in *S. aureus*-induced BSI mice.

| | | CRP | | PCT | | Score | |
|-------|---------|-------|-----------|-------|-----------|-------|-----------|
| | | r | p | r | p | r | p |
| IL-1β | Serum | 0.329 | 0.020 | 0.282 | 0.048* | 0.122 | 0.399 |
| | Liver | 0.294 | 0.038* | 0.343 | 0.015* | 0.361 | 0.010* |
| | Lungs | 0.312 | 0.032* | 0.383 | 0.006* | 0.066 | 0.648 |
| | Kidneys | 0.927 | < 0.001** | 0.940 | < 0.001** | 0.668 | < 0.001** |
| IL-6 | Serum | 0.317 | 0.025* | 0.442 | 0.001* | 0.055 | 0.730 |
| | Liver | 0.287 | 0.043* | 0.136 | 0.348 | 0.351 | 0.013* |
| | Lungs | 0.210 | 0.144 | 0.046 | 0.753 | 0.384 | 0.006* |
| | Kidneys | 0.090 | 0.535 | 0.236 | 0.099 | 0.054 | 0.709 |
| TNF-α | Serum | 0.215 | 0.133 | 0.330 | 0.019* | 0.063 | 0.664 |
| | Liver | 0.223 | 0.119 | 0.333 | 0.018* | 0.030 | 0.838 |
| | Lungs | 0.176 | 0.223 | 0.032 | 0.826 | 0.381 | 0.006* |
| | Kidneys | 0.085 | 0.555* | 0.198 | 0.168 | 0.288 | 0.042* |

CRP: C-reactive protein; PCT: procalcitonin; PIS: pathological inflammation scoring; BSI: bloodstream infection; *p < 0.05 **p < 0.001.

applied more specific evaluation parameters, and explored the correlation of various parameters, which will provide a more specific basis for future researchers. The above two methods have their respective characteristics. Sepsis is induced in the CLP models by a surgical procedure without the preparation of inoculum. Moreover, CLP models create a polymicrobial sepsis and deliver a variable microbiological dose. Therefore, it is difficult to control the magnitude of the septic challenge. Our sepsis model has several advantages. First, it mimics many of the features of extreme clinical sepsis such as these seen in bacteremia. Second, this model induces acute effects during short periods. Third, this model induces a specific bacterial sepsis rather than polymicrobial sepsis. In addition, our model can be used to study the interactions of different intraspecific or interspecific bacteria. The intravascular infusion of viable bacteria in this model is more accurate than in other models [13].

CRP and PCT

CRP is a classic acute phase inflammatory protein that has been used as a biomarker for the presence of inflammation and tissue damage [14]. In our study, CRP levels increased at 6 hours post-infection, and peaked at 24–48 hours post-infection (Table 2) in the BSIs group, compared to the control group ($p < 0.001$). These results are similar to a previous study, in which CRP levels were significantly elevated in BSI mice compared to placebo-inoculated mice at both 17 and 48 hours after infection [15]. PCT has also been proposed as a marker of bloodstream infections and sepsis [16]. Our results demonstrate that PCT levels increased significantly in BSI mice compared with PBS control mice ($p < 0.01$) (Table 2). PCT levels in the BSI mice were first detected at 2–3 hours and peaked at 12–48 hours post-infection, which is consistent with previous studies [17]. Moreover, prominent correlations were found between elevated CRP and PCT levels ($p < 0.001$) (Table 3), which may be due to the consistency of metabolism or kinetic characteristics between them [18].

IL-1 β , IL-6, and TNF- α

IL-1 β , IL-6, and TNF- α , the characteristic inflammatory markers of sepsis [15,19], increased significantly in our BSI mice. Van den Berg *et al.* [15] assessed the cytokine levels in a murine model with *S. aureus* bacteremia and showed that levels of IL-1, IL-6, and TNF- α in serum were significantly elevated. Ashare *et al.* [20] also suggested that levels of TNF- α and IL-1 β in liver homogenates were increased in septic

mice. However, the correlations among these cytokines in *S. aureus*-induced bacteremia are poorly understood. Our results demonstrate that *S. aureus* promotes the production of all the three cytokines in serum and tissue homogenates (lungs, liver, and kidneys) in BSI mice during the 7-day observation period (Figure 3). These results are similar to those of other studies, where most of the cytokines (IL-1 β , IL-6, and TNF- α) in serum and tissue homogenates began to increase at 1–3 hours post-infection and peaked at 12–24 hours post-infection. However, we also found that IL-6 in liver and kidney homogenates, and TNF- α in kidney homogenates increased persistently during the 7-day observation period. This may be due to the different characteristics of cytokine secretion in various tissues or a limited study time frame. A future study of the mechanism of cytokines will enhance our understanding of the pathogenesis of *S. aureus*-induced BSIs. Our results suggest that the levels of IL-1 β , IL-6 and TNF- α are indicative for the presence of *S. aureus* bacteremia [15].

Our study also demonstrates the significant positive correlations of TNF- α and IL-6 levels in serum, liver, lungs, and kidneys ($p < 0.001$) (Table 4). These results are consistent with a previous study described by Baghel *et al.*, in which the researchers found that TNF- α -308 polymorphism is significantly associated with the increased expression of TNF- α and IL-6 [21]. We also found the correlations of IL-1 β and IL-6 levels in serum, liver, and lungs ($p < 0.001$) (Table 4). IL-6 production is triggered by IL-1 both *in vitro* and *in vivo*, and the IL-6 levels in response to *S. aureus* are significantly decreased in the absence of IL-1R signaling [22]. Our study did not show any correlation of the cytokines in kidneys, which need to be further investigated.

Pathological changes and PIS

The inflammatory pathological changes in tissues and organs reflect the severity of sepsis. The histological changes in lungs, liver, and kidneys of mice with bacteremia were characterized by congestion, inflammatory cells, and abscess or necrosis [15], which are similar to a CLP-operated rat model described by Cadirci *et al.* [9]. Other studies also show tubular vacuolization of renal tubular in sepsis models [23], but no changes in the lungs and liver [24]. However, Fisher *et al.* did not find significant changes in the lungs or kidneys of FIP mice [25]. In the lungs, we found significant accumulations of inflammatory cells (neutrophils) in the air spaces and tissue necrosis, especially at 12–72 hours post-infection. In the liver, we also found the recruitment of neutrophils at the liver

surface and perivascular areas, especially at 12–72 hours post-infection. In the kidneys, we found abscess formed by neutrophils and tubular necrosis and calcification at 24–120 hours post-infection (Figure 4). The discrepancy between our research and other research may be due to the different sepsis models.

We used PIS to evaluate the extent of pathological severity that was reflected in the inflammatory responses during the acute phase of infection. A previous study showed that the PIS was significantly higher in CLP group, compared with the control group, reflecting the inflammation severity in the sepsis group [9]. Our results also indicate that PIS was higher in the BSI group than in the control group ($p < 0.001$) (Figure 4).

Inflammatory proteins, cytokines, and PIS

CRP is regulated by cytokines such as IL-6, IL-1 β , and TNF- α . IL-6 is the major stimulant in CRP synthesis during acute-phase responses [26]. The maximum IL-6 plasma levels precede the maximal concentrations of PCT, suggesting that the synthesis of PCT might be induced by IL-6. However, there was no correlation between IL-6 plasma levels and systemic PCT levels [27]. Among inflammatory cytokines, IL-1 β is a particularly potent stimulant for PCT synthesis, whereas TNF- α and IL-6 are only moderate stimulants [28].

In our study, we performed the linear correlation analysis among cytokines (IL-6, IL-1 β , and TNF- α), CRP, PCT, and PIS. We found the correlations between IL-1 β and CRP or PCT in both serum and tissues (liver, lungs, and kidneys) ($p < 0.05$) (Table 5). The correlation of IL-6 and CRP was only found in serum and liver. The correlation of IL-6 and PCT was only found in serum. The correlation of TNF- α and PCT was only found in serum and liver ($p < 0.05$) (Table 5). These results may indicate that IL-1 β is the major pro-inflammatory cytokine, while IL-6 and TNF- α are both pro- and anti-inflammatory cytokines [29]. Therefore, the immune regulation mechanisms of IL-6 and TNF- α are complex and need to be further studied. Our study also shows the correlations among cytokines (IL-6, IL-1 β , and TNF- α), inflammatory proteins (CRP or PCT), and PIS in serum and most of the tissues in *S. aureus*-induced BSI mice ($p < 0.05$) (Tables 3 and 5), which are consistent with previous studies [9].

Bacterial counts

Our results show that bacterial growth is observed in blood samples and tissue homogenates (lungs, liver, and kidneys) in BSI mice. The highest bacterial counts

in the blood (92.34 ± 2.03) $\times 10^4$ /mL were less than that in the tissue homogenates (51.5 ± 2.41) $\times 10^6$ /organ in liver, (17.6 ± 2.62) $\times 10^6$ /organ in lungs, and (20.8 ± 5.20) $\times 10^6$ /organ in kidneys (Figure 2). This may be due to the complement system and lysozyme in the blood that defend against infection directly by their lytic activities [30].

Weight loss

Weight loss is regarded as the result of undernourishment in sepsis or sepsis shock in mice. Colavite-Machado *et al.* described the weight loss after 3, 7, and 14 days of infection in *S. aureus*-infected mice [31]. Similarly, we observed a significant weight loss after 24 hours post-infection in BSI mice ($p < 0.001$) (Table 1). In contrast, the mice in the PBS control group showed an increase in body weight during the 7-day observation period. The early weight loss in BSI mice was associated with the intensity of inflammatory response at 24–48 hours post-infection, during which the inflammatory cytokines elevated significantly (Table 1). We speculate that IL-1 β exerts a direct down-regulatory effect on food intake [32], while IL-1R signaling might have indirect effects on food intake in the first 24 hours after inoculation of *S. aureus*. Moreover, TNF- α is also involved in the regulation of body weight [33] and its production is regulated by IL-1R expression. In addition, IL-6 induces fever and is recently shown to be an important mediator in controlling body weight [34].

Conclusions

In this study, we created a Gram-positive *S. aureus* BSI model to mimic human sepsis. BSI mice showed strong inflammatory responses and significant histopathological changes compared to PBS control mice. Correlations were found among the inflammatory proteins (CRP and PCT), cytokines (IL-6, IL-1 β , and TNF- α), and the pathological inflammation scores in *S. aureus*-induced BSI mice. However, underlying mechanisms related to the inflammatory responses and histopathological changes in BSIs still need to be further explored.

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