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Comparison of serum cytokines for bacteremia and fungemia in rat bloodstream infection model

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Abstract

Objective: We aim to measure the serum levels of cytokines/chemokines in cases of candidemia and bacteremia using mouse model. Methods and Materials: Mice models of bloodstream infection (BSI) with *Staphylococcus aureus* (Sa), *Enterococcus faecalis* (Ef), *Escherichia coli* (Ec), *Klebsiella pneumonia* (Kp), *Candida albicans* (Ca) and *Candida parapsilosis* (Cp) were established. Cytokines and chemokines including TNF-α, IL-1β, IL-10, IL-17, MIP-1β, MIP-2 and G-CSF in serum were detected by Luminex xMAP assay and were compared among different groups at different time points. Results: TNF-α, IL-1β and G-CSF first increased and then decreased after infection in all infection groups, while IL-10 decreased to the minimum levels 12h after injection and then gradually increased. As for IL-17, MIP-1β and MIP-2, they increased more rapidly than the other cytokines and then also decreased. The maximum levels of TNF-α, IL-1β, IL-17 MIP-1β and MIP-2 were all higher in GN group, when compared with GP groups and fungemia groups. Peak level of G-CSF in GP groups was higher than the other two groups, while IL-10 was the highest in fungemia groups. Conclusion: Our study indicates that variation trend of serum cytokines and chemokines were different in bacterial or fungal BSI models.

Keywords: bacteremia; candidemia; cytokine; chemokine; animal model.

Practical Application: Cytokines and chemokines may be used in the early diagnosis for BSI.

1 Introduction

Bloodstream infections are a major cause of death with increasing incidence and severity. They are often associated with severe diseases with high morbidity and mortality (Chen et al., 2017). The worldwide increase in incidence emerges as one of the leading causes of death, especially in critically ill patients (Xie et al., 2018). Increased mortality of BSIs is often related to delayed, insufficient, or inappropriate anti-infective treatment (Lahmer et al., 2016). Therefore, the diagnosis and treatment of BSI requires great concern. Since the primary disease variety and clinical manifestations vary widely, clinicians need to make appropriate diagnosis based on the early symptoms and laboratory tests, which highlight the clinical significance of early laboratory tests.

Blood culture has been used as the reference standard in clinical microbiology diagnosis. The single most important factor for achieving a sensitivity of over 95% is the sufficient volumes, sometimes two to three sets, filled with 10 mL per bottle. Since 50% of the patients present with a bacteremia of lower than 1 colony-forming unit (CFU)/m (Florio et al., 2018). Major drawbacks of blood culture include rather long turnaround time, failure to identify slow growing microorganisms and delay or even failure to detect microorganisms after receiving antimicrobials (Barenfanger et al., 2008).. Matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) is a highly accurate analytical technique that enables the identification and quantification of polypeptides, as well as a variety of other compounds. In recent years, MALDI-TOF has been widely used for microorganism identification, but still requires blood culture in advance (Barnini et al., 2015). Molecular methods including PCR assays and next generation sequencing (NGS) have also been used for pathogen identification during the past years (Bauer et al., 2010).. Due to multiple manual operations in the whole process, lack of conclusive validation for commercially available assays and variety of methodologies, these methods have not been recommended for routine use in clinical practice (Wang et al., 2020).. In addition, there are no clinical characteristics that could differentiate between candidemia and bacteremia (Gram-negative and Gram-positive).

Serological marker detection allows for a short turnaround time and cytokine analysis has been performed in bacteremia (Thamphiwatana et al., 2017). It has been reported that the concentrations of cytokines/chemokines may increase earlier than currently available inflammatory proteins during bacteremia. However, the dynamic changes of these cytokines/chemokines during bacteremia and candidemia have not yet been clearly demonstrated. Therefore, our study aims to use mouse models to investigate the serum levels of cytokines and chemokines during both bacteremia and candidemia.

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2 Material and methods

2.1 Preparation of standard strains and experimental mice

The standard strains *Staphylococcus aureus* ATCC 25923, *Enterococcus faecalis* ATCC 29212,*Klebsiella pneumoniae* ATCC 700603, *Escherichia coli* ATCC 25922,

Candida albicans ATCC 10231 and *Candida parapsilosis* ATCC 22019 were donated by the Department of Microbiology from our hospital. Bacterial strains were incubated in LB media (Oxide Microbiology Products, England) for 16~18 h at 37 °C. The standard strain of *Candida albicans* and *Candida parapsilosis* were separated on a sand-protected medium and then purified by LB liquid medium. The resuspended bacterial and fungal solution was formulated according to McFarley turbidity and diluted to different concentration gradients.

460 SPF male ICR mice weighing 25-27 g were purchased from Weitong Lihua Experimental Animal Technology Co., Ltd. Among them, 180 mice were used to determine the half lethal dose (LD50) and LD90. The other 280 mice were used for subsequently establishment of bloodstream infection model. Mice were housed in Grade 2 animal room and were adaptively fed for 1 week before the experiment. The experiment flowchart was shown in Figure 1.

2.2 Determination of median lethal dose

To ensure that the mice were alive under the infection condition during observation and blood collection, we chose 1/2 LD50 as the dose of bacterial infections. The resuspended bacteria or fung were first diluted to 3.9 Maxwell's turbidity. Then we performed a 10-fold dilution and diluted six concentrations sequentially. The diluted suspension was injected into the mice by the tail vein, and each concentration had 5 mice, with a total of 6 groups. The injection volume was 0.1 mL /10 g. Mouse actions, weight and death were observed and recorded daily for 7 days. The LD50 of was calculated by Karber method. LD50s of *Staphylococcus aureus*, *Enterococcus faecalis*,*Klebsiella pneumoniae*, *Escherichia coli*, *Candida albicans* and *Candida parapsilosis* were 8.1×10^8/mL, 9.6×10^8/mL, 8.1×10^8/mL, 1.1×10^8/mL, 1×10^8/mL and 2×10^8/mL, respectively.

2.3 Establishment of mice BSI model

Mice were weighed and randomly assigned to weight-matched groups in advance. The mice were separated into 7 groups treated with each type of pathogen and phosphate buffer saline (PBS) was considered as control group. Standard strain was activated on flat plates and cultured in the LB liquid nutrient medium for 12 h, and then 100 μL bacterial fluid was transferred into a new LB medium to culture for 4–6 h. Mice were injected in a volume of 0.1 mL/10 g by the intravenous tail with the concentration of half LD50 for each pathogen. Blood samples were collected from infected mice before injection and after 1, 3, 6, 12, 24, 48 and 72 h injection separately (n = 5 at each time point). Eyeball blood collection method was used to collect about 100 μL blood in EP tubes containing EDTA-K2. Then blood samples were centrifuged at 3000 g for 5 min and serum of each sample was transferred into a new tube and stored at −80 °C until use.

2.4 Measurement of the serum cytokine levels

Luminex® xMAP Bead Array Platform (Millipore Corporation, Germany) was used for measurement of cytokines including

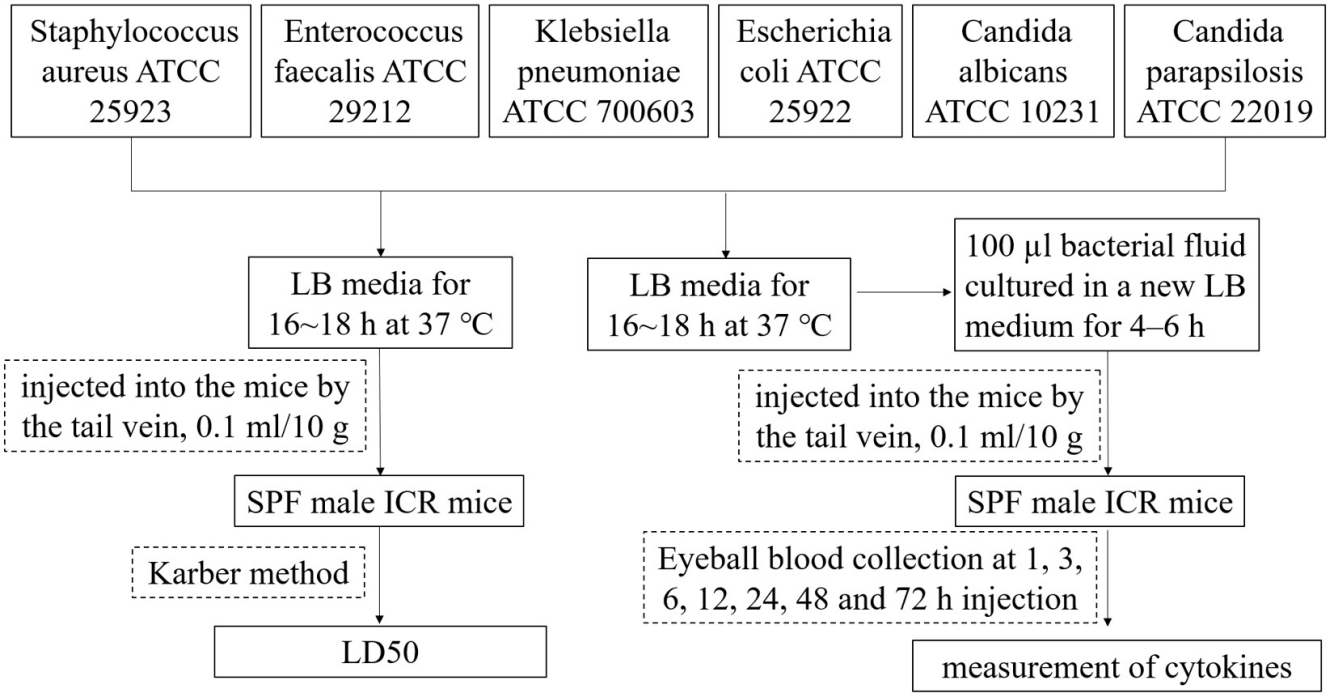

Figure 1. Flowchart.

tumor necrosis factor (TNF)-α, interleukin (IL)-1β, IL-10, IL17, macrophage inflammatory protein (MIP)-1β, MIP-2, IL-12p40 and IL-12p70. The assays were conducted according to the instruction of instrument, and data were analyzed with Milliplex analyte v5.1.0.0 software.

2.5 Statistical analysis

Data were analyzed with SPSS 22.0 and GraphPad Prism 5 (GraphPad Software, USA). Normality test was performed and data with normal distribution were expressed as mean ± SD and t-test was applied for comparison. One-way ANOVA was used to compare data among multiple groups. For non-normally distributed data, the median and quartiles were used to represent concentrated and discrete trends, and Mann–Whitney U test was used to compare samples. In order to determine the cut-off values for cytokines and chemokines, receiver operating characteristic (ROC) analysis was performed and an area under the curve (AUC), sensitivity and specificity values were calculated. $P < 0.05$ was considered as statistically significant.

3 Results

3.1 Kinetic changes of TNF-α, IL-1β and G-CSF in bacterial and fungal bloodstream infection models

TNF-α and IL-1β increased significantly during the first 48 h after injection in Gram-positive (GP) groups and Gram-negative (GN) groups and then decreased 72 h after infection. While in fungemia groups, these two cytokines increased gradually 72 h after injection (Figure 2ab). G-CSF increased during the first 24 h after infection in both GP and GN groups, while it reached the peak level 48 h after infection in fungemia groups (Figure 2c).

The highest level of TNF-α in *Staphylococcus aureus* and *Enterococcus faecalis* groups occurred at 24 h after injection, and reached 256.78 ± 32.42 pg/mL and 206.22 ± 20.42 pg/mL, respectively. While the highest level of IL-1β in these two GP groups occurred at 48h after injection, and reached 245.4 ± 19.3 pg/mL and 237.5 ± 15.4 pg/mL, respectively. In *Klebsiella pneumoniae* and *Escherichia coli* groups, TNF-α increased to 546.4 ± 28.4 pg/mL and 521.2 ± 21.2 pg/mL, respectively, 48 h after injection, while IL-1β also increased to the highest level at the same time point. As for *Candida albicans* and *Candida parapsilosis* groups, TNF-α continuously increased to 264.3 ± 20.22 pg/mL and 275.3 ± 19.44 pg/mL, respectively during the 72 h after injection, while IL-1β first increased to 252.3 ± 12.55 pg/mL and 241.5 ± 12.33 pg/mL, respectively 48 h after injection and then gradually decreased at 72 h. The maximum levels of TNF-α and IL-1β were significantly higher in GN group, when compared with both GP groups and fungemia groups ($p < 0.05$) (Figure 2d, 1e). G-CSF reached its peak levels of 19872 ± 198 pg/mL and 19442 ± 148 pg/mL, respectively in GP groups.

3.2 Kinetic changes of IL-10 in bacterial and fungal bloodstream infection models

As for the GN groups and fungemia groups, changes of IL-10 suffered from the same trend, first decreased to the minimum levels 12 h after injection and then gradually increased during the next 36 h, but again decreased after 48 h. For GP groups, after decrease for the first 12 h, IL-10 continues to increase in the following 60 h (Figure 3a). The minimum levels

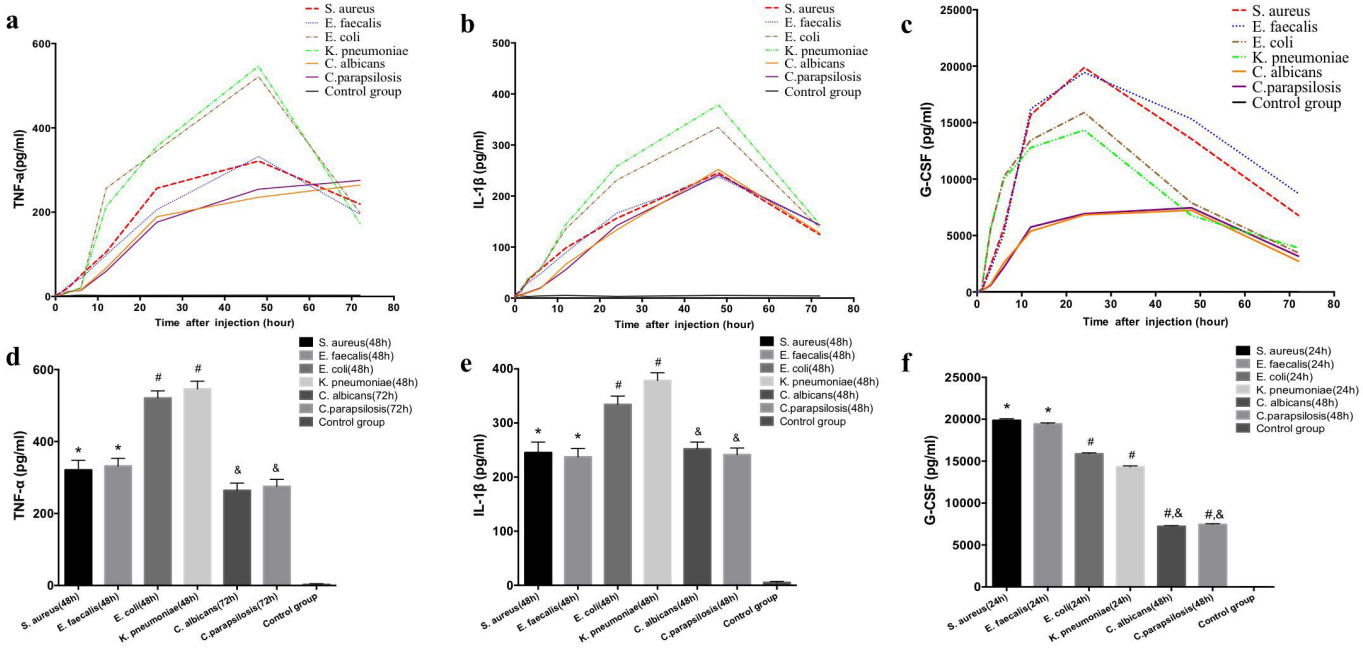

Figure 2. Kinetic changes and maximum levels of TNF-α (a, d), IL-1β (b, e), and G-CSF (c, f) at different time points in different bacteria and fungi bloodstream infection groups. *$p < 0.05$, compared with control group, #$p < 0.05$, compared with *S. aureus* and *E. faecalis* groups, &$p < 0.05$, compared with *E. coli* and *K. pneumonia* groups.

of IL-10 decreased to 10.6 ± 3.4 pg/mL and 13.4 ± 2.6 pg/mL, respectively, while the maximum levels of IL-10 were 96.9 ± 9.3 pg/mL and 98.6 ± 8.9 pg/mL, respectively in *Staphylococcus aureus* and *Enterococcus faecalis* groups. IL-10 increased to 105.4 ± 10.3 pg/mL and 121.7 ± 11.7 pg/mL, respectively in *Klebsiella pneumoniae* and *Escherichia coli* groups, both of which were significantly higher than GP groups. The highest levels of IL-10 in fungemia groups were 255.6 ± 26.3 pg/mL and 295.6 ± 28.7 pg/mL, respectively, which also occurred 48 h after infection and were significantly higher than those of GP and GN groups (Figure 3b).

3.3 Kinetic changes of IL-17, MIP-1 and MIP-2 in bacterial and fungal bloodstream infection models

IL-17, MIP-1β and MIP-2 increased more rapidly than the other cytokines. IL-17 increased to the maximum levels 6 h after infection in both GP and GN groups, while 12 h after infection in fungemia groups (Figure 4a). MIP-1β reached the highest levels at 6 h, 3 h and 12 h, respectively in GP, GN and fungemia groups (Figure 4b). It took 6 h for MIP-2 to reach the peak in GP and GN groups, while 48 h in fungemia groups (Figure 4c).

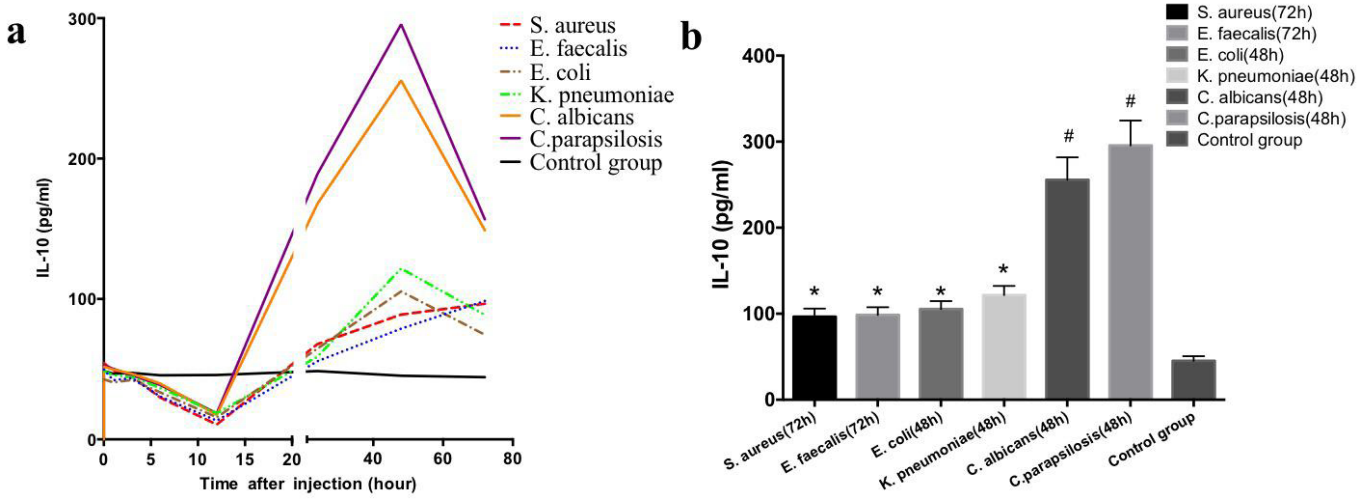

Figure 3. Kinetic changes and maximum levels of IL-10 at different time points in different bacteria and fungi bloodstream infection groups. *p < 0.05, compared with control group, # p < 0.05, compared with *S. aureus* and *E. faecalis* groups.

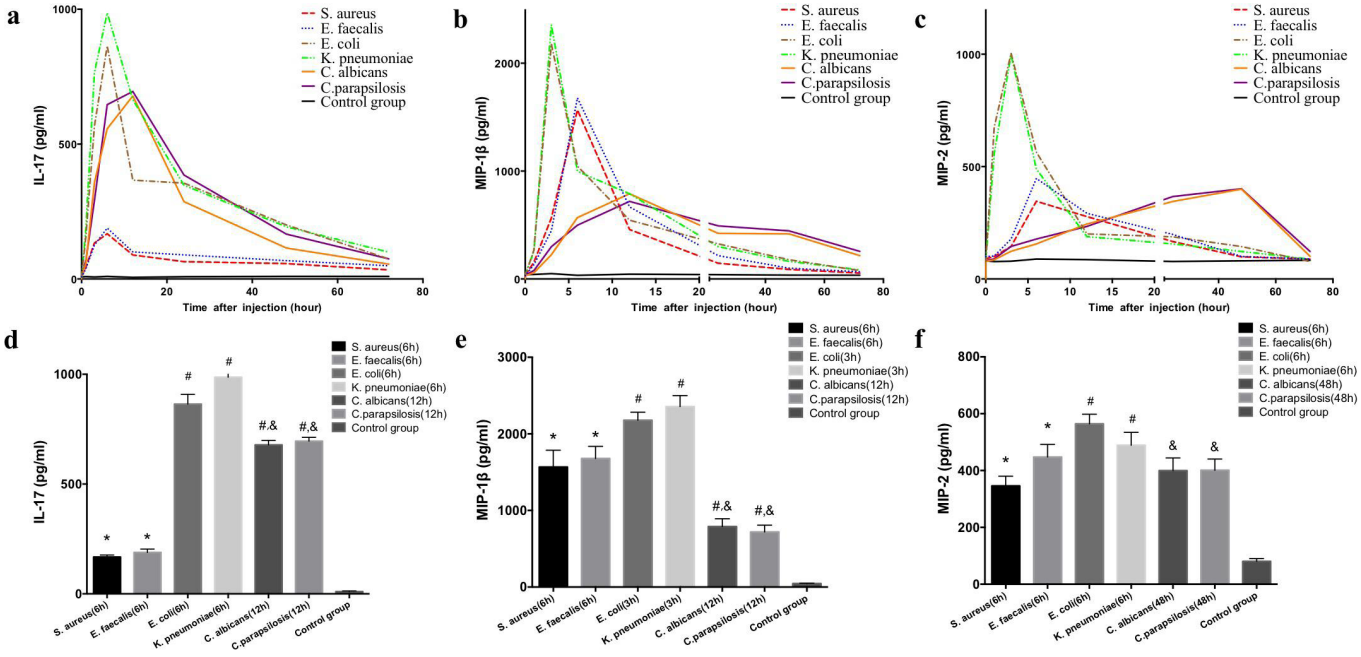

Figure 4. Kinetic changes and maximum levels of IL-17 (a, d), MIP-1β (b, e) and MIP-2 (c, f) at different time points in different bacteria and fungi bloodstream infection groups. *p < 0.05, compared with control group, #p < 0.05, compared with *S. aureus* and *E. faecalis* groups. &p < 0.05, compared with *E. coli* and *K. pneumonia* groups.

IL-17 increased to the maximum levels of 167.8 ± 17.6 pg/mL and 188.6 ± 15.3 pg/mL, respectively in *Staphylococcus aureus* and *Enterococcus faecalis* groups, both of which were significantly lower than the highest levels of IL-17 in both GN groups (864.3 ± 44.3 pg/mL and 986.8 ± 33.8 pg/mL, respectively). As for the fungemia groups, the maximum levels reached to 687.5 ± 30.3 pg/mL and 695.3 ± 27.5 pg/mL, respectively, both of which were lower than GN groups but higher than GP groups ($p < 0.05$) (Figure 4d).

MIP-1β increased to the highest levels of 1566.7 ± 219.2 pg/mL and 1667.8 ± 158.3 pg/mL, respectively in *Staphylococcus aureus* and *Enterococcus faecalis* groups. While in GN groups, the highest levels were 2177.8 ± 105.4 pg/mL and 2355.6 ± 144.5 pg/mL, respectively in *Klebsiella pneumoniae* and *Escherichia coli* groups, both of which were higher than those in GP groups. In fungemia groups, the peak levels were 789.3 ± 92.2 pg/mL and 719.5 ± 88.3 pg/mL, respectively, both of which were lower than those in GN and GP groups ($p < 0.05$) (Figure 4e).

MIP-2 increased to the peak levels of 345.6 ± 34.2 pg/mL and 447.3 ± 44.3 pg/mL, respectively in *Staphylococcus aureus* and *Enterococcus faecalis* groups. While in GN groups, the peak levels were 564.2 ± 44.8 pg/mL and 488.9 ± 41.3 pg/mL, respectively, both of which were significantly higher than those in GP groups ($p < 0.05$). As for fungemia groups, the highest levels were 399.8 ± 40.8 pg/mL and 401.9 ± 39.3 pg/mL, respectively, both of which were significantly lower than those in GN groups ($p < 0.05$) (Figure 4f).

4 Discussion

Blood stream infection (BSI) is associated with high mortality and high medical expenses (Moral et al., 2012). The identification of BSI mostly relies on blood culture conventionally, but this method has low positive rate, and is time-consuming. Innate immune cells including neutrophils and macrophages are the first line of host defense, which produce a number of pro-inflammatory cytokines and chemokines to eliminate pathogens upon their entry (Sugitharini et al., 2013). Though several studies have documented changes of cytokines and chemokines in bacteremia or sepsis, few studies were reported to compare their differences between fungemia and bacteremia. Thus, in our current study, we investigated the changes of 7 cytokines and chemokines during the first 72 h after bacterial or fungal BSI with mice models.

Immune response of the host differentiates among different types of bacteria. According to bacteria location in the host, this process can be divided into intracellular and extracellular immunity. In this study, mice were applied as experimental models. Through a large number of experiments in the early stage, four types of frequently occurred bloodstream infection models caused by *Staphylococcus aureus, Enterococcus faecalis, Escherichia coli*, *Klebsiella pneumonia, Candida albicans* and *Candida parapsilosis* were successfully established. Then we used liquid phase chip (Luminex) to explore discrepancies of cytokines and chemokines among infection of different types of bacteria or fungi. Through this study, we might provide auxiliary evidence for early diagnosis of bacterial bloodstream infection and basis for subsequent clinical validation.

Serum levels of TNF-α and IL-1β are important pro-inflammatory cytokines and are essential factors in innate immunity. Tumor necrosis factor alpha (TNF-α) is a fundamental cytokine in mounting immune response and its high levels are critical in inflammatory conditions (Tweedle & Deepe, 2018). TNF-α is stimulated to release after activation of phagocytosis and plays an important role in determining the development of subsequent adaptive immune responses (Wevers et al., 2013; Carvalho et al., 2012). In addition, anti-TNFα agents have been shown to be associated with increased infection risks for invasive fungal infections, particularly when given late in the overall course of treatment in pediatric patients (Tragiannidis et al., 2017). In our current study, kinetic changes of TNF-α and IL-1β were similar. Both these two cytokines were significantly higher in the two Gram-negative bacteremia groups, when compared with both Gram-positive bacteremia and fungemia groups. Thus, these two cytokines might be used to differentiate Gram-positive from Gram-negative bacteremia and bacteremia from fungemia.

G-CSF is a glycoprotein that stimulates the bone marrow to produce granulocytes and stem cells and release them into the bloodstream. It can be produced by endothelium cells, macrophage and several other immune cells from different tissues. It could not only act as a cytokine but also a hormone, and it stimulates the survival, proliferation, differentiation, and function of neutrophil precursors and mature neutrophils. In current study, G-CSF was significantly different among Gram-positive bacteremia, Gram-negative bacteremia and fungemia groups.

IL-10 is arguably the most potent anti-inflammatory cytokine, which is produced by almost all the innate and adaptive immune cells. Studies showed that multiple cell types are targets of IL-10 and that through its inhibitory effects on macrophages, IL-10 restrains immune responses to pathogens and microbial flora and prevents their pathologies (Saxena et al., 2015). Thus, according to our results, IL-10 decreased during the first 12 h after infection and then gradually increased to the peak level 48 h after infection.

IL-17 is produced by Th17 cells, a subset of CD4+ T-helper cells, which could induce the expression of other chemokines, cytokines and matrix metalloproteinases (Caron et al., 2014). It is a cytokine which elicits protection against extracellular bacterial and fungal infections and plays important roles in inflammation (Zielinski et al., 2021; Ismail et al., 2021; Ishimoto et al., 2020). IL-17 acts on various isolated cells in both humans and mice, such as endothelial cells, macrophages, fibroblasts, osteoblasts, and chondrocytes, which produce proinflammatory cytokines from monocytes such as TNF-α, IL-1β and IL-6 (Binger et al., 2017).

MIP-1β and MIP-2 belong to the chemokines, both of which play chemoattractant roles and are divided into four families based on the location of cysteine residues in the ligands: CXC, CC, C, and CX3C (Ciechanowska et al., 2020). During inflammation response, MIP-1β and MIP-2 can stimulate the accumulation of monocytes to the inflammation site, thereby exerting their anti-inflammatory effect. This study focuses on changes of MIP-1β and MIP-2 after different types of bacteria and fungi enter the bloodstream. Our results indicated that MIP-1β and MIP-2 increased to the peak levels three or six h after injection in Gram-positive or Gram-negative bacteremia

groups, while the maximum level postponed to 12 h or 48 h in fungemia groups. In addition, the peak levels in Gram-negative bacteremia groups were significantly higher than Gram-positive bacteremia and fungemia groups. Three h after the bacteria entered the blood, concentrations of both MIP-1β and MIP-2 in Gram-negative bacteremia groups were higher than those in Gram-positive groups. Therefore, higher concentration of MIP-1β might be indicative of Gram-negative bacteria infection such as Escherichia coli and Klebsiella pneumoniae. Otherwise, with relative low-level increase of MIP-1β, fungi tend to be the pathogens causing the bloodstream infection.

5 Conclusion

In conclusion, we investigated the kinetic changes of seven serum cytokines/chemokines in bacteremia and candidemia using the bloodstream infection mice model. Our study indicates that the immune response generated by mice infected with each bacterial or fungal infection varied, suggesting that multiple cytokines or chemokines should be combined together. Multiple time points could thus help to predict whether the bacteria or fungal bloodstream infection occurs or the type of pathogen that caused the infection. There might be differences in terms of cytokine profiles and concentrations between clinical isolates and standard strains of the same bacteria. Thus, changes of more cytokines and chemokines after bloodstream infection ought to be investigated and we will further explore cytokines and chemokines in follow-up clinical studies.

Conflict of interest

The author reports no conflicts of interest in this work.

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