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ORIGINAL ARTICLE

IL-6 and IL-10 Closely Correlate with Bacterial Bloodstream Infection

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ABSTRACT

Background: Given the high mortality of bacterial bloodstream infections (BSI), blood culture results do not meet clinical needs timely due to being time-consuming and having low positive rate. Whether we can identify the severity and type of bacterial infections by cytokines is a controversial issue. **Objective:** To investigate the dynamic change of cytokines in BSI. **Methods:** 55 patients with Gram-positive (GP) BSI, 64 patients with Gram-negative (GN) BSI and 52 healthy controls were enrolled. We quantitatively detected the cytokines interleukin (IL)-2, IL-4, IL-6, IL-10, tumor necrosis factor-alpha (TNF-α) and interferon-gamma (IFN-γ) by flow cytometry in the sera. The levels of procalcitonin, C-reactive protein, leukocytes and neutrophils were also detected simultaneously. **Results:** There were significantly up-regulated IL-6 and IL-10 expression in BSI patients, particularly in the GN-BSI, for instance Escherichia coli and Klebsiella pneumoniae infections; following the treatment, IL-6 and IL-10 decreased by 10-23 and 4-27 times, respectively. Additionally, IL-2, TNF-α and IFN-γ expression increased slightly in BSI patients and IFN-γ expression declined as GN-BSI progressed. **Conclusion:** IL-6 and IL-10 are closely associated with the severity and treatment efficacy of BSI, and can help to distinguish between GP-BSI and GN-BSI at an early stage.

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Keywords: Bloodstream Infection, Cytokines, Gram-Negative Bacteria, IL-6, IL-10

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INTRODUCTION

Bacterial bloodstream infections (BSI) have been defined by bacteria positive blood cultures in patients with systemic signs of infection and might be either secondary to a documented source or primary, meaning without an identified origin (1). Relevant studies have shown that in 2017, an estimated 48.9 million incident cases of sepsis were recorded worldwide and 11.0 million sepsis-related deaths were reported, representing 19.7% of the total global deaths (2). BSI have high mortality and could prolong hospital stays, increase hospitalization costs, and trigger serious harms to patients. The global economic burden of BSI is far beyond previously acknowledged. In 2017, an updated guideline suggested that microbiologic cultures should remain the gold standard for the diagnosis of sepsis and advocated empiric broad-spectrum therapy with one or more antimicrobials for patients presenting with sepsis or septic shock, to cover all likely pathogens (3). It is noteworthy that the speed of blood culture is far below the speed of the diagnosis we need; what is more, the empirical use of broad-spectrum antibiotics will greatly reduce the sensitivity of blood culture and increase the BSI morbidity. Obviously, early identification and appropriate management in the initial hours after BSI development, for sepsis in particular, could significantly improve prognosis. Cytokines, as rapid, simple, and convenient indicators for assessing body's immune status, have received increasing scientific attention regarding the diagnosis and treatment of infectious diseases. Under the stimulation of pathogens, $CD4^+$ T helper (Th) lymphocyte subsets would activate and differentiate into different subgroups, including Th1, Th2, Th17 and Treg cells, and do forth (4,5) to coordinate the immune response of the body. Th1 cells mainly produce interleukin (IL)-2, interferon-gamma (IFN-γ) and tumor necrosis factor-alpha (TNF-α) to induce the pro-inflammatory outcomes by cell-mediated immune responses. Th2 cells subset are believed to be able to produce IL-4, IL-5, IL-6, IL-9, IL-10, IL-13, IL-25 and amphiregulin, being mainly responsible for humoral-mediated immunity and having more anti-inflammatory response (6). A report claimed endotoxemia-induced suppression of pro-inflammatory Th cells might be considered as a contributing factor to immunoparalysis in sepsis (7). Concerning the above-mentioned matter, the optimal scenario would therefore seem to be that humans should produce a well-balanced $CD4^+$ Th lymphocyte subsets immune response, suited to the immune challenge (8). Shimizu et al. declared that pro-inflammatory cytokines, especially IFN-γ, TNF-α, and IL-18 are closely related to the occurrence of human parechovirus type 3 virus-induced sepsis (9). In addition, a prospective observational study asseverated that TNF-α, chemokine CCL4, E-selectin, vascular cell adhesion molecule-1, intracellular adhesion molecule-1 and tissue inhibitors of metalloproteinas-1 could be utilized in order to distinguish bacteremia patients from non-bacteremia ones (10). However, the relationship between Th1/Th2 cytokine profiles and the severity of BSI remains inconclusive, and the probability of distinguishing gram-positive (GP) and gram-negative bacterial bloodstream infections (GN-BSI) quickly by cytokines is obscure.

MATERIALS AND METHODS

Patients. The research protocol was approved by the Institutional Review Ethics Committee of the First Affiliated Hospital of Wenzhou Medical University. We enrolled

152 blood culture positive patients, who were hospitalized between the dates 09/2018 and 05/2019. Written informed consent was obtained from the studied patients or their authorized family members. Our patients were excluded from the study if they had any of the following conditions: contaminated blood culture specimens, non-single bacterial infections, with fungal or viral infections, treated with antibiotics before admission, using hormones or immunosuppressants during one month ahead of admission, severe immunodeficiency caused by various reasons or recent surgery. After screening through the above strict exclusion criteria, 33 unqualified cases were ruled out, including 17 complicated infection cases, among which more than one bacterial pathogen were found in blood culture, 11 cases with positive blood culture but highly suspected contamination, and 5 fungal infection cases. 119 cases of BSI patients were enrolled, divided into GP-BSI group (55 cases, 46.22%) and GN-BSI group (64 cases, 53.78%) by Gram staining. Additionally, 52 age-matched and sex-matched health individuals were enrolled as healthy controls (HC). Clinical baseline characteristics of the patients were collected at the time of admission (Table 1). No significant difference was observed concerning age ($p=0.961$), sex ($p=0.556$) and the distribution of major primary diseases (P, 0.061- 0.631) between the GP-BSI and GN-BSI groups. We reclassified the cytokines data of BSI patients according to the common primary diseases, and compared the significance of the cytokines IL-2, IL-4, IL-6, IL-10, TNF-α and IFN-γ in the tumor group ($p>0.138$), digestive system disease group ($p>0.114$) and unexplained fever group ($p>0.157$) with BSI group, and yet no significant statistical difference was found (data not shown). Moreover, through the classification of blood culture pathogens, we counted the bacteria type detected in each BSI patient's blood culture (Table 2), and found that GP bacteria were mainly *Staphylococcus epidermidis* (10, 18.18%) and *Staphylococcus hominis* (9, 16.36%), while GN bacteria were mainly *Escherichia coli* (21, 32.81%) and *Klebsiella pneumoniae* (21, 32.81%).

Laboratory Examination. At the peak of fever, before the patient started antibiotic treatment, two sets of whole blood were drawn for anaerobic and aerobic blood cultures. 3 ml peripheral venous blood was placed in a blood collection tube containing a separating gel, and no anticoagulant was added. Following the coagulation, the blood was centrifuged (3000 r/min, 5 min) and the serum was taken for cytokine, PCT and C-reactive protein (CRP) detection. 2 ml whole blood was collected with ethylenediaminetetraacetic acid anticoagulation tube for leukocytes (WBC) and neutrophils percentage (NEUT%) detection.

Blood Culture. The positive specimens cultured by the automatic blood culture instrument (BacT/Alert, America) were transferred to the blood plate and cultured for 12 to 24 hours in 37°C, 5% CO_2. The colonies were directly picked and coated on the target plate, and the bacteria were identified using the automatic mass spectrometer (Vitek 2-Compact, France).

Determination of Cytokine Profile. We quantitatively detected the cytokines IL-2, IL-4, IL-6, IL-10, TNF-α and IFN-γ with flow cytometer (FACSCanto™ plus, America) based on the cytometric bead array technique. Primarily, the capture beads, containing six kinds of capture bead mixtures with different fluorescence intensities and labeled allophycocyanin (APC)-conjugated specific antibodies of IL-2, IL-4, IL-6, IL-10, TNF-α and IFN-γ on the surface, bounded to the cytokines in the sample to be tested.

Table 1. Clinical baseline characteristics of enrolled BSI patients.

Characteristics	BSI	GP-BSI	GN-BSI
Total cases[N]	119	55	64
Age[median (IQR)]	63.00(52.00-71.00)	63.00(49.00-71.00)	63.00(54.25-70.00)
Female[N (%)]	40(33.61)	20(36.36)	20(31.25)
Primary disease[N]			
Tumor	30	15	15
Digestive system disease	25	13	12
Unexplained fever	24	7	17
Respiratory diseases	7	4	3
trauma	7	5	2
Cardiovascular diseases	5	3	2
Metabolic disease	4	0	4
Diseases of Urology	3	1	2
Dermatological diseases	3	0	3
Cerebrovascular disease	3	2	1
Hematologic diseases	3	2	1
After transplantation	2	1	1
Orthopedic diseases	2	1	1
Drug poisoning	1	1	0
Peak body temperature[1] [N]			
Low heat	11	8	3
Moderate heat	42	25	17
High heat	62	22	40
Ultra-high heat	4	0	4
Septic shock[N (%)]	30(25.21)	7(12.73)	23(35.94)
Hospital mortality[2] [N (%)]	17(14.29)	2(3.64)	15(23.44)
Pathogen detection specimen[N]			
Peripheral blood	104	53	51
Peripheral blood &Sputum	9	1	8
Peripheral blood & Drainage fluid	4	1	3
Peripheral blood & Urine	2	0	2

Abbreviations: BSI, bacterial bloodstream infections; GP-BSI, Gram-positive bacterial bloodstream infections; GN-BSI, Gram-negative bacterial bloodstream infections; N (%), sample size (percentage); IQR, interquartile range. Annotation: 1, Highest temperature recorded during the patient's hospital stay; 2, death from BSI, excluding death due to primary disease; Low heat, 37.3-38.0°C; Moderate heat, 38.1-39.0°C; High heat, 39.1-41.0°C; Ultra-high heat, >41.0°C.

Subsequently, phycoerythrin (PE)-conjugated detection antibodies were added to the above mixtures. Ultimately, double-antibody sandwich complexes were formed to analyze the mixture fluorescence intensity to obtain the cytokine content in the sample. Th1/Th2 cytokine kit Ⅱ was purchased from Becton Dickinson and Company (BD Biosciences, San Jose, CA, USA). We randomly measured the expression of cytokines in 28 patients (including 13 GP-BSI and 15 GN-BSI patients) after the treatment as blood cultures became negative.

Table 2. The bacteria classification of positive blood culture.

GP-BSI	N(%)	*GN-BSI*	N(%)
Staphylococcus epidermidis	10 (18.18%)	***Escherichia coli***	21 (32.81%)
Staphylococcus hominis	9 (16.36%)	***Klebsiella pneumoniae***	21 (32.81%)
Staphylococcus aureus	7 (12.73%)	***Acinetobacter baumannii***	4 (6.25%)
Staphylococcus capitis	6 (10.91%)	***stenotrophomonas maltophilia***	3 (4.69%)
Staphylococcus haemolyticus	3 (5.45%)	***Pseudomonas aeruginosa***	3 (4.69%)
Streptococcus anginosus	3 (5.45%)	***Salmonella enteritidis***	2 (3.13%)
Enterococcus faecium	3 (5.45%)	***Aeromonas sobria***	2 (3.13%)
Enterococcus faecalis	2 (3.64%)	***Burkholderia cenocepacia***	2 (3.13%)
Streptococcus agalactiae	2 (3.64%)	***Fusobacterium nucleatum***	1 (1.56%)
Bacillus cereus	1 (1.82%)	***Morganella morganii***	1 (1.56%)
Enterococcus gallinarum	1 (1.82%)	***Bacteroides fragilis***	1 (1.56%)
Coagulase-negative staphylococcus	1 (1.82%)	***Moraxella osloensis***	1 (1.56%)
Kocuria	1 (1.82%)	***Enterobacter aerogenes***	1 (1.56%)
Propionibacterium acnes	1 (1.82%)	***Roseomonas mucosa***	1 (1.56%)
Listeria monocytogenes	1 (1.82%)	***Total***	64 (100%）
Streptococcus oralis	1 (1.82%)		
Corynebacterium striatum	1 (1.82%)		
Streptococcus penumoniae	1 (1.82%)		
Staphylococcus saprophyticus	1 (1.82%)		
Total	55 (100%）		

Abbreviations: GP-BSI, Gram-positive bacterial bloodstream infections; GN-BSI, Gram-negative bacterial bloodstream infections; N (%), sample size (percentage).

Detection of Inflammation Indicators. Serum PCT was detected by a full-automatic electrochemical luminescence analyzer (Roche 602, Germany) with a normal reference range of 0.000-0.500 ng/mL. The detection of serum CRP was performed using the dry chemical analyzer (Johnson & Johnson, America), with a normal reference range of 0.00-10.00 mg/L. Full-automatic blood cells analyzer (Sysmex XE2100, Japan) was utilized in order to detect WBC and NEUT%, and the normal reference ranges were estimated to be (3.5-9.5) $\times 10^9$ /L, 40.0-75.0, respectively.

Statistical Analysis. GraphPad Prism software (GraphPad Prism 8.0.2, La Jolla, CA) was employed for statistical analysis. Quantitative variables that conformed to the normal distribution were expressed as mean ± standard deviation, and those that did not conform to the normal distribution were expressed as median and quartile. Qualitative variables were expressed as ratio or rate. The Mann-Whitney test and Kruskal-Wallis test were applied to compare continuous variables of different groups. The Wilcoxon

matched-pairs test was used for cytokines before and after the treatment. Qualitative data were tested with Chi-square test or Fisher's exact test. Spearman correlation test was used to assess the correlation between variables. Diagnostic value of each indicator was appraised employing the receiver operation characteristic (ROC) curves. The 95% confidence interval was utilized in order to calculate the area under curve (AUC), sensitivity (Se) and specificity (Sp), and the cut-off value was selected once the Jordan index was at its maximum. For all the tests, the definition of statistical significance, the two-tailed p-value<0.05, is represented as follows: ***p<0.001, **p<0.01 and *p<0.05.

RESULTS

Characteristics of cytokines expression in BSI patients before treatment.

We performed quantitative detection of cytokines. Figure 1a and Figure 1b represent the flow cytometer figures of an HC and a BSI patient, respectively, before the treatment selected randomly. Comparing cytokines expression between BSI, GP-BSI, GN-BSI and HC groups before the treatment, it is not difficult to discover that IL-6, IL-10, TNF-α and IFN-γ significantly increased in the three groups of BSI, GP-BSI and GN-BSI as compared with HC (Figure 1e-h); IL-2 also significantly increased in the BSI and GN-BSI groups while for IL-4, the only reduced expression was observed in the GP-BSI group (Figure 1c, d). Interestingly, the expression of IL-6 (p=0.010), IL-10 (p<0.001), TNF-α (p=0.019) and IFN-γ (p=0.002) in the GN-BSI group was significantly higher than that in the GP-BSI group (Figure 1e-h). The above-mentioned results are consistent with our statistics on cytokines in different type of bacterial infections (Figure 2). Patients with *Escherichia coli* or *Klebsiella pneumoniae* infections expressed higher IL-6 (4-31 times, median) and IL-10 (6-14 times, median) compared to those infected with *Staphylococcus hominis* or *Staphylococcus epidermidis* (Figure 2a). Compared with *Staphylococcus epidermidis* infection, *Escherichia coli* and *Klebsiella pneumoniae* infected patients also expressed higher TNF-α (2-4 times, median), and *Klebsiella pneumoniae* infected patients expressed even higher IL-2 (2 times, median). No significant difference was found in other cytokines between the previously mentioned four common bacterial infections.

Cytokines expression levels can assess the severity and prognosis of BSI patients.

In order to realize the cytokine changes as the progress of BSI, we focused on the cytokine expression in the patients with septic shock and death due to BSI, the highest body temperature and hospitalization time of each patient were recorded during hospitalization to explore their relationship with cytokine expression. Among all the enrolled patients, 30 (25.21%) cases experienced septic shock during hospitalization and 17 (14.29%) died, which included 23 cases with GN-BSI getting septic shock, and 15 died (Table 1), and 14 (82.35%) cases had septic shock in 17 died patients (data not shown). In the BSI group, compared with unseptic shock patients, despite no statistical difference in IL-2, IL-4, TNF-α and IFN-γ expression of the patients with septic shock (Figure 3a), IL-6 and IL-10 expression were both approximately 3 times (median) higher in the patients with septic shock than in those without septic shock (Figure 3b). In the GN-BSI group, patients with septic shock and death had less IFN-γ secretion compared to those without septic shock and death (Figure 3c).

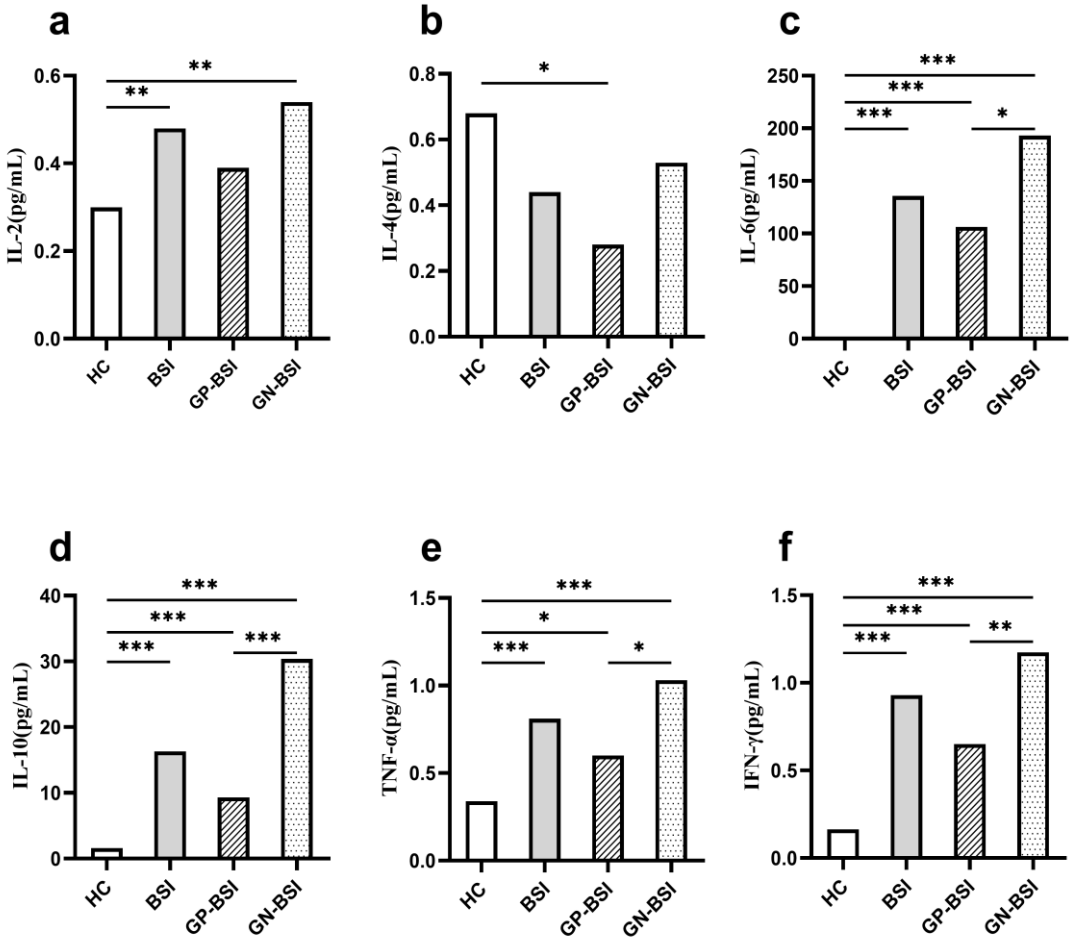

Figure 1. Cytokine expression levels in BSI patients before the treatment. We tested the cytokines of 52 HCs and 119 BSI patients with flow cytometry, including 55 GP-BSI and 64 GN-BSI patients. Each sample was measured 3 times. The minimum value in the above test was 0.01 pg/mL and the maximum value was 117447 pg/mL. The Mann-Whitney test was used to compare the differences among different groups. Figure 1a, b represented the flow cytometer figures of an HC and a BSI patient before the treatment, which was selected randomly, respectively. Figure 1c-h shows the expression of cytokines in different groups by box and whisker plot. Abbreviations: APC-A, allophycocyanin -conjugated specific antibodies; PE-A, phycoerythrin-conjugated detection antibodies; HC, healthy control; BSI, bacterial bloodstream infections; GP-BSI, Gram-positive bacterial bloodstream infections; GN-BSI, Gram-negative bacterial bloodstream infections; ***p< 0.001, **p< 0.01 and *p< 0.05.

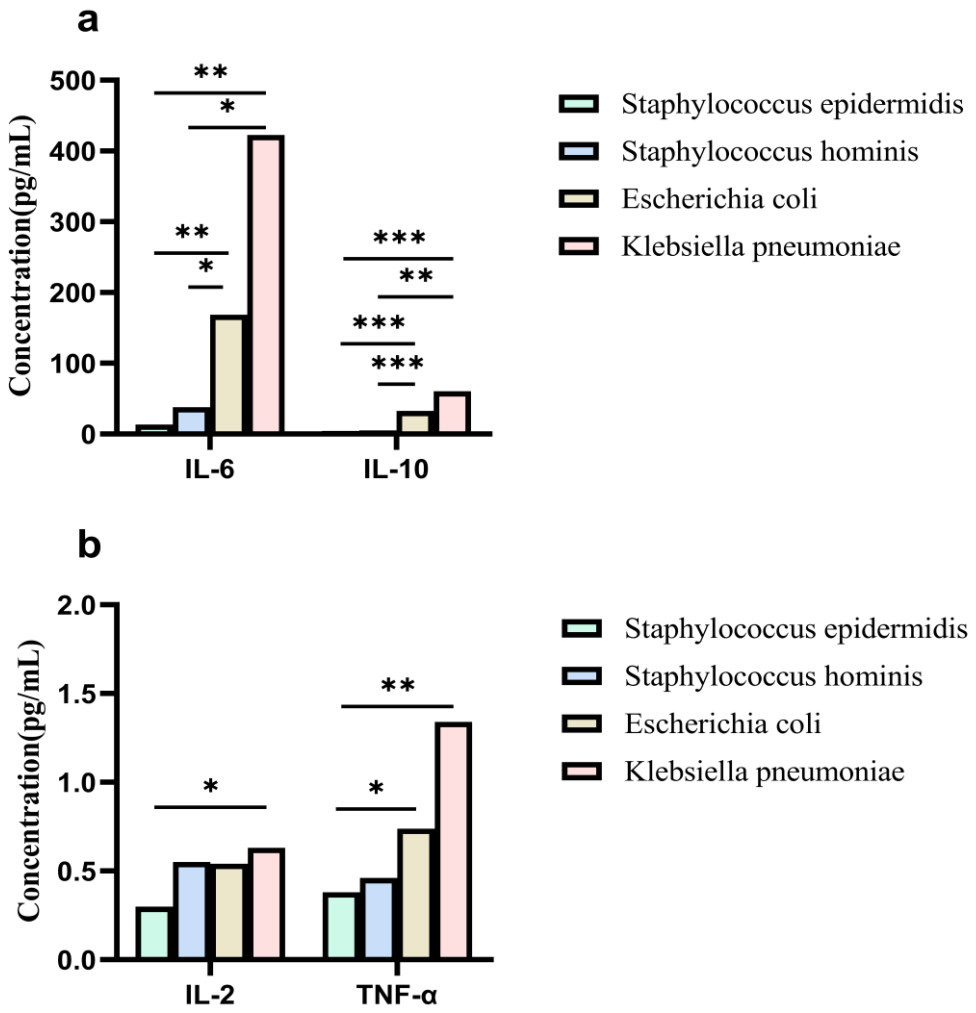

Figure 2. Cytokine expression levels in different pathogen types of BSI. Common GP bacteria included *Staphylococcus epidermidis* (10 cases) and *Staphylococcus hominis* (9 cases), common GN bacteria included *Escherichia coli* (21 cases) and *Klebsiella pneumoniae* (21 cases). The differential expression of IL-6 and IL-10 (Figure 2a), IL-2 and TNF-α (Figure 2b) in different groups were represented as box and whisker plot. The expression of IL-4 and IFN-γ were not statistically significant (data not shown in the figure). Abbreviations: BSI, bacterial bloodstream infections; GP, Gram-positive; GN, Gram-negative; ***p<0.001, **p<0.01 and *p<0.05.

According to these results, we found that the expression of IL-6 and IL-10 changed most significantly among the 6 kinds of cytokines. Therefore, we made the following further analysis of IL-6 and IL-10, based on septic shock rate and mortality. In the BSI group, we were pleasantly surprised to discover that all the patients had no septic shock or death when IL-6 ≤ 20 pg/mL before the treatment (Figure 3d). With the increase in IL-6 secretion, the septic shock rate and mortality increased correspondingly; when IL-6 > 1000 pg/mL, the incidence of both septic shock and mortality were as high as 50.00% and 38.89%. Nevertheless, IL-10 ≤ 20 pg/mL, septic shock and death also occurred in BSI patients, whose incidence rates were 12.12% and 9.09%, respectively (Figure 3e). Once

20 < IL-10 ≤ 50 pg/mL, the septic shock rate increased up to 40.00%, and the mortality increased to 15.00%. When IL-10 > 50 pg/mL, the BSI patients' septic shock rate reached 42.42%, and the mortality increased to 24.24%. Consequently, we speculate that IL-6 and IL-10 are closely associated with the severity and prognosis of BSI, and IFN-γ might help to judge the severity of GN-BSI. Therefore, we did ROC curve analysis on IL-6, IL-10 and IFN-γ with BSI to confirm our speculation. When IFN-γ < 0.70 pg/mL, there would be higher risk of septic shock (AUC = 0.663, Se = 43.5%, Sp = 82.9%) or death (AUC = 0.738, Se = 60.0%, Sp = 83.7%) in GN-BSI group. For the BSI group, once IL-10 took 23.03 pg/mL as the cut-off value, the diagnostic efficacy for septic shock was 0.679, and the sensitivity and specificity were 73.3% and 68.5%, respectively. While IL-6 > 534.70 pg/mL, the AUC for the diagnosis of septic shock was 0.637, the sensitivity was 43.3%, and the specificity was 84.3% (Figure 4).

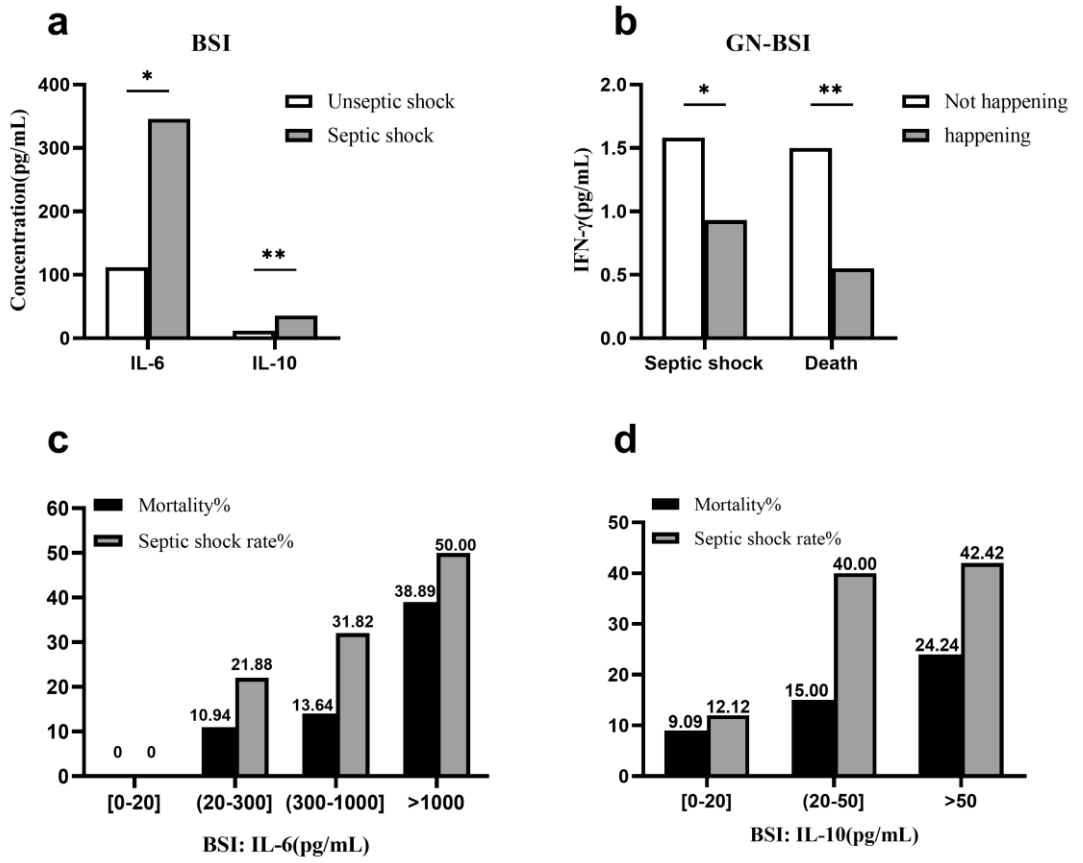

Figure 3. The changes of cytokine expression levels with the severity of BSI. Among 119 BSI patients, 30 (25.21%) cases experienced septic shock during hospitalization and 17 (14.29%) died, which included 23 cases with GN-BSI occurred septic shock and 15 died. Figure 3a, b represents the cytokine expression levels in BSI patients with septic shock and those without septic shock as box and whisker plot. In GN-BSI, grouping was based on the occurrence of septic shock or death. IFN-γ decreased significantly with the severity of the disease (Figure 3c, box and whisker plot). There was no statistical difference in other cytokine changes (data not shown in the figure). Figure 3d, e illustrates the changes in the incidence of septic shock and death with increasing IL-6 and IL-10 expression levels. Abbreviations: BSI, bacterial bloodstream infections; GN-BSI, Gram-negative bacterial bloodstream infections; ***p<0.001, **p<0.01 and *p<0.05.

Arranging the highest temperature of the patients with BSI during hospitalization, it is not hard to find that the temperature of the patients is mainly concentrated in moderate (42, 35.29%) and high (62, 52.10%) fever (Table 1). It is well known that antipyretic treatment will be actively carried out clinically if the body temperature is higher than 39.0°C. Accordingly, we arranged low heat and moderate heat into one group (T≤39°C; T, temperature), and high heat and ultra-high heat into another group (T>39°C). With the increase in the body temperature up to higher than 39°C, IL-2, TNF-α and IFN-γ increased significantly in BSI group (Figure 5a). Moreover, IL-4 and TNF-α had a significant increase in the GN-BSI group (Figure 5b) whereas other cytokines showed no difference between the above two groups. In addition, we also analyzed the changes in cytokines with the length of hospitalization of BSI patients, and found no significant correlation.

IL-6 and IL-10 may help to determine the efficacy of BSI treatment.

Through the comparison of cytokine expression before and after treatment in 28 patients, we found that the biggest change was still dominated by IL-6 and IL-10 (Figure 6). In terms of IL-6, the BSI and GP-BSI groups decreased by about 10-fold after the treatment, and for the GN-BSI group, IL-6 seemed to be more sensitive, with a decline of about 23-fold after the treatment (calculating multiples as median, $p<0.001$).

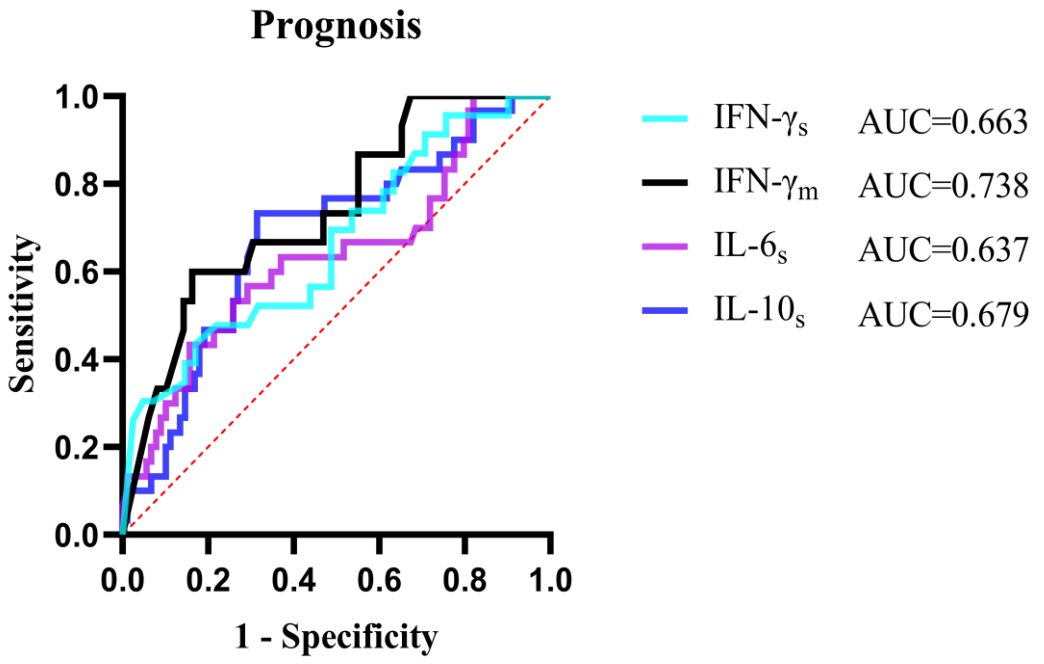

Figure 4. The ability of cytokines to predict the prognosis of BSI. We performed ROC curve analysis on the cytokines of 52 HCs and 119 BSI patients before the treatment. IFN-γs and IFN-γm, refer to the diagnostic efficacy of IFN-γ in the GN-BSI group for septic shock and death, respectively. IL-6s and IL-10s, refer to the diagnostic efficacy of IL-6 and IL-10 in the BSI group for septic shock, respectively. This figure only shows ROC curves with $p<0.05$. Abbreviations: BSI, bacterial bloodstream infections; ROC curve, receiver operation characteristic curve; HC, healthy control; GN-BSI, Gram-negative bacterial bloodstream infections; AUC, area under the curve.

IL-10 in the BSI group also decreased down to about 4 times after the treatment. In the GN-BSI group, IL-10 even reduced by about 27 times following the treatment (calculating multiples as median, $p<0.001$), yet there was no significant difference for IL-10 in the GP-BSI group before and after the treatment.
Meanwhile, none of IL-2, IL-4, TNF-α and IFN-γ revealed statistical significance in the three groups of BSI, GP-BSI and GN-BSI before and after the treatment.

Table 3. Correlation analysis between cytokines and clinical infection indicators.

Groups	Biomarkers(N[1])	r (IL-2)	r (IL-6)	r (IL-10)	r (TNF-α)
BSI	PCT(102)	0.260[b]	0.615[a]	0.657[a]	0.221[c]
	CRP(85)	0.228[c]	0.496[a]	0.400[a]	
	WBC(119)		0.181[c]	0.227[c]	
	NEUT%(108)		0.486[a]	0.555[a]	
GP-BSI	PCT(46)		0.662[a]	0.762[a]	0.308[c]
	CRP(44)		0.573[a]	0.451[b]	0.322[c]
	WBC(55)			0.405[b]	
	NEUT%(51)		0.546[a]	0.588[a]	
GN-BSI	PCT(56)	0.301[c]	0.563[a]	0.527[a]	
	CRP(41)		0.415[b]		
	WBC(64)				
	NEUT%(57)		0.419[b]	0.514[a]	

Abbreviations: BSI, bacterial bloodstream infections; GP-BSI, Gram-positive bacterial bloodstream infections; GN-BSI, Gram-negative bacterial bloodstream infections; r, correlation coefficient, the above table only shows correlation coefficients with significant differences ($P < 0.05$). Annotation: 1, number of cases actually used for data analysis after excluding PCT, CRP, WBC and NEUT% data incomplete cases; a, ***$p<0.001$; b, **$p<0.01$; c, *$p<0.05$.

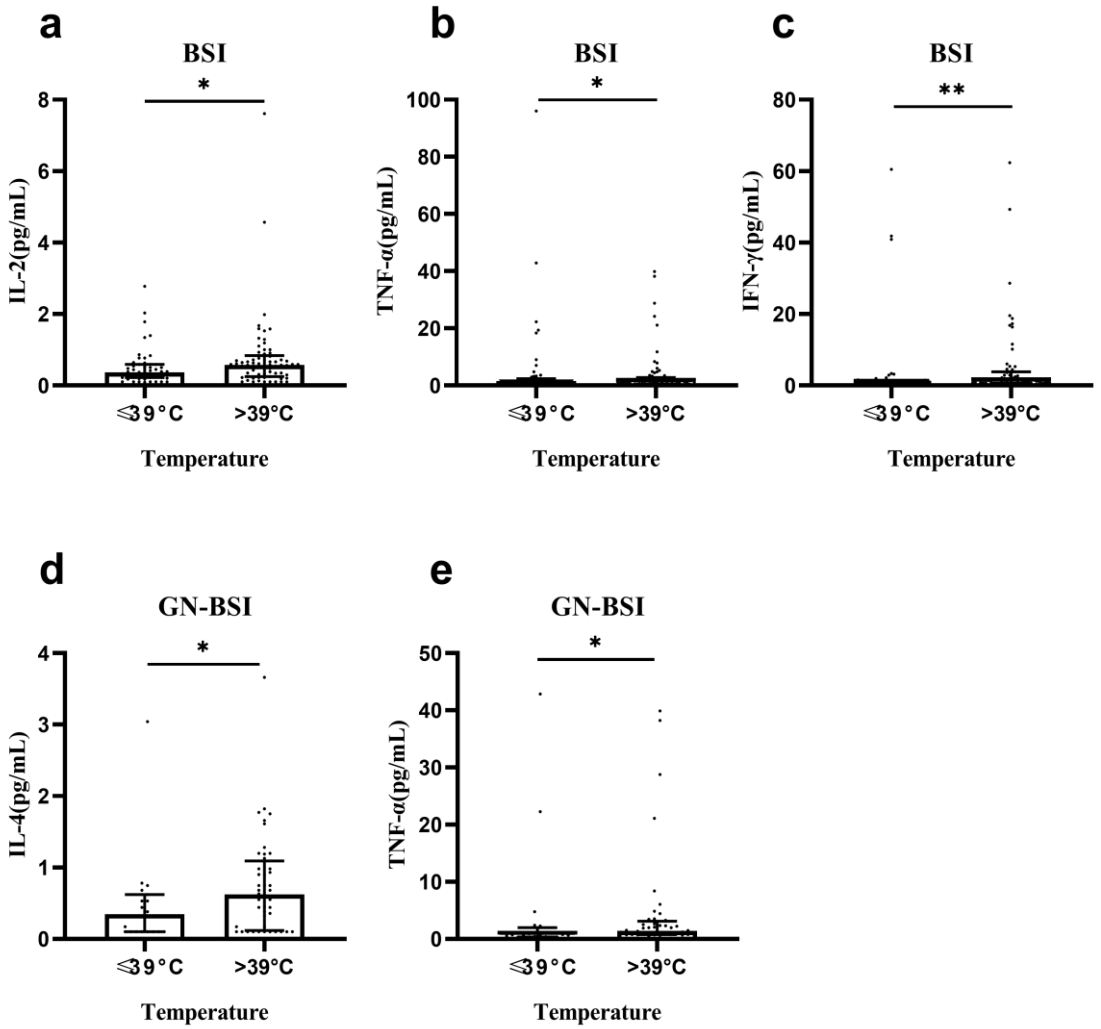

Figure 5. The box and whisker plot demonstrate the changes in cytokines of BSI and GN-BSI patients with increasing body temperature by median and interquartile range. We arranged low heat and moderate heat into one group (T ≤ 39°C), high heat and ultra-high heat into another group (T > 39°C). The Mann-Whitney test was employed to compare the differences in different groups. Abbreviations: BSI, bacterial bloodstream infections; GN-BSI, Gram-negative bacterial bloodstream infections; T, temperature; ***p<0.001, **p<0.01 and *p<0.05.

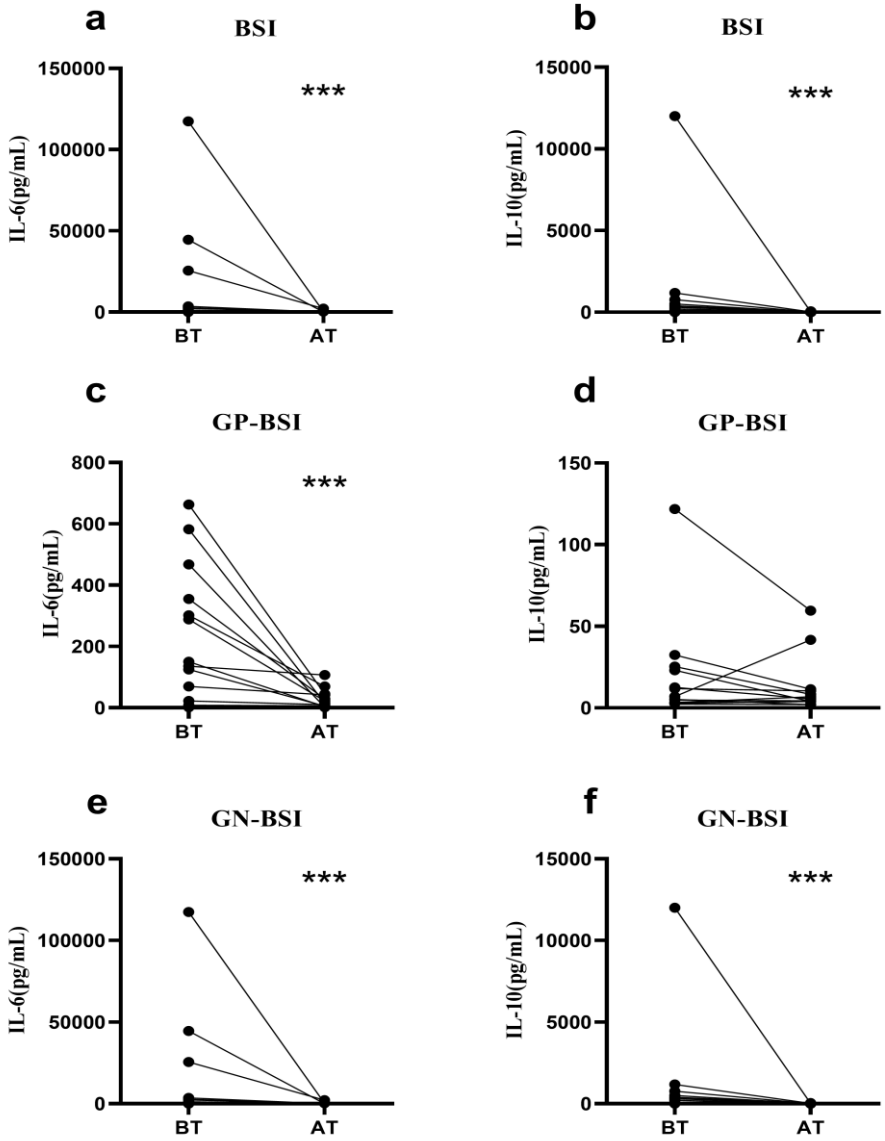

Figure 6. This line graph represents the changes in cytokines before and after the treatment. We randomly selected 28 BSI patients (including 13 GP-BSI and 15 GN-BSI patients) and tested the expression of cytokines after the treatment. Each sample was measured 3 times. The Wilcoxon matched-pairs test was used to compare the differences in different groups. Figure 6a-c respectively represents the differences of cytokine in BSI, GP-BSI and GN-BSI groups. Abbreviations: BSI, bacterial bloodstream infections; GP-BSI, Gram-positive bacterial bloodstream infections; GN-BSI, Gram-negative bacterial bloodstream infections; BT, before treatment; AT, after treatment; ***p<0.001, **p<0.01 and *p<0.05.

IL-6 and IL-10 are closely related to clinical infection indicators.

We performed Spearman correlation test of the cytokine IL-2, IL-4, IL-6, IL-10, TNF-α and IFN-γ with the four clinical infection indicators PCT, CRP, WBC and NEUT%, respectively (Table 3). The results demonstrated that IL-6 ($r_{BSI} = 0.615$, $r_{GP\text{-}BSI} = 0.662$, r refers to the correlation coefficient) and IL-10 ($r_{BSI} = 0.657$, $r_{GP\text{-}BSI} = 0.762$) in the BSI and GP-BSI groups had a strong correlation with PCT while in the GN-BSI group,

IL-6 (r = 0.563) and IL-10 (r = 0.527) were moderately related to PCT. Additionally, IL-6 and IL-10 were moderately correlated with CRP and NEUT% in each group, except IL-10 and CRP in the GN-BSI group. IL-10 and WBC were also moderately related in the GP-BSI group. Others were weakly related or unrelated ($r < 0.400$ or $p<0.05$), particularly for IL-2, IL-4, TNF-α and IFN-γ.

IL-6 and IL-10 could help to diagnose BSI and distinguish GP-BSI and GN-BSI.

We finally analyzed the diagnostic efficacy of cytokines in BSI. Through the ROC curve below, we found that cytokines can distinguish the patients with BSI from healthy people, particularly IL-6 (AUC = 0.994, $p<0.001$) and IL-10 (AUC = 0.987, $p<0.001$). Considering the cut-off value of IL-6 as 2.97 pg/mL, the sensitivity and specificity of forecasting the BSI occurrence were 94.2% and 99.2%, respectively. Meanwhile, when the cut-off value of IL-10 was 2.92 pg/mL, the sensitivity of predicting BSI occurrence was the same as that of IL-6, and the specificity was 93.3%. The diagnostic efficacy of TNF-α (AUC = 0.729, $p<0.001$) and IFN-γ (AUC = 0.798, $p<0.001$) was second, mainly due to the low specificity (67.2%, 63.0% respectively). The diagnostic efficacy of IL-2 (AUC = 0.627, $p=0.008$) was quite low, and the sensitivity was only 46.2% (Figure 7a). Furthermore, for distinguishing GN-BSI from GP-BSI, our statistics (Figure 7b) indicated that once the Jordan Index reached its largest, the diagnostic specificity of IL-6 (AUC = 0.637, $p=0.010$) reached its highest, which was 85.5%, but the sensitivity was at its lowest, 39.1%, the cut-off value was 418.90 pg/mL. TNF-α (AUC = 0.625, $p=0.019$) was of the highest sensitivity at 73.4%, yet the lowest specificity, 60.0%, to diagnose by taking 0.71 pg/mL as cut-off value. IFN-γ (AUC = 0.665, $p=0.002$) was observed to have lower sensitivity (67.2%) and specificity (61.8%) as compared with other cytokines. In terms of comprehensive selection, the IL-10 (AUC = 0.725, $p<0.001$) index would be the best choice, when the cut-off value is 17.26 pg/mL, the AUC is at its largest, sensitivity and specificity are 64.1% and 70.9%, respectively. However, the diagnostic efficacy of IL-10 was not perfect. We attempted to improve the efficacy by combining diagnosis, in addition to 6 kinds of cytokines; we also combined clinical indicators PCT, CRP, WBC and NEUT% to perform logistic regression analysis and draw ROCs. Unfortunately, no combined index was found to be superior to a single IL-10 diagnostic efficacy (data not shown).

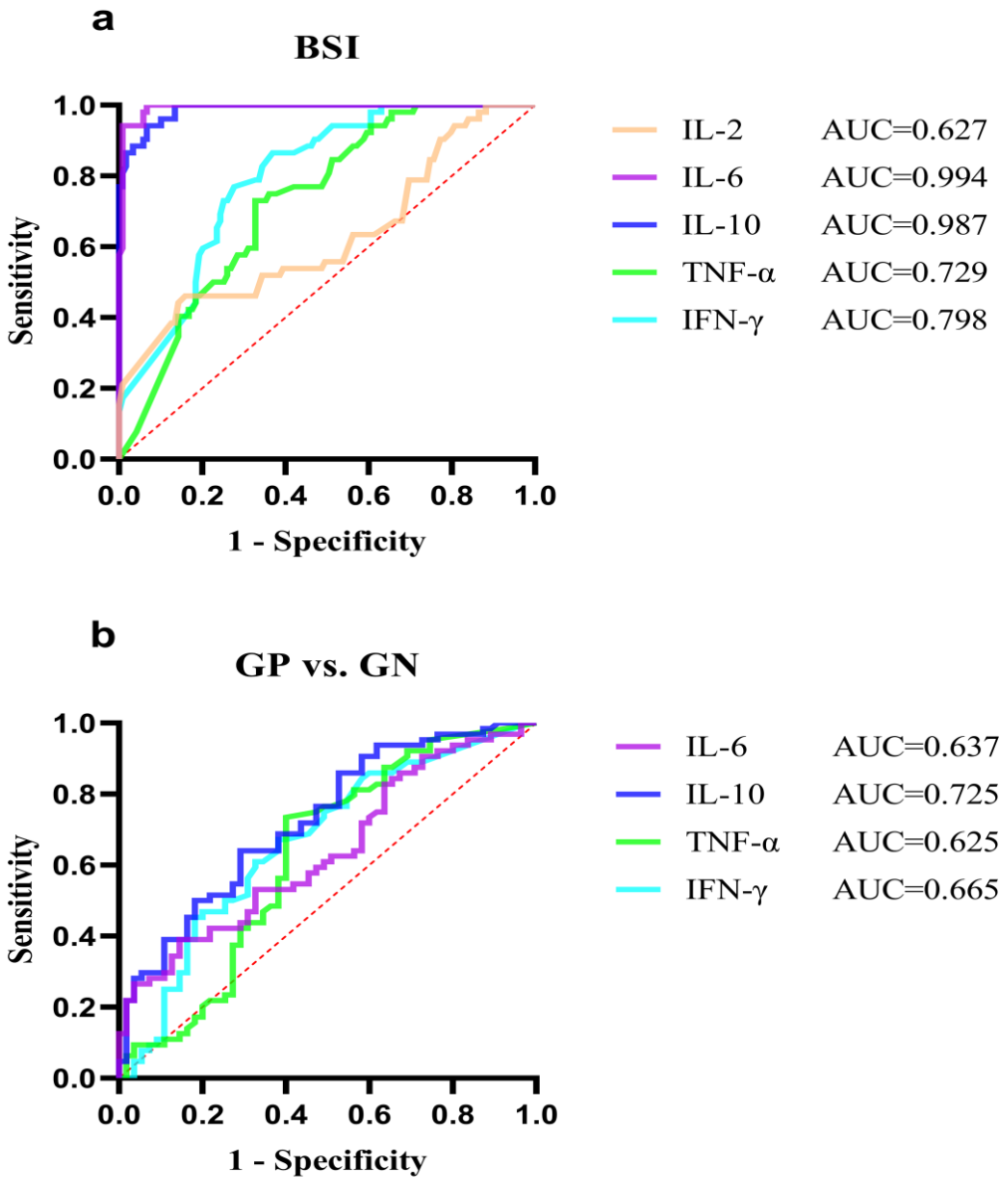

Figure 7. The ability of cytokines to distinguish BSI and HC, GP-BSI and GN-BSI. Figure 7a indicates the ROC curve for predicting BSI from HC. Figure 7b shows the ROC curve for distinguishing GN-BSI and GN-BSI. This figure only shows ROC curves with $P < 0.05$. Abbreviations: BSI, bacterial bloodstream infections; HC, healthy control; GP-BSI, Gram-positive bacterial bloodstream infections; GN-BSI, Gram-negative bacterial bloodstream infections; ROC curve, receiver operation characteristic curve; AUC, area under the curve.

DISCUSSION

IL-6 and IL-10 are highly expressed in case of infections, which have been reported in the patients with neutropenic pediatric cancer and hemophagocytic lymphohistiocytosis (11-15). This is in line with our findings in BSI in adults. However, it is unclear whether Th1/Th2 cytokine profiles are capable of identifying the severity and the type of bacterial infections. In this study, we performed a systematic and comprehensive

assessment of cytokine expression in GP-BSI and GN-BSI. We observed that under the stimulation of bacterial pathogens, Th1/Th2 cytokines exhibited an upward trend, except for IL-4, and the increase was mostly significant in the GN-BSI group.

This might be attributed to the inflammatory immune response through the nuclear factor-E2-related factor 2 (Nrf2) signaling pathway induced by lipopolysaccharide (LPS) of GN bacteria (16-19). IL-4 has always been considered to play a role in allergies and parasitic infections. A recent study has suggested that IL-4 could enhance IFN production by virus-infected mast cells (20). In a review about Th1/Th2 disorders in human diseases, Daniel R. Lucey *et al.* reported that IL-4 could inhibit the expression of IFN-γ from Th1 clones, while IFN-γ could downregulate the expression of IL-4 from Th2 clones (21). We observed an increase in the trend of IFN-γ and a decrease in the trend of IL-4 in BSI. The detailed regulation mechanism has yet to be clarified by experiments. In addition, it has been previously found that when exposed to tumor and viral antigens, IL-2 had the ability to regulate naïve T cell differentiation into Th1/Th2 subsets and promote the cytolytic activity of $CD8^+$ T cell and natural killer cell (22). In GN-BSI, the importance of IL-2 elevation in the Th1/Th2 inflammatory regulatory network is still inconclusive. Superantigens, as one of the most potent toxins induced by Staphylococcus aureus and Staphylococcus pyogenes, triggered the production of large amounts of proinflammatory Th1 cytokines, containing IL-2, TNF-α and IFN-γ (23). In mouse mammary tumor virus infection, the commensal microbiota-derived LPS stimulated the secretion of IL-10 in an IL-6-dependent manner (24). At the same time, it has been reported that IL-6 can directly promote the production of IL-10 in mouse induced arthritis (25). Interestingly, in a comparison of cytokines of four common BSI, we found that patients infected with GN bacteria *Klebsiella pneumoniae* and *Escherichia coli* expressed higher levels of IL-6, IL-10 and TNF-α. IL-2 is also elevated in patients with *Klebsiella pneumoniae* infection. These changes seemed to be partly consistent with the trends of cytokine changes in other studies discussed above, but the relevant change mechanisms in human BSI remain controversial. The Th1/Th2 cytokine regulatory network is complex and changeable; there are even cross-regulatory relationships between different Th subgroups, which are mysterious and fascinating aspects of this type of research. With the increase in the secretion of IL-6 and IL-10, the probability of septic shock and death in BSI patients also increases correspondingly. Once IL-6 > 1000 pg/mL, the septic shock rate was 50% and the mortality rate was 38.89%; when IL-10 > 50 pg/mL, the probability of these two groups were 42.42% and 24.24%, respectively. However, in the septic shock and death groups, the level of IFN-γ in GN-BSI patients actually declined. Studies have demonstrated that IFN-γ could reprogram host mitochondrial metabolism through inhibition of complex II to control intracellular bacterial replication (26). We conjectured that in GN-BSI, specifically when occurring septic shock or even death, the body's IFN-γ may no longer adequately control bacterial replication. It might be consumed that in large quantities, or insufficient supply, or both, and then the result of IFN-γ decline as the disease progresses was observed in this study. The above-mentioned change of IFN-γ was in accordance with the view of Jekarl et al. (27). Of course, it cannot be ruled out that in BSI, the upstream or downstream signal of the antibacterial mechanism of IFN-γ might change indirectly, leading to the change of IFN-γ, which needs to be studied further by experiments in the future. In our research, 82.35% of the dead patients died of septic shock, so septic shock should be worth paying attention to as a high-risk form of BSI. According to the ROC curve, in the BSI group, IL-6 < 534.70pg/mL (AUC = 0.637, Se

= 43.3%, Sp = 84.3%) can be used as an exclusion index for septic shock, and IL-10 > 23.03pg/mL (AUC = 0.679, Se = 73.3%, Sp = 68.5%) tends to have a high probability of septic shock. At the same time, the clinical manifestations of patients and other auxiliary examinations must be combined. Regarding the GN-BSI group, IFN-γ > 0.70 pg/mL could be more efficient for excluding septic shock (AUC = 0.663, Se = 43.5%, Sp = 82.9%) and death (AUC = 0.738, Se = 60.0%, Sp = 83.7%), but due to the high risk and high economic burden of GN-BSI, once IFN-γ < 0.70pg/mL, we should be attentive of the occurrence of poor prognosis. Obviously, cytokines could be quickly detected to predict the severity and prognosis, so that we could take preventive and curative measures as quickly as possible. During the disease remission period after the treatment, we also observed a significant decrease in the cytokines IL-6 and IL-10. Even though IL-10 expression in the GP-BSI group did not decrease significantly, this was exactly fit as the cytokine expression in GN-BSI more sensitive as described above. Infectious fever is a reaction that is conducive to the body's immune regulation. In the current work, it was also found that when the body temperature ≥ 39°C, the Th1 cytokines of patients in the BSI group significantly increased. In the GN-BSI group, only anti-inflammatory cytokine IL-4 and pro-inflammatory cytokine TNF-α increased with body temperature. Relevant reports stated that fever acts through a SMAD4-dependent mechanism associated with the Th17 cell immune axis (28). We suspect that the fever caused by BSI may be related to the regulatory network of Th1/Th2 and Th17 cytokines caused by T cell polarization. This needs further study to be confirmed by eliminating confounding factors. However, the analysis of cytokines and length of hospitalization did not reveal significant differences, which might be attributed to several other factors, for instance the patients' economics, nutritional status and personal wishes. The relationship between PCT and infection has been relatively clear (29-32). A meta-analysis has also stated that PCT and GN-BSI are relevant, but comparisons of different clinical backgrounds should be performed (33). In the same period, Jereb M's study asserted that there was no statistical difference in CRP and PCT concentration in patients with GN-BSI or GP-BSI (34). Clinical studies on neutrophil CD64 have demonstrated that nCD64 is a valuable parameter for early diagnosis and prognostic evaluation of infection in patients with sepsis (35). According to our Spearman correlation analysis, PCT is strongly correlated with IL-6 and IL-10 in GP-BSI, and weakly correlated with GN-BSI. CRP and NEUT% also showed certain correlation with cytokines, especially IL-6 and IL-10, but the intensity was less than that of PCT, and the correlation with WBC was the weakest. Tang YM and his colleagues insist that the performance of IL-6 and IL-10 is better than that of PCT and CRP in identifying the patients with high risk and high fever of pediatric cancer (15). We also confirmed the diagnostic value of IL-6 and IL-10 for BSI through ROC curve. Simultaneously, the ROC curve showed that IL-10 has a higher diagnostic efficacy on GN bacteria in BSI; patients with IL-10 > 17.26pg/mL (AUC = 0.725, Se = 64.1%, Sp = 70.9%) are more likely to be a GN-BSI. In spite of the poor sensitivity of IL-6 (AUC = 0.637, Se = 39.1%, Sp = 85.5%), it has higher specificity, so we can consider IL-6 as an exclusion indicator; BSI patients with IL-6 < 418.90 pg/mL are more likely to exclude GN-BSI. Nevertheless, PCT has not been found to be significantly better than IL-10 in either single diagnosis or combined cytokines through logistic binary regression and ROC curve analysis. On the premise that blood culture takes a long time and has the low positive rate, we provide the possibility of early detection of GN-BSI, and more reasonable use of antibiotics before blood culture results are clear. In summary, for BSI

patients, IL-6 < 534.70 pg/mL could be taken into consideration as a reference indicator to exclude septic shock, and IL-10 > 23.03 pg/mL is at greater risk of septic shock; IL-6 < 418.90 pg/mL is more likely to exclude GN-BSI, IL-10 > 17.26 pg/mL tends to be GN-BSI; IL-6 and IL-10 are also strongly related to the treatment efficacy of BSI, especially for GN-BSI. Likewise, once GN-BSI patients have IFN-γ < 0.70 pg/mL, we had better be attentive to the occurrence of poor prognosis. These findings indicated the importance of Th1/Th2 cytokine profiles based on IL-6 and IL-10 in the diagnosis and treatment of BSI.

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