T-helper-17, Regulatory T-helper Cells Related Serum Markers and IL-13 in the Outcome of Polytraumatic Patients with Bacteremia

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ABSTRACT

Background: Bacteremia and sepsis are associated with high mortality, increased hospital stays, and associated costs, especially in trauma patients. Sepsis is a fatal immunological disorder and its pathophysiology is still poorly understood. **Objective:** To ascertain the role of T-helper lymphocyte-related inflammatory serum cytokines in trauma patients with blood culture positive with Gram-negative bacteria. **Methods:** Peripheral blood samples (5 ml) were collected from 40 trauma patients on the day of obtaining positive blood culture (i.e., day 0), followed by an appropriate antimicrobial treatment and sample acquisition on day 4 and only once from 40 age-matched healthy controls. Bead-based cytometric analysis was used to quantify extracellular levels of 16 serum cytokines. The cytokine profiles were compared with those in healthy controls and then correlated to clinical outcomes. **Results:** A total of 40 patients were enrolled during the study period. Of these, 24 patients (60%) were discharged while 16 (40%) had a fatal outcome. Statistically significant elevated levels of serum IL-6, IFN-γ, TNF-α, IL-17A, IL-17F, and IL-4 were observed in septic patients, while lowered IL-13 levels correlated significantly with a favorable outcome. **Conclusion:** Sepsis following trauma elicits a heightened immune response in the body and provokes the production of a diverse array of cytokines that is both pro-inflammatory and anti-inflammatory. However, the unique cytokine profile of septic trauma patients is still not well understood.

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Keywords: Anti-inflammatory, Cytokine, Pro-inflammatory, Sepsis, Trauma, Antimicrobials

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INTRODUCTION

Trauma patients who survive the early phase after injury may suffer from complications during the subsequent treatment phase (1). The initial trauma and subsequent operative interventions encourage an exaggerated pro-inflammatory response, which may lead to organ injury including acute respiratory distress syndrome (ARDS) and multiple organ failure (MOF). Meanwhile, an anti-inflammatory response is involved in reducing the potentially harmful effects of the pro-inflammatory cytokines (2).

Sepsis and its associated multi-organ failure pose a major challenge to clinicians as well as scientists. Because they are associated with a high morbidity and mortality, they introduce a tremendous burden for healthcare systems. Despite the vigorous basic research and clinical studies, the pathophysiology of sepsis is still not well understood. It is increasingly being established that sepsis is a heterogeneous and dynamic disorder caused by the imbalances in the inflammatory network (3).

Sepsis results from the complex interactions between the infecting bacteria or viruses and the host immune system. A high burden of infection, compromised immune responses, and antibiotic resistance lead to the progression of sepsis when the host cannot inhibit the infection. Such infections also trigger a cytokine storm, which is often detected in patients with sepsis (4).

Both pro-inflammatory and anti-inflammatory cytokines constitute a double-edged sword in sepsis; on one hand, they are vital to eliminate the infection while, on the other, excessive production can result in tissue and organ damage (5). The major pro-inflammatory cytokines that regulate early responses include interleukin-1β (IL-1β), IL-6, and tumor necrosis factor-α (TNF-α). Other pro-inflammatory mediators include interferon-γ (IFN-γ), IL-12, and IL-17. Pro-inflammatory cytokines act as endogenous pyrogens (IL-1, IL-6, and TNF-α), up-regulate the synthesis of secondary mediators and other pro-inflammatory cytokines by both macrophages and mesenchymal cells, such as epithelial and endothelial cells, fibroblasts, and stimulate the production of acute phase proteins, or attract inflammatory cells(6). Host immunosuppression may be responsible for late deaths in patients with sepsis. Anti-inflammatory cytokines may also play a role in sepsis. Bacteremia combined with the well-known damage that occurs to the immune system in traumatized patients leads to sepsis and multiorgan failure. This issue is often seen in our intensive care units (7). Cytokine profiling pre- and post-treatment of patients with sepsis may have implications in diagnosis and prognostication of disease severity, and eventually, in better patient management. This may represent a valuable tool for delineating different patterns of immunological response and allowing prophylactic administration of cytokines leading to favorable patient outcomes.

A reliable biomarker for the diagnosis and prognosis of sepsis is critical and still unidentified. Using such biomarkers would not only provide early diagnostic accuracy and prognostic information on sepsis but also would predict the responsiveness to treatment interventions, especially in high-risk trauma patients.

Thus, the present study was conducted to ascertain the profile of T-helper lymphocyte and macrophage-related inflammatory serum cytokines in trauma patients with culture-confirmed bacterial bloodstream infections using the multiplex assays through flowcytometry and correlate the serum-cytokine levels with the clinical outcome of the trauma patients.

MATERIALS AND METHODS

Patients. This prospective cohort study was conducted from June 2017 to December 2017 at a 186 bedded, level-1 Trauma Centre, comprised of 40 trauma patients admitted to the ICU, with positive blood culture for Gram-negative bacteria along with 40 age-matched healthy controls. Only the patients who had monomicrobial or bimicrobial Gram-negative bacteremia were included in the study. Patients were excluded if they had any of the following conditions: diabetes mellitus; renal failure; history of the neoplasm; major cardiovascular or cerebrovascular disease; and blood transfusion. The healthy controls included the laboratory staff at the Trauma Centre. The study was approved by the ethics committee of the institute and written informed consent was provided by all participants. Clinical Follow-up: All patients were clinically followed-up daily upon obtaining a positive blood culture. The development of sepsis and the clinical course were evaluated by the critical care specialists in-charge of the patients, as per standard protocols.

Specimen collection. In all cases, the peripheral venous blood samples (5 ml) were collected on the day of obtaining a positive signal for a blood culture with Gram-negative bacteria (identified by Gram staining) (day 0), followed by a sample acquisition on the 4th day of culture-specific treatment and only once from the healthy control individuals. Following centrifugation for 5 min at 2100 rpm and 4℃, the serum samples were stored at -80℃ until further analysis. Multiplexed bead-based immunoassays were performed to assess the serum cytokine levels in the sera of 40 cases and each control. LEGENDplex™ Human Th Cytokine Panel (13-plex) (Biolegend, San Diego, CA, USA) and BD Cytometric Bead Array (CBA) (BD Biosciences, San Jose, CA, USA) assay were used to assess the levels of the 16 cytokines (Table 1).

Table 1: The bead positions and experimental panels to ascertain the cytokine concentrations using the Legendplex™ # & Cytometric Bead Array (CBA)* assay.

S.No.	Cytokine	Function	Secreting cell
1	**IL-5**#	anti-inflammatory	Th2, Th17
2	**IL13**#	anti-inflammatory	Th2, Th 17
3	**IL-2**#	pro-inflammatory	Th 2
4	**IL-6**#	both	Th2, monocyte
5	**IL-9**#	pro-inflammatory	Th 9
6	**IL-10**#	anti-inflammatory	Th2, Th 9, T regulatory
7	**IFN-γ**#	both	Th 1, , Tfh, NKT, monocyte
8	**TNF-α**#	pro-inflammatory	Th 1, Th17, Th 22, monocyte
9	**IL-17A**#	pro-inflammatory	Th17, Th22, Tfh, NKT
10	**IL 17F**#	pro-inflammatory	Th17, Tfh
11	**IL 4**#	anti-inflammatory	Th2, Tfh, NKT
12	**IL-21**#	anti-inflammatory	Th17, Tfh,
13	**IL 22**#	anti-inflammatory	Th17, Th 22
14	**IL-12***	pro-inflammatory	Th1
15	**TGF-β***	anti-inflammatory	Th 9, T regulatory
16	**IL-1β***	pro-inflammatory	Monocyte & neutrophils

Th; T helper cells; NKT: Natural Killer T cells; T fh: T follicular helper cells.

Protocol for the multiplex assays and acquisition strategy. The appropriate cytokine standards were serially diluted and samples (50 µl) were diluted to a concentration of 1:4 in the dilution buffers provided by the manufacturers. The multiplex assays were performed as the manufacturer's instructions. After the instrument setup and verification check, the BD FACS Diva™ software was used for data acquisition on the BD FACS Aria III flowcytometer (BD Biosciences, San Jose, CA, USA).

The results were generated using the softwares; Legendplex Version 7.0.0.1 software, VigeneTech Inc. (LEGENDplex™ Human Th Cytokine Panel) and FCAP Array™ Version 3.0.1 software (BD Cytometric Bead Array assay). The cytokine profile was correlated with clinical outcomes. In addition, pre- and post-treatment serum procalcitonin (PCT) levels were assessed in all patients using MINI VIDAS®(bioMérieux, SA, France).

Statistical analysis. Statistical analyses were performed using SPSS 12.0 for Windows 8.0 (SPSS Inc., Chicago, IL, USA). Numeric variables are expressed as mean (± standard error mean). The case-control comparison was done using the Mann-Whitney U test. The independent samples t-test was applied for comparing pre- and post-treatment cytokine values with the final outcome and equal variance was not assumed if a significant Levene's test value was obtained. A P-value of <0.05 was considered as statistically significant.

RESULTS

Among the 40 trauma patients with positive blood culture for Gram-negative bacteria, 28 (70%) were males while 12 (30%) were females. The age of the trauma patients with positive blood culture positive with Gram-negative bacteria ranged from 18 and 62 years (median: 27years). Antimicrobial therapy was based on the culture reports. The most common organism was *Acinetobacter baumannii*, isolated from 22(55%) patients, followed by *Pseudomonas aeruginosa* isolated from 5 (12.5%) patients, *Klebsiella pneumonia* from 4 (10%) patients, and *Stenotrophomonas maltophilia* from 2 (5%) patients. *Burkholderia cepaciae, E coli, Proteus mirabilis,* and *Providencia spp*. were isolated in one patient each. Three patients had bimicrobial bloodstream infection. Statistically significant elevation of IL-6, IL-10, IFN-γ, TNF-α, IL-17F, IL-21, IL-12, and TGF-β levels, and a decrease in levels of IL-13 were observed compared to healthy controls. On both sampling days, the titer of IL-17A and IL-1β were observed to be significantly higher on day 0 as compared to controls, while IL-2 and IL-4 were elevated in the post antimicrobial treatment phase (Table 2).

The expression of regulatory T-helper related cytokines viz. IL-10 and TGF-β were observed to be inversely associated with post-treatment levels. A similar inverse association was overserved for T-helper 17 related serum cytokines viz. IL-17A and IL-17F. The levels of serum IL-10 & IL-17F were observed to be lower post-treatment, while the serum TGF-β & IL-17A were observed to be higher than their respective levels pre-treatment.

An interesting finding of prognostic value is that lowered serum IL-13 levels post-treatment significantly correlated to a favorable patient outcome. The level of pre-treatment serum IL-13 (15.44 ± 8.14 pg/ml dropped down to 0.09 ± 0.9pg/ml to 0.09 +- 0.9pg/ml, post-treatment, in patients who were discharged (P value<0.005).

The mean value of PCT levels in the discharged patients, pre-treatment was 22.4 ng/ml, which declined to a mean of 6.5 ng/ml, post-treatment (Paired sample T-test P-value< 0.005). These results suggest that both sera- IL-13 and PCT have predictive values and may have implications in the prediction of disease progression and mortality.

Table 2: Comparison of serum cytokine titer (pg/ml) of cases pre- and post-treatment with controls

Serum cytokine	Controls	Cases (Pre-treatment)		Cases (Post-treatment)	
	Mean± S.E.M	Mean± S.E.M	*P* value	Mean± S.E.M	*P* value
IL-5	1.0± 0.2	6.67± 6.28	0.49	3.02± 1.92	0.52
IL13	18.54± 5.1	18.26± 5.15	0.94	17.38± 5.55	0.97
IL-2	0	0.34± 0.34	0.20	4.60± 2.77	**0.02***
IL-6	0.15± 0.0	713.3± 240.04	**0.00***	440.45± 224.25	**0.00***
IL-9	12.77± 5.9	10.86± 3.51	0.08	102.73± 95.10	0.54
IL-10	0.59± 0.2	49.15± 44.97	**0.02***	15.75± 12.05	**0.00***
IFN-γ	2.31± 0.7	7.61± 2.28	**0.02***	5.21± 1.12	**0.00***
TNF-α	2.28± 0.8	12.96± 3.25	**0.00***	58.53± 46.37	**0.00***
IL-17A	5± 1.4	16.63± 4.16	**0.00***	19.39± 7.92	0.07
IL-17F	0.24± 0.2	3.18± 1.16	**0.00***	2.21± 0.31	**0.00***
IL-4	0.56± 0.4	9.58± 4.78	0.06	9.18± 3.62	**0.00***
IL-21	52.68± 28.8	208.08± 67.91	**0.00***	219.59± 84.78	**0.00***
IL-22	6.37± 1.7	9.68± 2.16	0.47	9.46± 2.26	0.93
IL-12	1.69± 1.0	3.21± 1.00	**0.01***	3.94± 1.33	**0.01***
TGF-β	674.88± 316.6	2657.24± 355.18	**0.00***	8223.61± 5601.46	**0.00***
IL-1β	0.72± 0.5	16.76± 6.57	**0.02***	12.30± 6.36	0.08

*statistically significant.

DISCUSSION

Sepsis constitutes a systemic immune response to infection and elicits the production of a diverse array of cytokines that are pro-inflammatory and anti-inflammatory (8). While pro-inflammatory cytokines are essential for controlling infection, in excess doses they may lead to tissue and organ injury. Similarly, the anti-inflammatory cytokines are critical in establishing homeostasis and in regulating the overall immune response. Therefore, their dysregulation can also trigger pathogenesis (9).

In the present study, we assessed an array of 16 serum cytokines and observed a statistically significant predominance of T-helper-17 and regulatory T-helper cells related inflammatory serum markers; i.e., IL-17A, IL-21, and TGF-β. A significant decrease in T-helper-17 and regulatory T-helper cells related serum cytokines viz IL-17F and IL-10, respectively, was observed. However, we did not find any association between T-helper-22 related cytokines and sepsis or the outcome of our patients. The limitation of our study is that we did not use the intracellular cytokine-based assay, which would have provided cell-specific information in these patients.

A statistically significant association of IL-13 and PCT with a favorable outcome of polytrauma patients with septicaemia suggests that it could have prognostic value for sepsis in critically ill polytrauma patients. Further investigation into this issue using SOFA and APACHE II scores may enable us to develop a scoring system based on

these serum inflammatory markers and the severity of the disease. This would, in due course, lead to successful cytokine-based therapy in critically ill trauma patients.

Various studies have indicated that imbalanced pro- and anti-inflammatory cytokines produced during sepsis may play an important role in its pathogenesis. While many clinical and experimental studies have identified some crucial roles played by individual cytokines, a combined signature profile of cytokines implicated in sepsis is important to understand the pathophysiological mechanisms at play and development of effective treatment modalities against sepsis. Such a profile may also aid in the identification of biomarkers of sepsis and its prognosis. Effective removal of pathogenic cytokines and administration of protective cytokines may prove to be helpful in the successful treatment of sepsis (5).

A multiplex analysis evaluating plasma levels in patients with severe sepsis discovered that the concentrations of IL-1β, IL-6, IL-7, IL-8, IL-10, IL-13, IFN-γ, MCP-1,and TNF-α were significantly higher in patients with septic shock than in those with severe sepsis, and distinct cytokine profiles were associated with severity of sepsis, evolution of organ failure and death (10). Our study had similar findings and, additionally, we observed elevated levels of IL-17A and TGF-β at the onset of bacteremia.

It would be highly promising and beneficial to therapeutically target these mediators in order to decrease the unfavorable effects of sepsis-related host responses and to improve the overall outcome (11).

Trauma itself triggers a catastrophe of the immune response in the body and may play a confounding role while defining the cytokine profile in the septic trauma patients. It has been reported that a growing number of trauma patients survive sepsis but remain chronically critically ill. Greater elevations in inflammatory cytokines (IL-6, IL-8, and IL-10) have been demonstrated by such patients as compared to those who recover rapidly (12). The unique cytokine profile of septic trauma patients is still poorly understood. More studies with larger sample sizes and stringent exclusion criteria are needed for identifying a reliable cytokine profile for prognostication of high-risk septic trauma patients.

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