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Original Research Article

CHARACTERIZATION OF THE INFLAMMATION AND IMMUNE STATUS IN SEPSIS PATIENTS AND REGULATION OF THIS INFLAMMATION BY ULINASTATIN

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Abstract

Objective*:* Our study was designed to investigate the levels of the IL-17, IL-6, and IL-10 inflammatory mediators as well as of the T regulatory (Treg) and Th17 immune-regulation cells in patients with severe sepsis, and to observe the effect of Ulinastatin treatment on these levels. *Methods:* A total of 60 patients with severe sepsis hospitalized in our ICU during October 2011 to July 2012 served as the case group, and 20 healthy individuals served as the control group. The 60 patients were randomly divided into two groups: the routine group (n=30) received routine bundle treatment and the Ulinastatin group (n=30) received routine bundle treatment + Ulinastatin treatment. Patients in the Ulinastatin group received 300,000 U Ulinastatin 3 times/day in addition to routine bundle treatment. A single course of treatment lasted 3 days. IL-17, IL-6, IL-10, Treg, Th17, and HLA-DR expression were evaluated on the third day in the ICU and then every 3 days thereafter. The effect of Ulinastatin on the patients was evaluated. *Results:* The percentage of both Treg and Th17 cells in the periphery was significantly higher in the entire patient cohort than that in the control group (all $P<0.01$), while Treg cells were more significantly enhanced than Th17 cells. The expression rate of the IL-17 and IL-6 pro-inflammatory mediators and the IL-10 anti-inflammatory mediator was significantly higher in the entire patient cohort than that in the control group (IL-17: all $P<0.01$). The Treg/Th17 ratio was higher in the severe sepsis group than in the control group ($P<0.01$). In the severe sepsis group, immune analysis revealed that HLA-DR expression on $CD14^+$ monocytes was decreased compared to the control group ($P<0.01$]. Compared with the routine group, our study indicated that Ulinastatin treatment more effectively reduced the abnormal percentage of Treg and Th17 cells (all $P<0.01$) and decreased the Treg/Th17 ratio ($P<0.01$). Additionally, Ulinastatin ameliorated the elevated immune status of sepsis patients, as HLA-DR expression on $CD14^+$ monocytes improved ($P<0.01$). Compared with routine group, Ulinastatin treatment reduced the abnormal IL-17 and IL-6 expression (all $P<0.01$); while Ulinastatin also decreased IL-10 expression, this difference did not reach statistical significance. Ulinastatin also lowered the 28-day mortality of sepsis patients, but this difference did not reach statistical significance (18.7%

vs. 20%; *P*>0.05). *Conclusions:* In severe sepsis patients, the expression of the IL-10 anti-inflammatory mediator, the IL-17 and IL-6 pro-inflammatory mediators, and the percentage of Treg and Th17 cells in the periphery were abnormally enhanced. Additionally, the Treg/Th17 ratio was in disequilibrium, immune paralysis was enhanced, and HLA-DR expression decreased. Ulinastatin treatment decreased IL-17 and IL-6 expression, decreased the percentage of Treg and Th17 cells, inversed the Treg/Th17 ratio, ameliorated cellular immunity, and improved HLA-DR expression. Ulinastatin treatment can therefore improve the prognosis of patients with severe sepsis to a certain extent.

Key words: sepsis; Ulinastatin; Th17; Treg; HLA-DR

Introduction

The pathogenesis of sepsis is complex. It involves a range of tissue infections, inflammation, and immune responses, and is closely related to the pathophysiological changes in multiple organs and systems within the body [1]. Sepsis associated with organ dysfunction, tissue perfusion, or hypotension is deemed as severe sepsis. The resulting immune paralysis or imbalance of pro- and anti-inflammatory responses make severe sepsis the leading cause of patient death in the ICU [2, 3]. The features of immune paralysis include the following: increased anti-inflammatory mediators (such as IL-10), decreased cell surface expression of the HLA-DR MHCII molecule, decreased antigen-presenting function, decreased immune-cell proliferation, increased apoptosis, weakened hypersensitivity reactions, and increased secondary infections [4,5]. With the recent advances in our understanding of the pathophysiology and immune characteristics in sepsis patients, we predicted that early-stage intervention to block the occurrence of severe sepsis would improve the prognosis of patients with sepsis.

Materials and Methods

General patient information

Sixty patients admitted into the ICU with severe sepsis between October 2011 and July 2012 at our hospital was recruited for this study (average age: 54.3 ± 16.2 years). The patient population consisted of the following: 16 cases of infection with gastrointestinal perforation and intestinal necrosis, 25 cases of chest infection, 10 cases with perineum and urinary tract infection, 6 cases of blood-borne infections, and 3 cases presenting with other infections. Thirty of these cases were placed in the conventional (routine) treatment group and were given early recovery, rehydration according to lactate monitoring, antibiotic treatment, mechanical ventilation, blood purification, intestinal protection with glutamine and fish oil, etc., according to the 2008 Sepsis Treatment Guidelines and the classic treatment program found to be effective in our department. The other 30 cases placed in the Ulinastatin group were given 300,000 U of Ulinastatin every 8 hours in combination with conventional treatment, and were monitored once every 3 days. The 20 individuals in the healthy control group were recruited from health exams. There were no significant age differences among all groups. The experiment was approved by the hospital ethics committee.

Diagnostic criteria

According to the diagnostic criteria described by the 2001 International Sepsis Definitions Conference [6], we used the parameters outlined in each of the following categories to diagnose sepsis. (1) For infection, the patients need to be documented or suspected to have some of the following general parameters: fever

(core temperature >38.3°C); hypothermia (core temperature <36°C); heart rate >90 bpm or >2 SD above the normal value for age; tachypnea >30 bpm; altered mental status; significant edema or positive fluid balance (>20 mL/kg over 24 h); and hyperglycemia (plasma glucose >110 mg/dL or 7.7 mM/L) in the absence of diabetes. (2) Inflammatory parameters: leukocytosis (white blood cell count >12,000/μL); leukopenia (white blood cell count <4,000/μL); normal white blood cell count with >10% immature forms; plasma C-reactive protein >2 SD above the normal value; and plasma procalcitonin >2 SD above the normal value. (3) Hemodynamic parameters: arterial hypotension (systolic blood pressure <90 mmHg; mean arterial pressure <70; or a systolic blood pressure decrease >40 mmHg in adults or <2 SD below normal for age); mixed venous oxygen saturation >70%; and cardiac index >3.5 L min^{-1} m^{-2}. (4) Organ dysfunction parameters: arterial hypoxemia (PaO_2/FiO_2 <300); acute oliguria (urine output <0.5 mL kg^{-1} h^{-1} or 45 mM/L for at least 2 h); increased creatinine ≥0.5 mg/dL; coagulation abnormalities (international normalized ratio >1.5 or activated partial thromboplastin time >60 s); ileus (absent bowel sounds); thrombocytopenia (platelet count <100,000/μL); and hyper-bilirubinemia (plasma total bilirubin >4 mg/dL or 70 mmol/L). (5) Tissue perfusion parameters: hyper-lactatemia (>3 mmol/L); and decreased capillary refill or mottling. The exclusion criteria included various types of autoimmune diseases, acute cardiovascular diseases, HIV infection, viral infection, and use of drugs that affected immune function within the past 6 months.

Research Methods

Specimen collection

Venous blood (10 mL) from patients was collected with heparin within 2 h of diagnosis and then again 3 days after treatment; cultures and testing occurred within 4 h of diagnosis. Data for the APACHE II score, vital signs, arterial blood gases, and 28-day mortality were recorded during the treatment period. Specimens from individuals in the healthy control group were collected at the same time.

Flow cytometry and ELISA

Intracellular cytokine stain for IL-17A

(1) Stimulating agents (5 μL) (BD Biosciences, USA) were added into the mixture of peripheral blood (250 μL) and RPMI 1640 (450 μL), and cells were stimulated for 4–6 h at 37°C for 5% CO_2. (2) Cells were removed after stimulation and resuspended. CD3-PC5 (10 μL) and CD8-FITC (10 μL) mAbs (BD Biosciences) were incubated with the cells in the dark for 15–20 minutes. (3) The stained blood was aliquoted into two tubes labeled 'a' and 'b,' mixed, and incubated in the dark for 15 minutes at room temperature; 2 mL PBS was added, the cells were centrifuged at 1200 rpm for 5 min; supernatant was discarded. Cells were then resuspended in fixative and permeabilization reagents (100 μL), mixed, and incubated for 5 min. (4) Murine IgG1-PE (purchased from U.S. EB) was added to tube 'a,' IL-17A-PE (10 μL) was added to tube 'b,' and cells were incubated in the dark for 15–20 minutes. (5) Cells were washed with PBS (2 mL) and centrifuged for 1200 rpm for 5 min; supernatant was discarded. Cells were resuspended in 0.5 mL PBS, and cytokine expression was detected by a BD FACSCalibur flow cytometer. Th17% = $[(CD3^+CD8^+IL\text{-}17A^+ - CD3^+CD8^+IgG1^+)/CD3^+CD8^+] \times 100\%$.

Treg detection

(1) Peripheral blood (30 μL) was combined with 5 μL CD4-FITC, 5 μL CD25-PC5, and 5 μL CD127-PE and incubated in the dark for 15–20 minutes. (2) A hemolytic agent (1.2 mL) was added to the cells for 10 min. (3) Cells were then washed with PBS (2 mL)

and centrifuged at 1200 rpm for 5 min; supernatant was discarded. Cells were resuspended in 0.5 mL PBS, and Treg cells were detected by a BD FACSCalibur flow cytometer.

Detection of HLA-DR expression on $CD14^+$ monocytes

(1) Peripheral blood (100 µL) was stained with 10 µL of CD14-PC5. (2) HLA-DR–FITC (10 µL) and an isotype control were added to each tube, and cells were incubated at room temperature for 15 min. (3) Hemolysin (500 µL) was added for 15 min followed by the addition of an equal volume of PBS (500 µL), and cells were stored at 4°C. (4) Within 24 h, $CD14^+$ monocyte cells were analyzed for HLA-DR expression by flow cytometry.

Detection of the Th17-related cytokines, IL-17 and IL-6, as well as the Treg-related cytokine, IL-10, by ELISA

IL-17, IL-6, and IL-10 cytokines were measured by their respective ELISA kits (Biosource) strictly according to the manufacturer's instructions.

Statistical Analysis

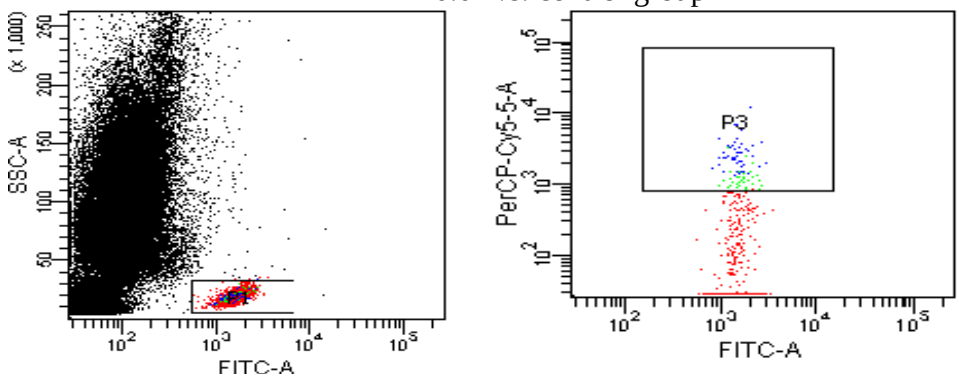
All data is expressed as $\bar{x} \pm s$. SPSS 17.0 statistical software was used for statistical analysis. Student's *t*-test was used to compare between groups, and correlation tests were performed using the Spearman rank correlation test. Differences achieving a value of $P<0.05$ were deemed statistically significant.

Results

Expression characteristics and changes in immune status of Treg and Th17 cells in the severe sepsis patients and in healthy controls.

Group	Cases	Treg (%)	Th17 (%)	Treg/Th17	HLA-DR (%)
Severe sepsis group	60	12.49 ± 1.67*	5.00 ± 0.53*	2.93 ± 0.32*	26.82 ± 4.65*
Control group	20	4.37 ± 0.35	2.43 ± 0.21	2.02 ± 0.23	74.89 ± 7.11

*$P<0.01$ *vs.* control group



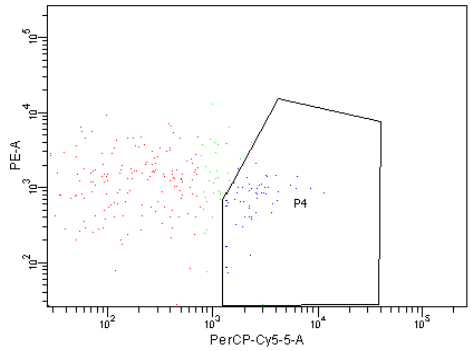


Figure 1. The percentage of Treg cells in the healthy control group.

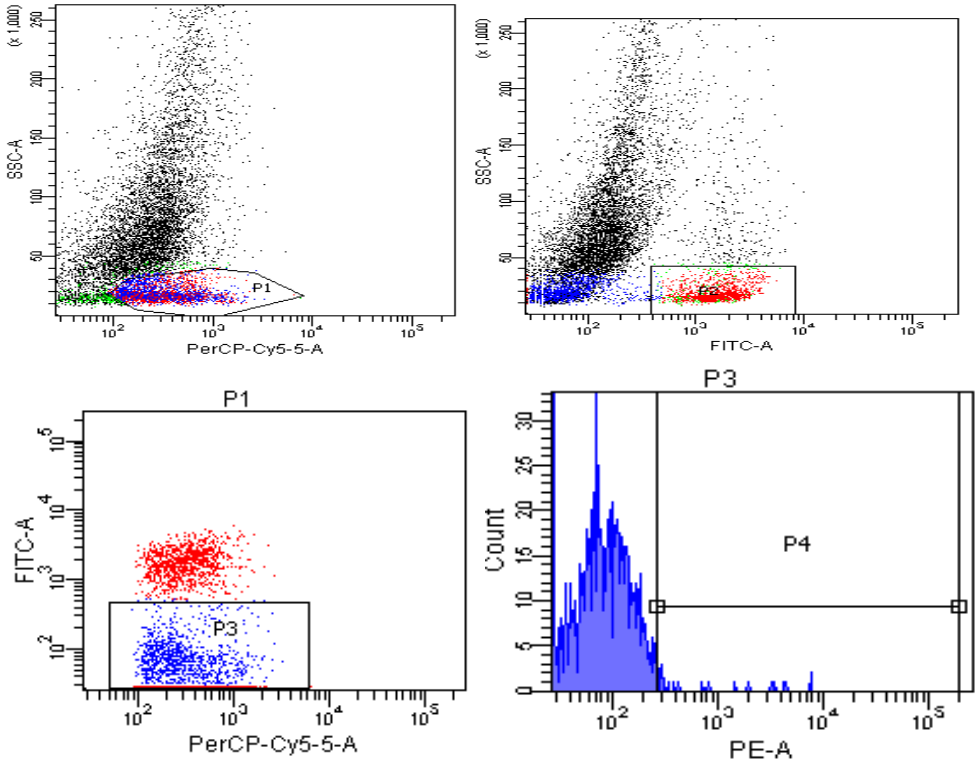


Figure 2. The percentage of Th17 cells in the healthy control group.

Table 2: Inflammatory mediator expression in severe sepsis patients and in healthy controls.

Group	Cases	IL-17 (pg/mL)	IL-6 (pg/mL)	IL-10 (pg/mL)
Severe sepsis group	60	36.07 ± 6.35*	118.43 ± 15.22*	42.03 ± 7.07*
Control group	20	12.14 ± 7.20	39.80 ± 11.81	14.48 ± 8.12

*P<0.01 *vs.* control group

Correlation among the percentage of Th17 and Treg cells, APACHE II score, and HLA-DR expression. There was no correlation among the percentage of Th17 expression, APACHE II score, and HLA-DR expression. The percentage of Tregs positively correlated with the APACHE II score (r = 0.93, P<0.01) and negatively correlated with HLA-DR expression on

$CD14^+$ monocytes (r = -0.89, *P*<0.01). HLA-DR expression on $CD14^+$ monocytes negatively correlated with the APACHE II score (r = -0.91, *P*<0.01).

Table3: Expression characteristics and changes in immune status of Treg and Th17 cells in the conventional and Ulinastatin treatment groups.

Group	Cases	Treg (%)		Th17 (%)		Treg/Th17		HLA-DR (%)	
		Before	After	Before	After	Before	After	Before	After
Conventional group	30	12.54±1.68	11.83±1.30	5.01±1.56	4.42+0.35[a]	2.95+0.33	2.82±0.29	27.49±4.86	36.44±8.14[a]
Ulinastatin group	30	12.45±1.68	9.05±1.27[ab]	4.99+0.51	3.20±0.33[ab]	2.90±0.31	2.22±0.28[ab]	26.15+4.41	49.34±11.34[ab]

Compared with before treatment,[a]*P*<0.01. Compared with conventional group, [b]*P* <0.01.

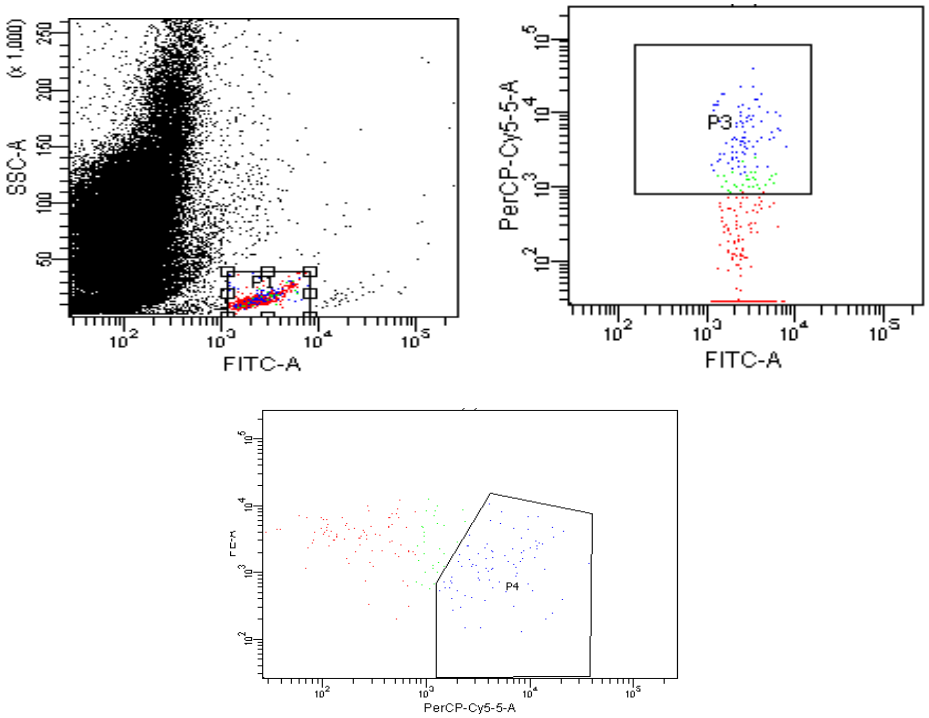
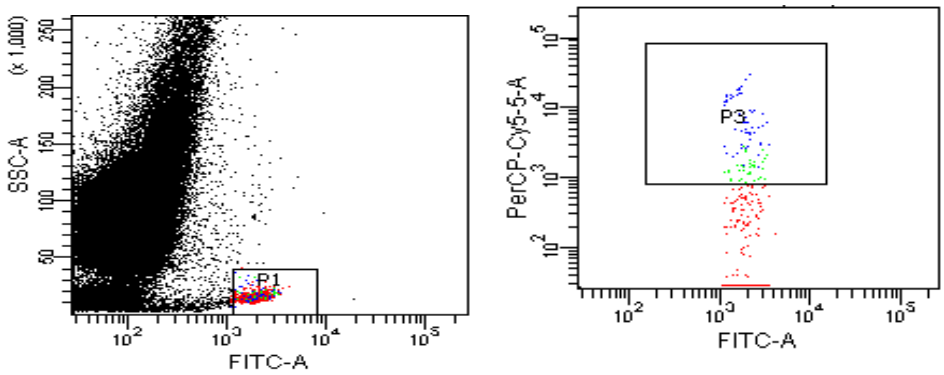


Figure 3. The percentage of Treg cells in the conventional treatment group.



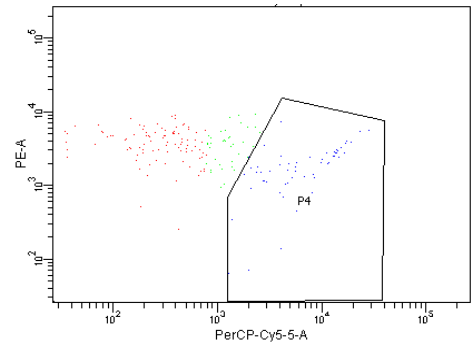


Figure 4. The percentage of Treg cells in the Ulinastatin treatment group.

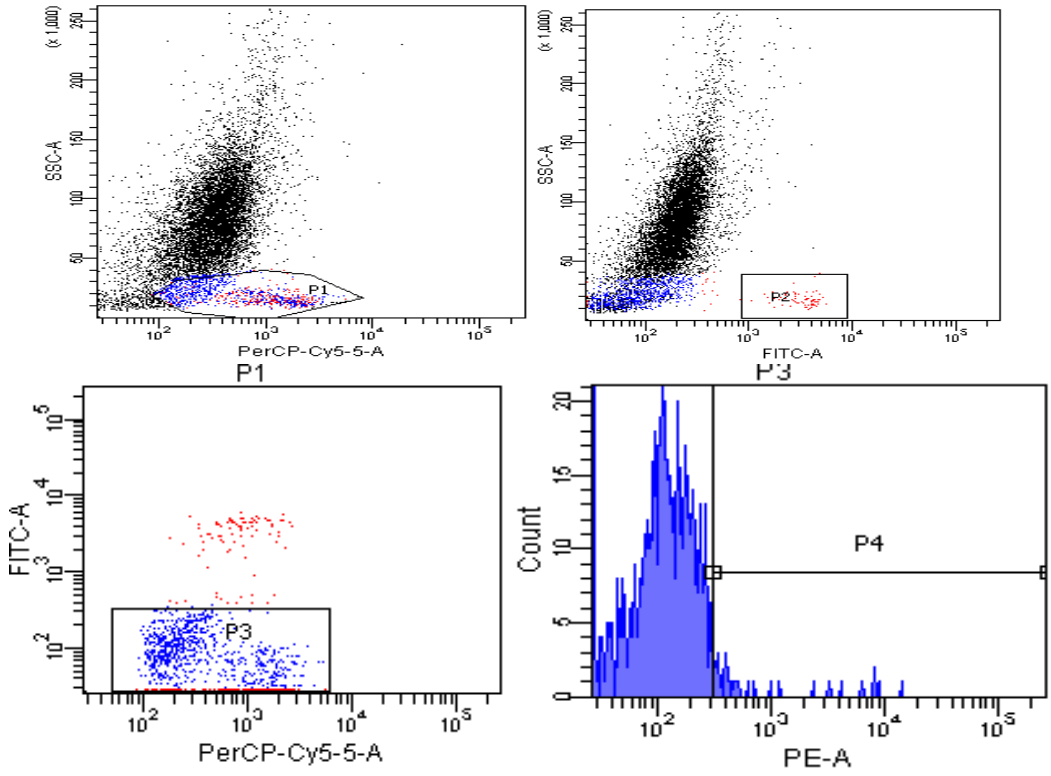
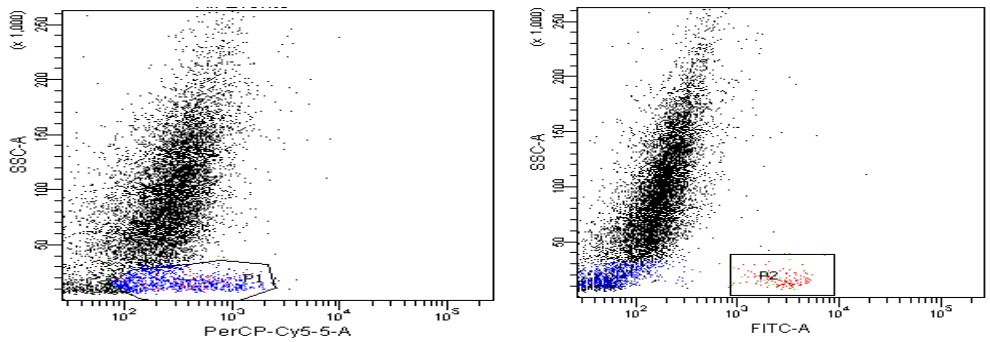


Figure 5. The percentage of Th17 cells in the conventional treatment group.



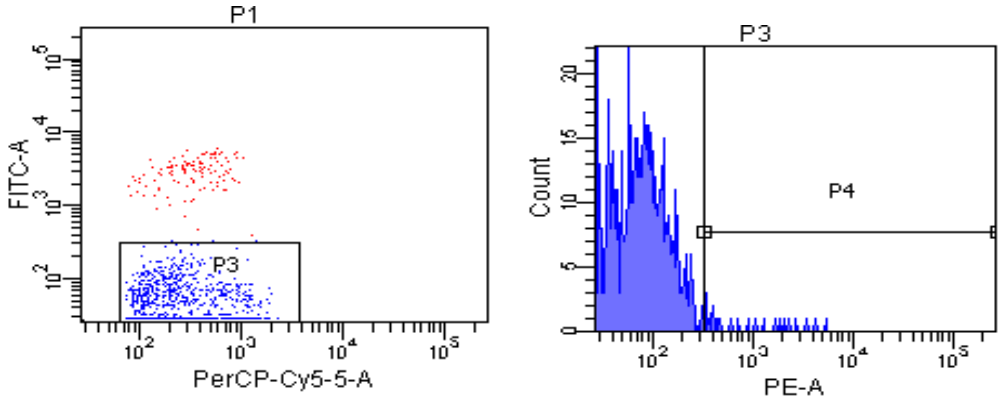


Figure 6. The percentage of Th17 cells in the Ulinastatin treatment group.

Table 4: Inflammatory mediator expression in the conventional and Ulinastatin treatment groups.

Group	Cases	IL-17 (pg/mL)		IL-6 (pg/mL)		IL-10 (pg/mL)	
		Before	After	Before	After	Before	After
Ulinastatin group	30	36.13±6.44	19.27±4.01[ab]	116.16±15.79	82.20±6.96[a b]	42.74±7.11	37.05±6.82[a]
Conventional group	30	36.02±6.36	33.70±5.35	120.70±14.53	96.67±10.01[a]	41.64±7.15	39.80±7.07

Compared with before treatment, [a]*P*<0.01. Compared with conventional group, [b]*P* <0.01.

The 28-day mortality rate was lower in the Ulinastatin treatment group than in conventional treatment group (18.7% *vs.* 20%, *P*>0.05).

Discussion

The pathogenesis of sepsis, involving the inflammatory response and cell-mediated immunity, is complex [7]. Mononuclear macrophages and other inflammatory cells are activated by pathogen-associated factors to produce and release large amounts of inflammatory mediators. These strongly promote both pro- and anti-inflammatory responses in the early phase of sepsis. In the late phase of sepsis, immune dysfunction predominates: on one hand, T cell dysfunction or inflammatory mediators drift toward an anti-inflammatory response, while on the other hand immune paralysis occurs. In recent years, studies have shown that immune Treg and Th17 cells have anti- and pro-inflammatory effects, respectively, by releasing the inflammatory mediators IL-17, IL-6, and IL-10. While the function of Tregs is contrary to that of Th17 cells, they have an interdependent relationship and maintain the immune equilibrium state [8]. A previous study found that an imbalance in the respective levels of these cells in the host during sepsis is involved in the observed immune dysfunction [9].

Ulinastatin is a glycoprotein hydrolase inhibitor extracted and refined from the fresh urine of healthy adult males. Also known as a urinary trypsin inhibitor, it scavenges oxygen free radicals and inhibits the release of inflammatory mediators. This effect enhances lymphocyte numbers and function, especially of NK and B cells, thereby improving overall immune function. A previous study confirmed that the Ulinastatin reduces the inflammatory response and improves the immune status and prognosis of patients with sepsis [10].

The present study found that the percentage of Treg and Th17 cells in the periphery was significantly higher in patients with severe sepsis compared to the healthy control group. Treg and Th17 both differentiate from $CD4^+$ T cells. Tregs exert anti-inflammatory effects through Foxp3 expression and the release of anti-inflammatory cytokines, such as IL-10 and TGF-β, to inhibit lymphocyte responses. Th17 cells exert pro-inflammatory effects through RORγt expression and IL-17, TNF-α, and IL-6 secretion. The significant elevation of both cell types in sepsis patients explained why the patients experienced such a strong pro-inflammatory response after sepsis. While the increased percentage of Th17 cells exerted a pro-inflammatory immune response to clear the pathogen from the host, a compensatory anti-inflammatory response, expressed as elevated Tregs, appeared at the same time. Consistent with this finding, IL-17, IL-6, and IL-10 expression was significantly higher in patients with sepsis than in the normal control group, indicating the presence of both strong pro- and anti-inflammatory responses in sepsis patients.

Further studies showed that the degree of elevation was more significant for Treg than Th17 cells. The normal Treg: Th17 ratio of 1.5–2.4 can reflect the status of an inflammatory response: whereas an elevated ratio prompts a dominant anti-inflammatory response, the pro-inflammatory response is otherwise dominant. Favre et al. previously found that a Treg/Th17 imbalance indicates the progression of an inflammatory disease [11]. In our study, Ulinastatin treatment reduced the percentage of both Treg and Th17 cells, driving the Treg/Th17 ratio toward normalization as compared to conventional treatment. Although Th17 and Treg cells have opposing functions, the coordination of their respective differentiation depends on the state of the innate immune system as well as the generation of acute phase proteins within the host. TGF-β produced by the immune system inhibits effector T cell proliferation to maintain immune tolerance in a stable state or in the absence of inflammation injury. However, if a large number of acute phase proteins are produced with the host, Treg cell proliferation is inhibited and Th17 cell differentiation is induced together with TGF-β, which mediates the pro-inflammatory response in the presence of infection or inflammation in the host. In response, the host then raises the number or function of Tregs to suppress this excessive Th17-mediated immune response against the pathogen during disease progression; however, this results in excessive inhibition, leading to secondary immunosuppression [12].

The ability of Ulinastatin to adjust the Treg/ Th17 balance during sepsis may occur by influencing inflammatory mediators. Ulinastatin improved patient immune status and elevated HLA-DR expression on $CD14^+$ monocytes, which may relate to the reduced percentage of Treg cells. Previous studies show that Ulinastatin could enhance host immunity by affecting lymphocyte number and function, especially of NK cells and B cells [13]. The 28-day mortality declined in the Ulinastatin group as compared to the conventional treatment group. This may relate to the effects of Ulinastatin on altering cytokine levels, inhibiting the generation of oxygen free radicals, stabilizing lysosomal membranes, improving microcirculation and tissue perfusion, and reducing reperfusion injury. Therefore, the ability of Ulinastatin to regulate cellular immune function and reduce immune paralysis likely can improve the prognosis of patients with severe sepsis.

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