

## EFFECT OF ULINASTATIN ON REGULATORY T CELLS AND T HELPER 17 CELLS IN PATIENTS WITH SEVERE SEPSIS

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### Abstract

The aim of this study was to assess the effect of ulinastatin (UTI) on the levels of immune regulatory cells, pro-inflammatory mediators, and the expression of severe sepsis in regulatory T cells (Treg) and T helper 17 (Th17) cells monocytes. Peripheral blood was obtained from severe sepsis patients. The blood was divided in two groups, the experimental group treated with ulinastatin and the control group without any treatment. The rate of CD4+ CD25+ Foxp3+ Treg cells in CD4+ T cells was analysed by flow cytometry. CD4+ CD25+ Foxp3+ Treg cells were purified by magnetic cell labelling (MACS) and expanded *in vitro* by culture with addition of CD3/CD8 Dynal beads and IL 2. The cells number, activity and purity after expanding the cells for 0 day, 7 days, 14 days and 21 days were determined. The rate of CD4+ CD25+ Foxp3+ and CD8+ CD25+ Foxp3+ cells in the control cell group was (6.41 ± 0.37)% and (6.67 ± 0.48)%. The rate of CD4+ CD25+ Foxp3+ and CD8+ CD25+ Foxp3+ cells gradually decreased with the increase of UTI concentration. When the concentration of UTI reached 1600 U/mL, the percentage of CD4+ CD25+ Foxp3+ and CD8+ CD25+ Foxp3+ cells were (3.40 ± 0.16)% and (3.53 ± 0.22)% respectively. UTI inhibited the differentiation of CD4+ CD25- cells into CD4+ IL-17 cells and CD4+ CD25+ Foxp3+ cells and the differentiation of CD8+ CD25- cells into CD8+ IL-17 cells and CD8+ CD25+ Foxp3+ cells. The inhibitory effects observed were dose dependent.

### Rezumat

Scopul acestui studiu a fost evaluarea efectului ulinastatinului (UTI) asupra celulelor implicate în reglarea imună, a mediatorilor pro-inflamatorii, precum și expresia sepsisului sever la nivelul celulelor T de reglatoare (Treg) și T helper 17 (Th17). A fost recoltat sânge periferic de la pacienți cu sepsis sever. Numărul celulelor CD4+ CD25+ Foxp3+ Treg din populația de limfocite T CD4+ a fost analizată prin citometrie în flux. Numărul celulelor, activitatea și puritatea acestora au fost determinate la momentul inițial, după 7 zile, 14 zile și respectiv 21 de zile. Procentul celulelor CD4+ CD25+ Foxp3+ și CD8+ CD25+ Foxp3+ în grupul de control a fost 6,41 ± 0,37% și, respectiv, 6,67 ± 0,48%. Procentul celulelor CD4+ CD25+ Foxp3+ și CD8+ CD25+ Foxp3+ a scăzut proporțional cu creșterea concentrației de UTI. Pentru concentrația de 1600 U/ml, procentul celulelor CD4+ CD25+ Foxp3+ și CD8+ CD25+ Foxp3+ a fost de 3,40 ± 0,16% și, respectiv, 3,53 ± 0,22%. UTI a inhibat diferențierea celulelor CD4+ CD25- în celule CD4+ IL-17 și celule CD4+ CD25+ Foxp3+ precum și diferențierea celulelor CD8+ CD25- în celule CD8+ IL-17 și celule CD8+ CD25+ Foxp3+. Efectele inhibitoare au fost dependente de doză.

**Keywords:** regulatory T cell, Foxp<sub>3</sub>, MACS, severe sepsis, ulinastatin

### Introduction

Sepsis is defined as life-threatening organ dysfunction caused by a dysregulated host response to infection and is a leading cause of death worldwide [1, 2]. Septic shock occurs in response to an infection with resistant bacterial germs [3, 4]. The inflammatory response to microbial invasion is a continuous process that can progressively evolve to worsening, if not treated [5, 6]. Ulinastatin (UTI), a drug used in acute inflammatory disorders, has been tested in animal models of autoimmune inflammatory diseases, such as ulcerative colitis, acute pancreatitis and crescentic glomerulo-nephritis [5, 7].

In a previous study, intravenous administration of ulinastatin reduced mortality in patients with severe sepsis in the modified intention-to-treat analysis [8]. In the current study, we used magnetic cell labelling (MAC<sub>S</sub>) for the enrolment and sorting the cells from the peripheral blood of patients with severe sepsis. We investigated the role of regulatory T cells (Treg) and T helper 17 (Th17) and related cytokines in patients with severe sepsis in order to determine the potential therapeutic effect of ulinastatin, its effect on Treg and Th17 cells, and to investigate the role of ulinastatin in the differentiation and function of Treg and Th17 cells *in vitro*.

## Materials and Methods

### Patients

All patients with severe sepsis were from Hang Zhou Red Cross Hospital, China. The experiments were approved by the hospital ethics committee. Written informed consent was obtained at the moment of admission from either the patient or, in the event that the patient lacked capacity, a personal or professional legal representative for participating in the study.

Patients were included in the study within 24 hours after meeting the inclusion criteria for severe sepsis. Exclusion criteria: basic disease prognosis was poor and in the short term may become the leading causes of death (such as stroke, craniocerebral injury, post-operative cardiopulmonary resuscitation (CPR) and late malignant tumour), as well as people with autoimmune diseases such as rheumatoid arthritis, multiple sclerosis and systemic lupus erythematosus (SLE), asthma and other diseases or patients that used within 3 months prior to admission corticosteroids or immunosuppressants.

The data regarding demographic data, sepsis diagnosis, intensive care unit (ICU) procedures, and a date-stamped log of all invoiced items, including medications, laboratory orders, diagnostic and therapeutic services were taken from medical records of each patient.

There were no significant differences in the blood pressure, lactic acid levels, urine volume, oxygenation index, serum creatinine levels, serum bilirubin, platelet count and blood coagulation among all the patients with sepsis. APACHE II and SOFA scores were no significant different among the patients.

Patients had received standard care according to the ACCP/SCCM: Surviving Sepsis Campaign 2001 guidelines. Antibiotics were administered to treat identified infections. In the study were included 120 cases of severe sepsis, 30 cases in the control group and 90 cases in the experimental group, with no significant difference regarding the clinical medication among all patients. Peripheral blood was collected from all the patients involved in the experiment. In the experimental group peripheral blood samples were treated with ulinastatin in doses of 0 U/mL, 100 U/mL, 400 U/mL and 1600 U/mL. The percentages of peripheral blood, lymphocyte subsets, Treg and Th17 cells were measured by flow cytometry and the ratio of the two was calculated for the values obtained in day 1, 3, 5 and 7.

### Sampling

Before the ulinastatin treatment, human CD3<sup>+</sup> CD25<sup>-</sup> T cells which include CD4<sup>+</sup> CD25<sup>+</sup> T cells and CD8<sup>+</sup> CD25<sup>-</sup> cells were determined in the peripheral blood of all patients. The purity of the obtained T cells was determined by the separation and enrichment of the magnetic beads and the enrichment of the cells. From each patient 120 mL of peripheral blood

was collected and mononuclear cells were obtained by density ladder centrifugation.

### Cell Sorting and T-cell Suppression

Using magnetic beads to select human CD3<sup>+</sup> T cells (Miltenyi Biotec), the CD3<sup>+</sup> T cells were enriched according to the instructions of the kit. The obtained CD3<sup>+</sup> T cells were incubated using anti-CD25APC (allophycocyanin) antibody (eBioscience), and the anti-CD25<sup>+</sup> cells were removed using anti-anti-APC magnetic beads, and CD3<sup>+</sup> CD25<sup>-</sup> T cells were obtained and counted.

The purity of CD3<sup>+</sup> CD25<sup>-</sup> T cells obtained by the magnetic bead separation and enrichment was determined. A quantity of  $5 \times 10^7$  cells were added to the 80  $\mu$ L PBS (phosphate buffer saline) and the 10  $\mu$ L CD3-PE (phycoerythrin) antibody (eBioscience) and the 10  $\mu$ L CD25-APC antibody (eBioscience). The mixture was incubated for 30 min. After centrifugation, the supernatant was discarded. 1 mL PBS was added and the sample was centrifuged at  $300 \times g$  for 10 min and then the above liquid was discarded. Finally, the concentration of CD3<sup>+</sup> CD25<sup>-</sup> T cells was detected by flow cytometry using the standard procedure.

### Treatment groups and cell culture

**Control group:** 30 samples of CD3<sup>+</sup> CD25<sup>-</sup> T cells treated with 0 U/mL UTI.

**Experimental groups:** 90 samples of CD3<sup>+</sup> CD25<sup>-</sup> T cells were divided in 3 subgroups treated with 100 U/mL, 400 U/mL and 1600 U/mL ulinastatin.

In each group it was evaluated the effect of the treatment on the differentiation of CD3<sup>+</sup> CD25<sup>-</sup> in Th17 T cells.

On the first day of culture, anti-CD3 (5  $\mu$ g/mL), anti-CD28 (5  $\mu$ g/mL), anti-IL-4 (5  $\mu$ g/mL) and anti-IFN- $\gamma$  (5  $\mu$ g/mL) as well as the cytokines TGF- $\beta$  (2 ng/mL) and IL-23 (10 ng/mL) were added in each plate. The cells were cultured for 72 h in 50 ng/mL PMA (phorbol-12-myristate-13-acetate) with 750 ng/mL ionomycin and 10 g/mL brefeldin A as stimulating factors. The cells were then separated through flow cytometry in CD4<sup>+</sup> IL-17<sup>+</sup> cells and CD8<sup>+</sup> IL-17<sup>+</sup> cells.

### Statistical analysis

All results were analysed using SPSS 13.0 software, and all quantitative data were presented as mean  $\pm$  standard deviation (SD). Statistical analysis was performed by use of one-way ANOVA. For other analysis, the SNK-q test was used for multiple comparisons between groups. A value of  $p < 0.05$  was considered statistically significant.

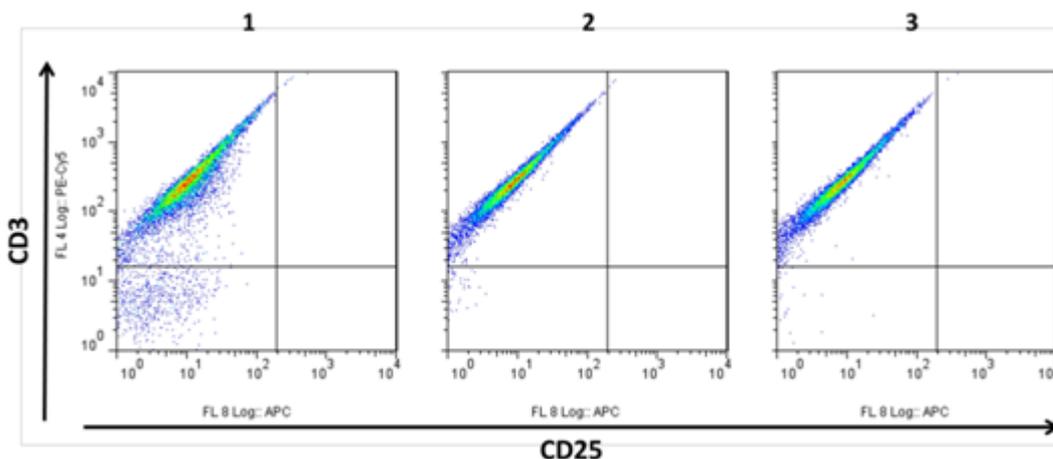
## Results and Discussion

### Human CD3<sup>+</sup> CD25<sup>-</sup> T cell enrichment.

Before the UTI treatment, for all the participants from which the blood was collected, CD3<sup>+</sup> CD25<sup>-</sup> T cells enrichment and sorting was performed in order to test the purity of the cells and it can be

seen in Figure 1 and Table I that the CD3+ CD25- cells were in a concentration of  $89.70 \pm 1.85\%$  and CD3- CD25- were in a concentration of  $10.23 \pm 1.87\%$  (Figure 1 and Table I). The levels of CD4+ CD25- T cells and CD8+ CD25- T cells were measured

from CD3+ CD25- T cells obtained. The percentage of CD4+ CD25- cells and CD8+ CD25- cells were  $60.24 \pm 0.24\%$  and, respectively,  $34.19 \pm 0.44\%$  (Figures 2, 3 and Table II).



**Figure 1.**

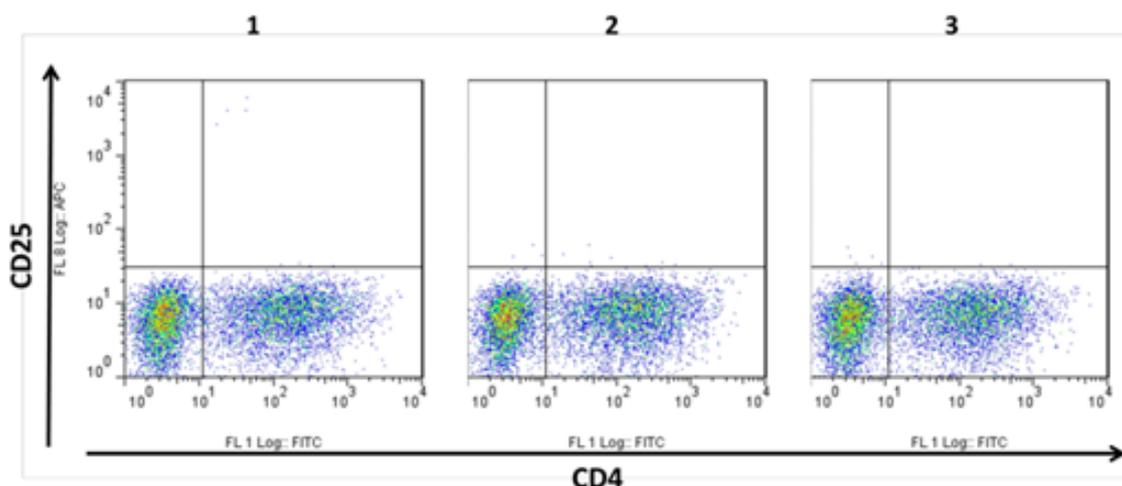
Detection of cells concentration by flow cytometry: CD3+ CD25- T cells

**Table I**

The evaluation of cells concentration by flow cytometry: the percentage of CD3+ CD25- T cells(% , mean  $\pm$  SD)

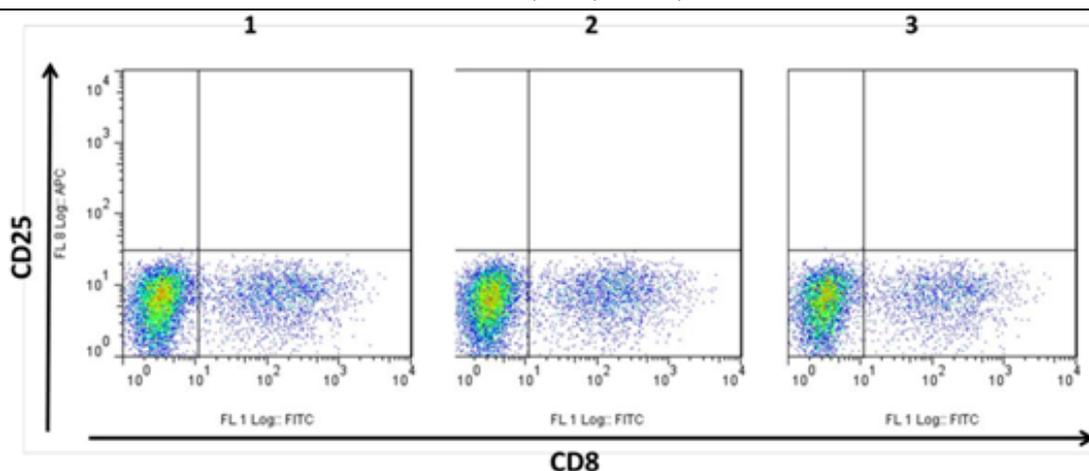
Antibodies	Cell type	Cell levels (%)
CD3-PE-Cy5/CD25-APC	CD3+ CD25-	$89.70 \pm 1.85$
	CD3+ CD25+	$0.07 \pm 0.05$
	CD3- CD25-	$10.23 \pm 1.87$
	CD3- CD25+	$0.00 \pm 0.00$

PE - phycoerythrin; Cy5 – Cyanine 5; APC- allophycocyanin



**Figure 2.**

Detection of cell concentration by flow cytometry: CD4+ CD25- T cells



**Figure 3.**

Detection of cell concentration by flow cytometry: CD8+ CD25- T cells

**Table II**

The evaluation of cells concentration by flow cytometry: concentrations of CD4+ CD25- and CD8+ CD25- (% , mean ± SD)

Antibodies	Cell type	Cell levels (%)
CD4-FITC/CD25-APC	CD4- CD25-	30.77 ± 0.29
	CD4+ CD25-	60.24 ± 0.24
	CD4- CD25+	0.03 ± 0.02
	CD4+ CD25+	1.12 ± 0.07
CD8-FITC/CD25-APC	CD8- CD25-	65.77 ± 0.43
	CD8+ CD25-	34.19 ± 0.44
	CD8- CD25+	0.02 ± 0.02
	CD8+ CD25+	0.02 ± 0.01

Note: FITC – fluorescein isothiocyanate; APC - allophycocyanin

*The effects of ulinastatin on CD3+ CD25 - T cells differentiation into Th17 cells.*

The results of this study showed that different concentrations of ulinastatin induce differentiation of CD3+ CD25- T cells into CD4+ IL 17 + cells (Figure 4) and CD8+ IL-17 + cells (Figure 5). Ulinastatin inhibited the differentiation of CD4+ CD25- T cells into CD4+ IL17 cells and CD4+ CD25+ Foxp3+ cells. The percentages of CD4+ CD25+ Foxp3+ and

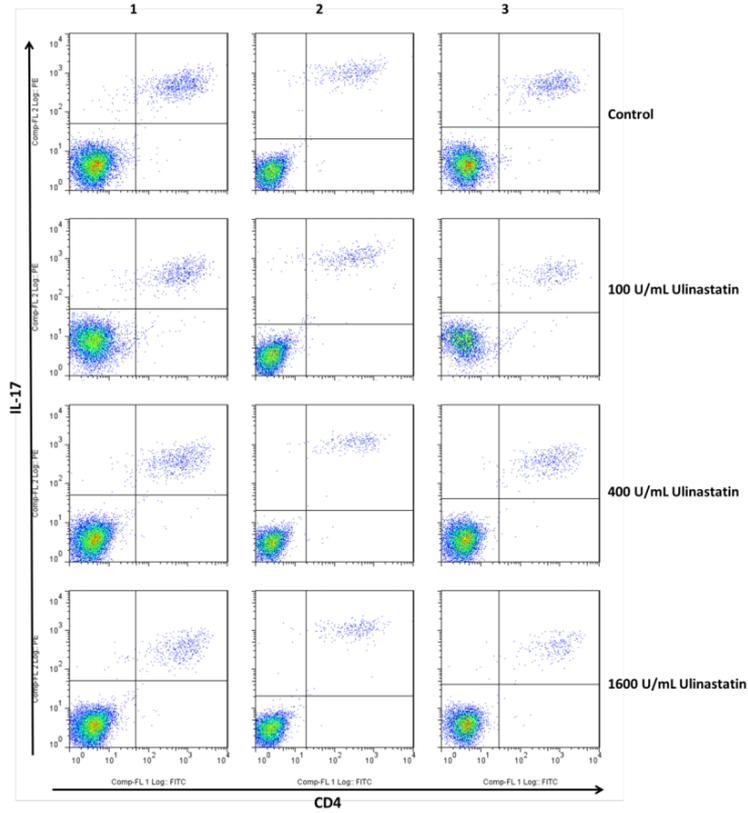
CD8+ CD25+ Foxp3+ cells in the control group were 6.41 ± 0.37% and 6.67 ± 0.48% respectively. The percentages of CD4+ CD25+ Foxp3+ and CD8+ CD25+ Foxp3+ cells gradually decrease with the increase of UTI concentration. For UTI concentration of 1600 U/mL, the percentage of CD4+ CD25+ Foxp3+ and CD8+ CD25+ Foxp3+ cells was of 3.40 ± 0.16 % and 3.53 ± 0.22 %, respectively (Figure 6, Table III).

**Table III**

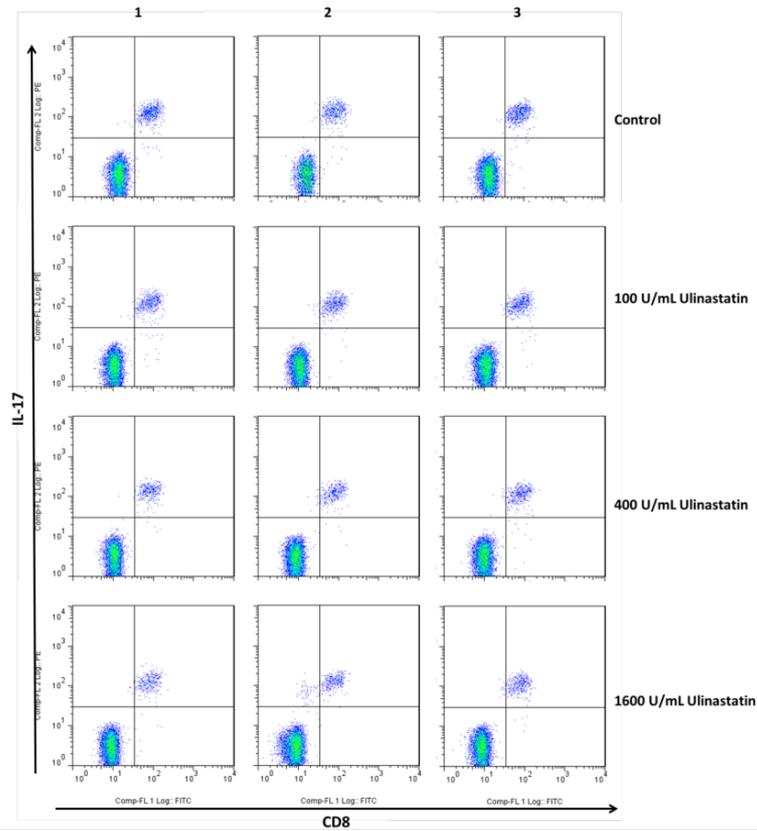
The effect of ulinastatin on the differentiation of CD4+ CD25-T cells into CD4+ IL17 cells and CD4+ CD25+ Foxp3+ cells (% , mean ± SD)

Antibodies	Goup	Cell concentration (%)
CD4-FITC/IL-17-PE	Control	6.41 ± 0.37
	100 U/mL UTI	5.13 ± 0.07
	400 U/mL UTI	4.96 ± 0.04
	1600 U/mL UTI	3.40 ± 0.16
CD8-FITC/IL-17-PE	Control	6.67 ± 0.48
	100 U/mL UTI	4.90 ± 0.10
	400 U/mL UTI	4.19 ± 0.08
	1600 U/mL UTI	3.53 ± 0.22

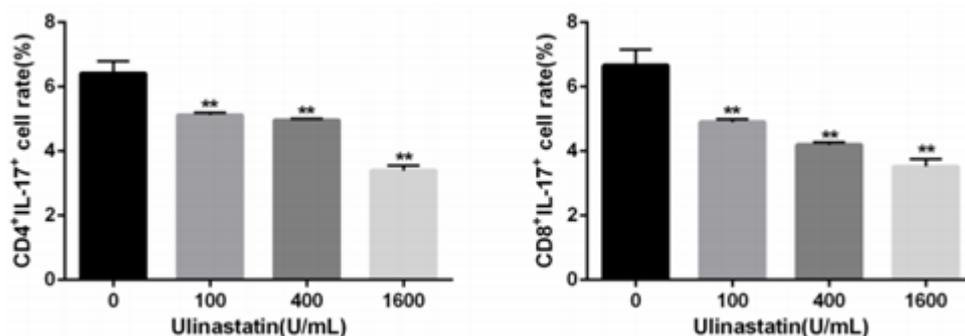
Note: FITC - fluorescein isothiocyanate; PE - phycoerythrin; UTI - Ulinastatin



**Figure 4.**  
Detection of CD4+ IL-17+ cells by flow cytometry



**Figure 5.**  
Detection of CD8+ IL-17+ cells by flow cytometry



**Figure 6.**

The evaluation of cells concentration by flow cytometry: the levels of CD4+ IL-17 and CD8+ IL-17 (\*\* p < 0.01 vs. 1600 U/mL Ulinastatin)

**Table IV**

The effect of ulinastatin on the differentiation of CD4+ CD25-T cells into CD4+ IL17 cells and CD4+ CD25+ Foxp3+ cells (% , mean ± SD)

Antibodies	Group	Cell concentration (%)
CD4-FITC/IL-17-PE	Control	6.41 ± 0.37
	100 U/mL UTI	5.13 ± 0.07
	400 U/mL UTI	4.96 ± 0.04
	1600 U/mL UTI	3.40 ± 0.16
CD8-FITC/IL-17-PE	Control	6.67 ± 0.48
	100 U/mL UTI	4.90 ± 0.10
	400 U/mL UTI	4.19 ± 0.08
	1600 U/mL UTI	3.53 ± 0.22

Note: FITC - fluorescein isothiocyanate; PE - phycoerythrin; UTI - Ulinastatin

Severe sepsis has become one of the most common causes of death in ICU [9]. Cellular immune disorders and acute inflammatory reaction are the two key factors that favour severe sepsis. In the early stage, immune cells and inflammatory response cells can be activated, producing a large number of inflammatory mediators, so that the body produces a strong pro-inflammatory and anti-inflammatory response, which cannot be well controlled and eventually will lead to immune dysfunction. Traditionally, it was considered that the host immune response to sepsis is characterized by an initial hyper-inflammatory phase that evolves over several days into a more protracted immunosuppressive phase [10, 11]. Many patients develop severe systemic oedema. Pleural and peritoneal effusion, lower blood pressure, hypoxia, even circulating and respiratory failure. The mechanism of capillary leakage is not completely clear. Most researchers believe that endotoxins and some damaging factors factors can activate the immune system to release pro-inflammatory cytokines (TNF $\alpha$ , IL-1-, IL-6) that activate the effector cells and release inflammatory mediators such as TXA2 and PGI2. Despite improvements in access to care, imaging and interventional techniques, severe sepsis continues to be associated with significant morbidity and mortality. Wang's study found a role for ulinastatin in limiting the acidosis state and attenuating the coagulation disbalance induced by coecal ligation and puncture

(CLP) [12]. Such results suggested that ulinastatin's protection was also related to its positive influence on coagulatory disorders.

Similar to our study, Huang's study suggests that ulinastatin is useful for bronchial asthma, it can effectively relieve clinical symptoms, raises the seric levels of IL-2 and IL-4 and regulates the immune function by changing the relative percentages of T cells subsets [13]. In Karnad's trial, the addition of ulinastatin was associated with severe organ dysfunction in adult patients with severe sepsis [14]. Ulinastatin can also decrease the expression of Treg and Th17, inverse the ratio of Treg/Th17, and ameliorate the immune status, which can improve the prognosis of patients with severe sepsis [15].

The main limitation of this study is that it was performed in a single institution with a limited number of patients. The total number of study participants could be increased by including multiple units; however, the level of complexity, the required personnel to consistently perform the analyses and the required places limits the widespread of the units participation. Another limitation of this study is the incomplete data at later time points as patients were discharged; collection of blood after hospital discharge would allow for a more robust generalizable analysis.

## Conclusions

In conclusion, our study showed that Treg and Th17 cells play an important role in the occurrence and development of severe sepsis. Ulinastatin inhibited the differentiation of CD4+ CD25- T cells into CD4+ IL17 cells and CD4+ CD25+ Foxp3+ cells. Ulinastatin can influence the differentiation of Treg and Th17 cells *in vitro*. When the concentration of ulinastatin reached 1600 U/mL, the inhibitory effect of ulinastatin was more obvious. Overall this study provided new research ideas and experimental evidences for the treatment of sepsis by intra-intestinal administration of UTI.

## Acknowledgement

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## Conflict of interest

The authors declare no conflict of interests.

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