

# Characteristics of CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> regulatory T cells in patients with multiple organ dysfunction syndrome

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**Abstract.** Multiple organ dysfunction syndrome (MODS) is a systemic inflammation. The aim of the present study was to evaluate the role of CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> regulatory T cells (Treg) in patients with MODS and to determine the association between Treg cells and serum cytokine levels. The percentage of Treg in 42 MODS patients and 10 healthy subjects was evaluated using flow cytometry. Serum levels of cytokines were measured using an enzyme-linked immunosorbent assay. The percentage of Treg cells was significantly elevated in patients with MODS on Day 1 ( $P < 0.05$ ). At Day 7, the percentage of Treg cells in MODS patients was reduced, but remained higher in comparison with the control group ( $P < 0.05$ ). The CD4<sup>+</sup>/CD8<sup>+</sup> ratio and the levels of tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), interleukin (IL)-2, IL-4, IL-6, IL-8, IL-10 and IL-1 $\beta$  were significantly enhanced in patients with MODS by Day 1. TNF- $\alpha$ , IL-2, IL-4 and IL-10 levels were gradually reduced to normal by Day 7, whereas the IL-6, IL-8 and IL-1 $\beta$  levels remained higher compared with the healthy subjects ( $P < 0.001$ ). The present results demonstrated an elevated percentage of CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> Treg cells in patients with MODS. Therefore, the proinflammatory cytokines TNF- $\alpha$ , IL-2, IL-6, IL-8 may promote MODS development, whereas the anti-inflammatory cytokines IL-4 and IL-10 may protect against MODS.

## Introduction

Multiple organ dysfunction syndrome (MODS) is a systemic inflammation resulting from infectious or non-infectious stimuli, leading to the dysfunction of two or more organs and the failure of multiple systems (1,2). MODS remains the most common cause of mortality in intensive care units, and may be triggered by a variety of stimuli, including severe

trauma, infection, shock, cardiopulmonary resuscitation and surgery (3). The mechanism underlying MODS is complicated; however, it has been widely accepted that uncontrolled inflammatory response contributes to the rapidly progressive development of MODS (4).

In 1995, Sakaguchi *et al* identified a subpopulation of CD4<sup>+</sup> T lymphocytes with high cell surface expression of interleukin (IL)-2 receptor  $\alpha$  chain (CD25); namely, CD4<sup>+</sup>CD25<sup>+</sup> regulatory T (Treg) cells (5). These cells are crucially involved in the regulation of autoimmune diseases, transplant tolerance, and infectious and anti-tumor immune responses (6-9). Using the CD4<sup>+</sup>CD45RB high T cell mice transfer model of inflammatory bowel disease, Mottet *et al* (10) showed that CD4<sup>+</sup>CD25<sup>+</sup> cells, but not CD4<sup>+</sup>CD25<sup>-</sup> CD45RB low T cells, were able to cure intestinal inflammation, indicating the critical role played by CD4<sup>+</sup>CD25<sup>+</sup> Treg cells in the prevention of the T cell-mediated immune response. Furthermore, the forkhead family transcriptional regulator Foxp3, a lineage-specific marker of CD4<sup>+</sup>CD25<sup>+</sup> Treg cells, has been recognized to be critical for the development and function of Treg cells (11). Notably, FOXP3 expression regulates the activity of the Treg cells *in vitro* and *in vivo* (11). Depletion of CD4<sup>+</sup>CD25<sup>+</sup> Treg cells from melanoma patients resulted in enhanced immune responses and substantial development of antigen-specific CD8<sup>+</sup> T cells in peptide-vaccinated individuals (12). In addition, the presence of CD4<sup>+</sup>CD25<sup>+</sup> Treg cells in patients with active rheumatoid suppresses the proliferation of autologous T cells from synovial and peripheral blood by promoting the production of IL-10 and transforming growth factor- $\beta$  (TGF- $\beta$ ) secreted by Treg cells (13).

In patients with MODS, proinflammatory and anti-inflammatory cytokine levels are imbalanced and abnormal cytokine secretion may lead to exaggerated inflammation (14). Application of monoclonal antibodies for tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) or soluble TNF- $\alpha$  receptor suppresses the elevated expression of IL-1 and IL-6 via the reduction of the release of TNF- $\alpha$ , and thus protects against MODS (3). Additionally, specific IL-1 receptor antagonists have been shown to reduce the mortality of endotoxin-induced shock in rabbits (15). However, such anti-inflammatory therapies may act as a double-edged sword, as they may suppress the damage caused by excessively-activated inflammation while simultaneously eliminating the benefits of the inflammatory response. Therefore, efficient therapy for the regulation of inflammation is required.

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Due to its powerful effect in suppressing immune responses in various human diseases, the application of CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> Treg cells may provide a possible therapy for the treatment of MODS. However, the potential role for these cells in MODS is not well understood. In the present study, the role of CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> Treg cells in MODS was investigated.

## Materials and methods

**Reagents.** Ficoll was purchased from Tianjin Hao Yang Biological Manufacture Co., Ltd. (Tianjin, China). Phosphate-buffered saline (PBS) was obtained from Beijing Chemical Works (Beijing, China).

**Patients.** A total of 42 patients (age range, 15-82 years) were selected from the Intensive Care Unit of the First Hospital of Jilin University (Changchun, China) between January 2009 and February 2010. All patients met MODS diagnostic criteria (16) and 27 patients remained in the hospital for over one week. These 42 patients were assigned into 2 groups; Survival >15 days (n=15) and Survival <15 days (n=27), as 15 patients survived and 27 patients succumbed to MODS in the hospital between days 1 and 5 following admission. In addition, MODS was complicated by various disease states, as follows: Five cases had septic shock; 11 cases received cardiopulmonary resuscitation; 11 cases had surgical palliation for gastric cancer, gallbladder carcinoma, colon or rectum; 7 cases had multiple injuries; 3 cases had allergic shock; 3 cases had severe pancreatitis; 1 case had cesarean section; and 1 case had ectopic pregnancy. All patients had injuries in between 2 to 6 organs. Ten healthy physical examinees aged 20-28 years were selected as control subjects. Informed consent was provided by all participants who met eligibility criteria. Ethical approval for this study was provided by the Ethics Committee at First Hospital of Jilin University.

**Peripheral blood collection.** Blood samples (2 ml) were collected from study cohorts at indicated time points and were allowed to clot for 2 h at room temperature. Serum was isolated by centrifugation (1,100 × g for 30 min at 20°C) and stored at -20°C until further use.

**Antibodies and flow cytometric analysis.** Mouse anti-human IgG (PAB9307), fluorescein isothiocyanate (FITC)-conjugated anti-CD25 mAb (ANC-174-040), anti-CD4 mAb (ANC-148-040), phycoerythrin (PE)-conjugated anti-CD8 mAb (ANC-154-070), anti-Foxp3 mAb (AG-20A-0025-C050), PerCP5 conjugated anti-CD4 (CYT-4C3), anti-CD3 (CYT-3C4), and isotype control antibodies were purchased from Caltag Laboratories (Carlsbad, CA, USA). Peripheral blood samples (2 ml) were collected for flow cytometric analysis, and the levels of CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup>, CD3<sup>+</sup>, CD4<sup>+</sup> and CD8<sup>+</sup> were determined as described previously (9,11).

Percentage of Tregs in gated lymphocytes was measured based on a method previously described by Liu *et al* (17). Briefly, lymphocytes were washed and resuspended in PBS with 1% bovine serum albumin (BSA; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) and incubated with PerCP-conjugated anti-CD4 (550631), PerCP-conjugated anti-CD25 (558689) and FITC-conjugated anti-CD25 (124774)

(all dilution 1:50; all purchased from BD Biosciences, San Jose, CA, USA) for 20 min at 4°C. Cells were fixed and permeabilized with Fix/Perm buffer (005523; eBioscience, San Diego, CA, USA) followed by washing and blocking with normal rat serum (eBioscience). Cells were then stained with PE-conjugated anti-Foxp3 (Foxp3 Staining Set, clone PCHI01; eBioscience) for 60 min, and analyzed using a FACSCalibur flow cytometer (BD Biosciences). The data were analyzed using FlowJo software (version 7.6.1; Tree Star, Inc., San Carlos, CA, USA).

**Enzyme-linked immunosorbent assay (ELISA).** ELISA kits were used to determine the concentration levels of TNF-α (EK0986), IL-2 (EK0397), IL-4 (EK0404), IL-6 (EK0410), IL-8 (EK0413), IL-10 (EK0416) and IL-1β (EK0392), measured in triplicate according to the manufacturer's instructions (Wuhan Boster Biological Technology, Ltd., Wuhan, China). The optical density values were detected using an ELISA reader (Anthos 2010; Biochrom Ltd., Cambourne, UK) at a 450-nm wavelength and calculated in the linear part of the curve.

**Statistical analyses.** Data were analyzed using SPSS software, version 11.5 (SPSS, Inc., Chicago, IL, USA) and are presented as the mean ± standard deviation. Comparisons between the two groups of subjects were made using Student t-test or  $\chi^2$  test.  $P < 0.05$  was considered to indicate a statistically significant difference.

## Results

**Increased percentage of CD4<sup>+</sup>CD25<sup>+</sup>FOXP3<sup>+</sup> Treg cells in patients with MODS.** To investigate the potential role of CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> Treg cells in patients with MODS, a total of 27 patients that fulfilled the MODS diagnostic criteria were recruited and the percentages of CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> Treg cells in total lymphocytes in the patients' peripheral blood were evaluated using flow cytometry on Days 1 and 7 following administration. Meanwhile, ten healthy subjects were selected as controls. As shown in Fig. 1, a markedly elevated CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> Treg cell number was detected in patients with MODS on Day 1 compared to the healthy control group ( $P < 0.05$ ). Notably, although it was reduced on Day 7 as compared with Day 1, the percentage of CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> Treg cells in MODS patients remained higher than the control group on Day 7 ( $P < 0.05$ ; Fig. 1B). These results suggest that CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> Treg cells may play a functional role during the disease progression of MODS.

**Prognostic value of percentage of CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> Treg cells in MODS.** To investigate the possible prognostic value of CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> Treg cells in MODS, a total of 42 patients with MODS were divided into two groups: Survival >15 days, including patients that survived over 15 days in hospital (n=15); and Survival <15 days, including patients that succumbed to MODS within 15 days (n=27). Consistent with our previous results, the percentage of CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> Treg cells was very low in healthy subjects (0.77±0.09%) (Fig. 2). An increased percentage of CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> Treg cells in total lymphocytes was observed in patients with MODS on Day 1. In addition, the

Table I. Ratio of CD4<sup>+</sup>/CD8<sup>+</sup> in the various study cohorts.

Group	n	Percentage of total gated lymphocytes (%)		
		CD3 <sup>+</sup> CD4 <sup>+</sup>	CD3 <sup>+</sup> CD8 <sup>+</sup>	CD4 <sup>+</sup> /CD8 <sup>+</sup>
Healthy subjects	10	37.61±10.56	24.64±6.54	1.55±0.81
Patients with MODS	27	52.43±14.78 <sup>a</sup>	22.50±8.62 <sup>a</sup>	3.33±3.54 <sup>a</sup>

<sup>a</sup>P<0.05 vs. healthy subjects. MODS, multiple organ dysfunction syndrome.

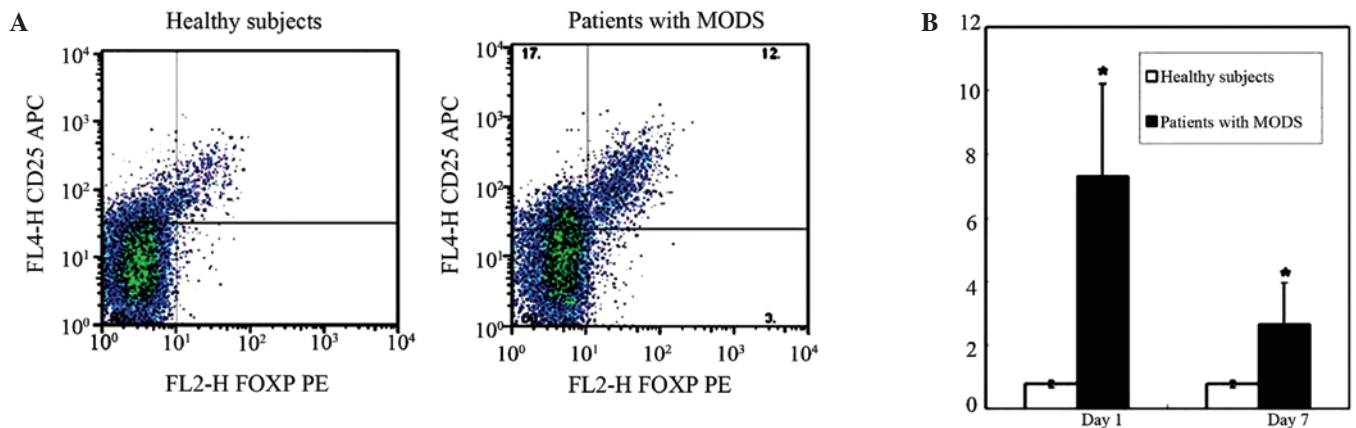


Figure 1. Percentages of CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> Treg cells in healthy subjects and patients with MODS analyzed using flow cytometry. (A) Representative flow cytometry dotplots showing % of CD25<sup>+</sup>Foxp3<sup>+</sup> cells in gated CD4<sup>+</sup> lymphocytes. (B) Composed data from flow cytometry analysis showing the ratio of CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> and CD3<sup>+</sup>CD4<sup>+</sup> T cells in MODS patients determined on days 1 and 7 following admission. \*P<0.05 vs. healthy subjects. MODS, multiple organ dysfunction syndrome; Treg, regulatory T cells.

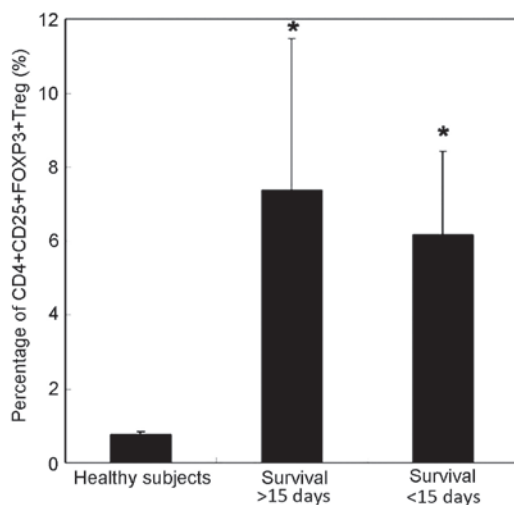


Figure 2. Analysis of the ratio of CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> and CD3<sup>+</sup>CD4<sup>+</sup> T cells in patients that survived or succumbed to multiple organ dysfunction syndrome within 15 days. Percentages of CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> Treg cells in various study cohorts were determined using flow cytometry on Day 1 of admission. \*P<0.05 vs. healthy subjects.

percentage of CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> Treg cells was lower in the Survival <15 days group (6.16±2.25%) compared with the Survival >15 days group (7.37±4.08%); however, the difference did not reach significance (P>0.05). These results indicate that Treg frequency may have no prognostic value in patients with MODS.

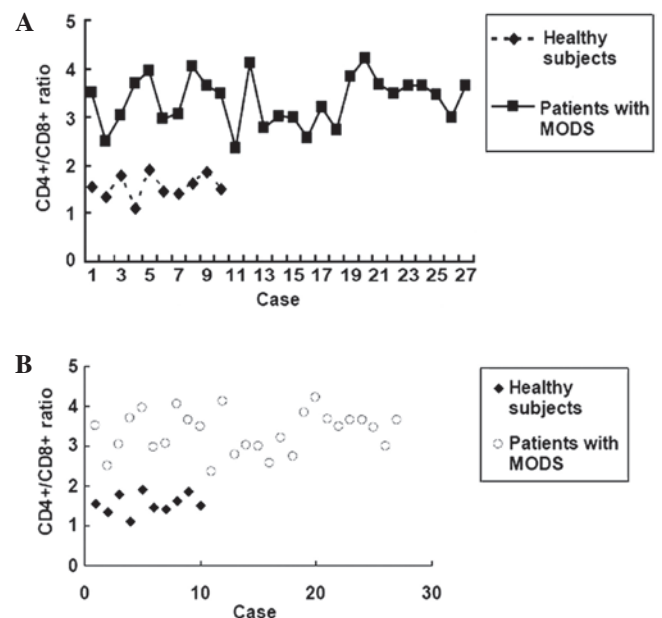


Figure 3. (A and B) Ratio of CD4<sup>+</sup>/CD8<sup>+</sup> in various study cohorts. Ten cases of healthy subjects and 27 cases of patients with MODS were recruited for analyzing the ratio of CD4<sup>+</sup>/CD8<sup>+</sup>. MODS, multiple organ dysfunction syndrome.

*T*-lymphocyte subsets CD4<sup>+</sup> and CD8<sup>+</sup> and the CD4<sup>+</sup>/CD8<sup>+</sup> ratio. The CD4<sup>+</sup>/CD8<sup>+</sup> ratio is a key indicator of immune function. Therefore, the levels of the T-lymphocyte subsets CD4<sup>+</sup> and CD8<sup>+</sup> in peripheral blood from patients with MODS were

Table II. Levels of cytokines in patients with MODS as compared with healthy subjects.

Group	Day	TNF- $\alpha$ (ng/l)	IL-2 (ng/l)	IL-4 (ng/l)	IL-6 (ng/l)	IL-8 (ng/l)	IL-10 (ng/l)	IL-1 $\beta$ (ng/l)
Healthy subjects	1	28.63 $\pm$ 4.51	28.63 $\pm$ 4.51	18.90 $\pm$ 4.11	46.97 $\pm$ 8.16	37.83 $\pm$ 5.61	17.14 $\pm$ 7.76	38.13 $\pm$ 5.14
Patients with MODS	1	108.47 $\pm$ 6.34 <sup>a</sup>	246.38 $\pm$ 19.29 <sup>a</sup>	78.29 $\pm$ 3.78 <sup>a</sup>	774 $\pm$ 87.41 <sup>a</sup>	2654.89 $\pm$ 34.56 <sup>a</sup>	62.62 $\pm$ 6.34 <sup>a</sup>	72.93 $\pm$ 8.50 <sup>a</sup>
Patients with MODS	7	24.21 $\pm$ 3.45 <sup>b</sup>	55.00 $\pm$ 5.88 <sup>b</sup>	18.38 $\pm$ 5.67 <sup>b</sup>	206.42 $\pm$ 18.86 <sup>a,b</sup>	559.83 $\pm$ 56.21 <sup>a,b</sup>	16.55 $\pm$ 4.56 <sup>b</sup>	59.9 $\pm$ 7.23 <sup>a,b</sup>

<sup>a</sup>P<0.05 vs. healthy subjects; <sup>b</sup>P<0.001 vs. patients with MODS determined on day 1. (healthy subjects, n=10; patients with MODS, n=27). MODS, multiple organ dysfunction syndrome; TNF- $\alpha$ , tumor necrosis factor- $\alpha$ ; IL, interleukin.

evaluated at day 1 of administration. As shown in Table I, the percentage of CD4<sup>+</sup> was significantly increased, whereas the percentage of CD8<sup>+</sup> was decreased in MODS patients (n=27) as compared with healthy control subjects (n=10) (P<0.05). Furthermore, the CD4<sup>+</sup>/CD8<sup>+</sup> ratio was calculated, and an elevated CD4<sup>+</sup>/CD8<sup>+</sup> ratio was observed in patients with MODS (3.33 $\pm$ 3.54) as compared with healthy subjects (1.55 $\pm$ 0.81) (Table I and Fig. 3), indicating the elevated immunoactivity in patients with MODS. No significant difference was detected in the CD4<sup>+</sup>/CD8<sup>+</sup> ratio between the survival >15 days group and the survival <15 days group (P>0.05).

*Cytokines in the blood of patients with MODS.* Serum blood concentrations of cytokines, including TNF- $\alpha$ , IL-1 $\beta$ , IL-2, IL-4, IL-6, IL-8 and IL-10, were determined in the various patient groups. As shown in Table II, compared with the healthy subjects, the levels of all seven cytokines were elevated in patients with MODS on Day 1. On Day 7, levels of TNF- $\alpha$ , IL-2, IL-4 and IL-10 were gradually reduced to similar levels as the healthy controls, while IL-6, IL-8 and IL-1 $\beta$  levels were remained at significantly higher levels compared with the healthy controls (P<0.001) (Table I). No significant difference was detection in the levels of these cytokines between the survived and deceased patients (P>0.05).

## Discussion

MODS has been recognized as a severe human disease, with massive inflammation and a high mortality rate (1,2). The cellular mechanisms underlying the development and progression of MODS are complicated, and pathological processes including uncontrolled inflammation, systemic inflammation, imbalanced immune activity, tissue hypoxia, dysregulated apoptosis, microvascular coagulopathy and endothelial activation have been suggested to lead to the clinical manifestations of this disease (18). Among all these processes, an uncontrolled inflammatory response is known to contribute to the rapidly progressive development of MODS (3). The dynamic balance between pro- and anti-inflammatory cytokines is critical to maintaining normal function of the immune system (19). However, during the progression of MODS, an imbalanced generation of anti-inflammatory cytokines (including IL-4, IL-10, IL-13, TGF- $\beta$  and soluble TNF- $\alpha$  receptor) and proinflammatory cytokines (including IL-1 $\beta$ , IL-6, IL-8 and TNF- $\alpha$ ) has become the key event (19).

Previous studies indicated that the naturally arising CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> Treg cells, the majority of which are produced by the normal thymus as a functionally mature T-cell subpopulation, are crucially involved in the maintenance of physiological and pathological immune responses (20,21). In this study, a marked elevation in CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> Treg cells was detected in patients with MODS on Day 1 of admission as compared with the healthy subjects (Fig. 1), demonstrating that CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> Treg cells may be involved in the development of MODS.

Furthermore, alterations in the levels of various pro- and anti-inflammatory cytokines were observed in the present study. Increased concentrations of proinflammatory cytokines, such as IL-2, TNF- $\alpha$ , IL-1 $\beta$ , IL-6 and IL-8, were found in the early



stages of this disease (Table II), suggesting that inflammation plays a dominant role in the progression of MODS. Similarly, the levels of the anti-inflammatory cytokines IL-4 and IL-10 in patients with MODS were increased on Day 1 of admission, which was parallel to the increase in CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> Treg cells. It is possible that CD4<sup>+</sup>CD25<sup>+</sup>FOXP3<sup>+</sup> Treg cells protect against MODS via the secretion of anti-inflammatory cytokines, thereby inhibiting the exaggerated inflammation. The present results demonstrated that during the early stages of MODS, the production of proinflammatory and anti-inflammatory cytokines was elevated, and these cytokines interact with each other, playing pivotal roles in the regulation of the disease process.

The CD4<sup>+</sup>/CD8<sup>+</sup> ratio is widely accepted as an indicator of immune activity (22), and also provides valuable prognostic information for patients with certain type of tumor, such as renal cell carcinoma (23) and metastatic melanoma (24). In the present study, an increased CD4<sup>+</sup>/CD8<sup>+</sup> ratio was detected in patients with MODS, indicating elevated immunoactivity in these patients.

The results suggest that the percentage of CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> Treg cells and the levels of pro- and anti-inflammatory cytokines may be used as a biomarker for evaluating the status of disease, in addition to prognosis. Furthermore, the suppression of the secretion of proinflammatory cytokines, such as TNF- $\alpha$ , IL-2, IL-6 and IL-8, may provide a valuable tool for the clinical therapy of MODS.

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