

Original Article

Changes of cytokines levels after decreasing regulatory T cells activity during sepsis

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Received November 22, 2016; Accepted January 7, 2017; Epub March 1, 2017; Published March 15, 2017

Abstract: Background: Regulatory T cells have unique immunological characteristics compared with other regulatory or suppressor T cells, immunosuppressive cytokines produced by Tregs, including transforming growth factor (TGF)- β and IL-10, might also play a role in the suppression of effector T cells. To investigate in vivo and in vitro the changes of cytokines levels after decreasing regulatory T cells activity in mouse after sepsis. Material/Methods: In this study, Abdominal hypertension and peritonitis-induced sepsis was affected by cecal ligation and puncture surgery (CLP), PC61 and anti-Transforming growth factor beta (TGF)- β antibody were used to decrease CD4⁺CD25⁺ Treg cell levels and inhibit TGF- β activity in vivo and vitro, respectively. Interleukin (IL)-2, IL-4, IL-10, interferon (IFN)- γ , and TGF- β in the peripheral blood and in the culture supernatants were determined with enzyme-linked immunosorbent assay (ELISA) kits. Results: We found that mortality rate was significantly decreased in CLP animals treated with PC61 group compared with CLP group. Meanwhile, administration of PC61 or anti-TGF- β antibody, IL-2 and IFN- γ levels were significantly increased in comparison to the CLP induced sepsis group, thus levels of IL-4, IL-10 and TGF- β markedly decreased. Conclusions: These findings suggest that decreasing regulatory T cells activity might affect cytokine levels in mouse after sepsis, possibly through TGF- β signaling.

Keywords: Regulatory T cells, sepsis, cytokines, transforming growth factor- β

Introduction

To date, sepsis is a complex clinical syndrome that results in both the widespread activation and dysfunction of the innate and adaptive branches of the immune system. CD4⁺CD25⁺ Tregs, as a class of mature T cell subsets with immune function, play important roles in the maintenance of immunologic self-tolerance and in down-regulation of various immune responses [1-3].

Recently, there has been an increasing interest in investigating the biology of CD4⁺CD25⁺ Tregs and their role as well as regulatory mechanism in the immune functions of T lymphocyte [4]. Tregs have unique immunological characteristics compared with other regulatory or suppressor T cells, immunosuppressive cytokines produced by Tregs, including transforming growth factor (TGF)- β , and IL-10, might also play a role in the suppression of effector T cells by Tregs [5-8]. TGF- β 1 was proved to possess the distinct property in normal tissue homeostasis by

regulating diverse functions such as cellular differentiation, apoptosis, cell-cycle arrest, and cellular migration, superfamily consists of structurally and functionally related cytokines that signal through a pair of transmembrane serine-threonine kinase receptors known as the type I and type II receptors [9-11]. Other studies suggested that TGF- β 1 signal could be involved the apoptosis of CD4⁺CD25⁺ T cells promoted by CD4⁺CD25⁺ Tregs, therefore inhibition of TGF- β 1 expression may provide a novel strategy for the improvement of host immunosuppression following sepsis [12]. Therefore, in the present study, we investigated the changes of cytokine levels in mouse after decreasing regulatory T cells activity and inhibiting TGF- β activity during sepsis.

Materials and methods

Medium and reagents

Thiazolyl blue (MTT) and Triton X-100 were purchased from Sigma, St. Louis, MO. RPMI 1640,

The changes of cytokines levels during sepsis

fetal calf serum (FCS), glutamine, penicillin, streptomycin, and HEPES were purchased from TianRunShanda Biotech Co. LTD, Beijing, China. Anti-mouse TGF β 1^{m+} was purchased from Sigma, St. Louis, MO. PC-61 and HRPN were purchased from BioXcell, West Lebanon, NH.

Animal's cecal ligation and puncture (CLP) model

All experimental manipulations (Male C57BL/6 mice) were purchased from Shandong University Animal Ethical Committee (Jinan, China), and were 8-10 weeks old at the time of entry into the study. All mice were housed in separate cages in a temperature-controlled room with a 12-hour light-dark cycle. Polymicrobial sepsis was induced by the cecal ligation and puncture (CLP) procedure described by Wichterman and colleagues [13]. Briefly, after being anesthetized, the mice were placed in supine position and their abdomens shaved. An abdominal midline incision of 1 cm was made to expose the cecum. According to Rittirsch et al, the cecum was ligated at the middle and punctured twice with a 21-gauge (0.723-mm) needle to induce a moderate severity sepsis. Sham-operated mice underwent the same laparotomy procedure with the exception of the ligation and perforation.

Experimental design

One hundred and sixty-five mice were used for both in vivo and in vitro experiments, respectively. In vivo study, 75 mice were randomly divided into 5 groups as follows: normal control group, sham group, CLP group, CLP with PC61 treatment group, and CLP with HRPN treatment group, and each of these groups consisted of 15 mice. PC61 (the specific antagonist for Tregs) and HRPN (rat immunoglobulin G1) were intraperitoneally injected to mice 5 days before CLP operation, respectively in CLP+ PC61 group and in CLP+HRPN group. In the in vitro study, 90 mice were randomly divided into 6 groups, namely the normal control group (15 mice), sham group (15 mice) and CLP-24-h group (15 mice), control treated with anti-TGF- β antibody group (15 mice), sham treated with anti-TGF- β antibody group (15 mice), CLP-24-h treated with anti-TGF- β antibody group (15 mice), and finally, CD4⁺CD25⁺ Tregs were isolated in all mice.

Cell isolation and purification

Spleens were obtained from healthy BALB/C mice, and they were teased in 5 ml RPMI 1640. Mononuclear cells were separated using Ficoll-Paque density gradient centrifugation, and CD4⁺CD25⁺ Tregs were then purified. Non-CD4⁺ T cells were stained using biotin antibody (Ab) cocktail (10 μ l/10⁷ total cells) and incubated for 15 min at 4°C. They were then magnetically labeled with antibiotin microbeads (20 μ l/10⁷ total cells) and CD25-phycoerythrin (PE) Ab (10 μ l/10⁷ total cells), incubated for 10 min at 4°C (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany), and depleted over a magnetic cell sorting (MACS) column (purity of purified CD4⁺ T cells >90%). CD25⁺ and CD25⁻CD4⁺ T cells were further isolated by MACS enriched with anti-CD25 PE microbeads (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany). Purity of CD4⁺CD25⁺ Tregs was greater than 98% as determined by flow cytometry.

Cytokine measurements with ELISA

To analyze the secretion of cytokines, IL-2, IL-4, IL-10, IFN- γ , and TGF- β in the peripheral blood and in culture supernatants were determined. The levels of IL-2, IL-4, IL-10, IFN- γ , and TGF- β were measured using commercially available ELISA kits according to the manufacturer's instructions.

Statistical analysis

All data in this study were represented as mean \pm standard deviation (SD). Statistical evaluation of continuous data was examined by one-way ANOVA. The individual group means were then compared with Student paired t test. Statistical software SPSS 17.0 was applied to perform these statistical analysis. A *P* value of 0.05 or less was considered to indicate statistical significance.

Results

The survival rate of the mice subjected to thermal injury

Mice in CLP group performed a series of manifestations of septic symptoms, and death occurred after 12 h. The mortality of CLP at 72 h was about 40% (**Table 1**), and the immune

The changes of cytokines levels during sepsis

Table 1. Postoperative survival rate of each group

| GROUP | Survival rate |
|----------|---------------|
| CLP | 60% |
| CLP+PC61 | 93.3% |
| CLP+HRPN | 60% |

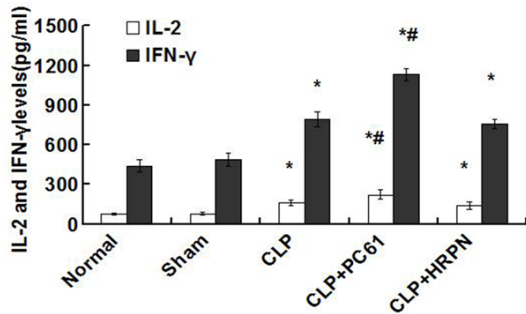


Figure 1. IL-2 and IFN- γ production in vivo. Enzyme-linked immunosorbent assay analysis for IL-2 and IFN- γ was used in the present study (n=15, in each group). Levels of IL-2 and IFN- γ in CLP+PC61 group were much higher than that in CLP group. Statistical significance: *P<0.05 as CLP group versus sham group, #P<0.01 as PC61 group versus CLP group.

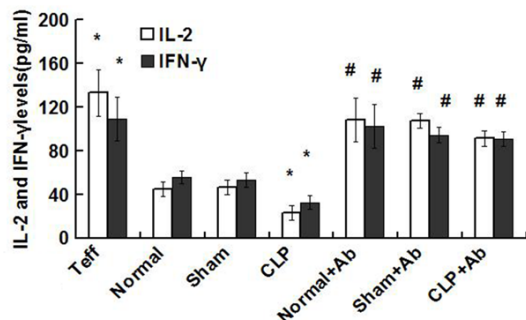


Figure 2. IL-2 and IFN- γ production in vitro. Enzyme-linked immunosorbent assay analysis for IL-2 and IFN- γ was used in the present study (n=15, in each group). In vitro, treatment with anti-TGF- β antibody, the levels of IL-2 and IFN- γ were markedly increased in comparison to without anti-TGF- β antibody treatment group. Statistical significance: *P<0.05 as CLP group versus sham group, #P<0.01 as anti-TGF- β antibody group versus without anti-TGF- β antibody treatment group.

dysfunction occurred 24 h after CLP, indicating that the sepsis model was stable and could be used for experiments.

IL-2 and IFN- γ production

IL-2 is a potent T-cell growth factor that acts upon itself in an autocrine fashion, level of IL-2 in the peripheral blood or in culture superna-

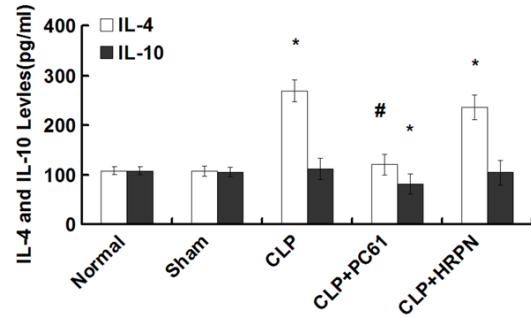


Figure 3. IL-10 and IL-4 levels in vivo. Enzyme-linked immunosorbent assay analysis for IL-10 and IL-4 was used in the present study (n=15, in each group). IL-4, IL-10 and TGF- β in peripheral blood in CLP group after 24 h were higher than that of normal group and sham group, while Levels of IL-4 and IL-10 in CLP+PC61 group showed a different magnitude of decline in comparison to CLP group. Statistical significance: *P<0.05 as CLP group versus sham group, #P<0.01 as PC61 group versus CLP group.

tants of CD4⁺CD25⁻ T cells were measured by ELISA. As shown in **Figure 1**, IL-2 and IFN- γ levels in the CLP group were significantly higher than that of normal group and sham group (P<0.01), while the levels of IL-2 and IFN- γ in CLP+PC61 group were much higher than that in CLP group (P<0.01). In vitro, IL-2 and IFN- γ levels in culture supernatants of CD4⁺CD25⁻ T cells was decreased after CLP, thus treatment with anti-TGF- β antibody, the levels of IL-2 and IFN- γ were markedly increased in comparison to without anti-TGF- β antibody treatment group (**Figure 2**, P<0.01).

IL-10, IL-4 and TGF- β production after sepsis

To determine the levels of IL-10, IL-4 and TGF- β , both *in vivo* and *in vitro* experiments, changes in levels of IL-10, IL-4 and TGF- β in both normal group and sham group showed no statistically significant difference. It was shown in **Figure 2**, IL-4, IL-10, TGF- β in peripheral blood in CLP group after 24 h were higher than that of normal group and sham group (**Figure 3**, P<0.01), only IL-4 level increased significantly in CLP group, compared with normal group and sham group (P<0.01), while the levels of IL-10 and TGF- β were not improved obviously. Levels of IL-4, IL-10 and TGF- β in CLP+PC61 group showed a different magnitude of decline in comparison to CLP group (P<0.05, or P<0.01). Further, As shown in **Figure 4**, levels of IL-4, IL-10 and TGF- β in culture supernatants of CD4⁺CD25⁻ T cells was increased after CLP, treatment with anti-TGF- β antibody, the levels

The changes of cytokines levels during sepsis

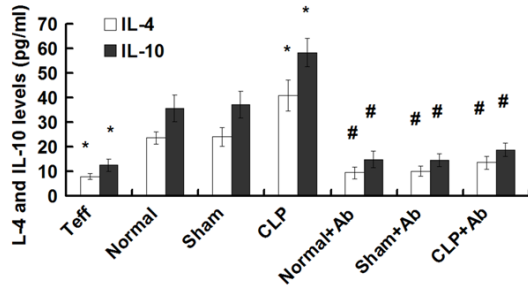


Figure 4. IL-10 and IL-4 levels in vitro. Enzyme-linked immunosorbent assay analysis for IL-10 and IL-4 was used in the present study (n=15, in each group). Levels of IL-4 and IL-10 in culture supernatants of CD4⁺CD25⁻ T cells was increased after CLP, treatment with anti-TGF- β antibody, the levels of L-4 and IL-10 in CLP group were markedly decreased in comparison to without anti-TGF- β antibody treatment group. Statistical significance: *P<0.05 as CLP group versus sham group, #P<0.01 as anti-TGF- β antibody group versus without anti-TGF- β antibody treatment group.

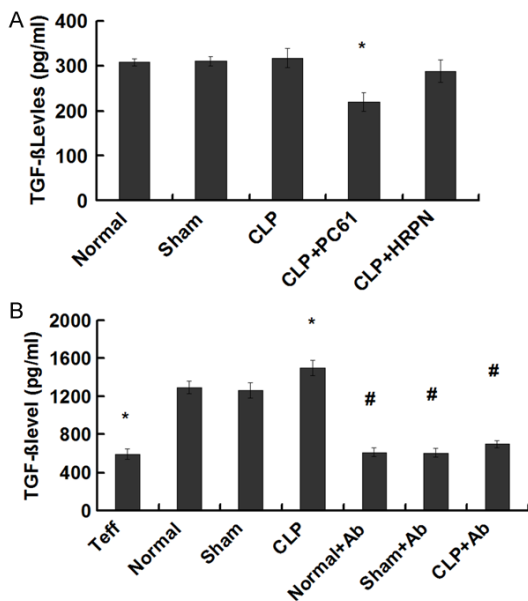


Figure 5. TGF- β levels in vitro and in vivo. Enzyme-linked immunosorbent assay analysis for TGF- β was used in the present study (n=15, in each group). In vivo, levels of TGF- β in CLP+PC61 group showed decline in comparison to CLP group. In vitro, levels of TGF- β in culture supernatants of CD4⁺CD25⁻ T cells was increased after CLP, treatment with anti-TGF- β antibody, TGF- β levels in CLP group were markedly decreased in comparison to without anti-TGF- β antibody treatment group. Statistical significance: *P<0.05 as CLP group versus sham group, #P<0.01 as PC61 group versus CLP group, or anti-TGF- β antibody group versus without anti-TGF- β antibody treatment group.

of L-4, IL-10 and TGF- β in CLP group were markedly decreased in comparison to without anti-TGF- β antibody treatment group (Figure 5, P<0.01).

Discussion

Sepsis represents a complex clinical morbidity that results from a harmful or devastating host response to infection. It has been proposed that Tregs play a central role in the maintenance of immune tolerance and immune balance in the peripheral lymphatic system [14-16]. Recently, TGF- β 1, a pleiotropic cytokine secreted by Th2 or Tregs, was found to produce both membrane-bound (TGF- β 1^{m+}) and secreted TGF- β 1 under certain conditions. Experiments in animal models have demonstrated that CD4⁺CD25⁺ Tregs could suppress the proliferation of CD4⁺CD25⁻ T cells via cell surface TGF- β 1^{m+} in a cell contact dependent fashion [17, 18]. Therefore, we would expect that decreasing regulatory T cells activity might affect cytokine levels in mice after sepsis, through TGF- β signaling.

Because IL-2 is a key regulator of the immune response secreted by activated T lymphocyte, and it is essential to activate T lymphocyte proliferation [19, 20]. A significant up-regulation in IL-2 production was observed in the peripheral blood from CLP mice compared with those from sham-injured mice. However, the IL-2 levels in CLP+PC61 group was much higher than that in CLP group *in vivo*. In vitro, treatment with anti-TGF- β antibody, the levels of IL-2 were markedly increased in comparison to without anti-TGF- β antibody treatment group. Therefore, decreasing CD4⁺CD25⁺ Treg cell levels or inhibiting TGF- β activity appeared to promote IL-2 secretion in the setting of acute insult and further modulate activation of T lymphocytes.

It was shown that proliferation of T lymphocytes was suppressed and modulation of Th1 as well as Th2 was shifted in sepsis [21, 22]. Indeed, in animal models of injury, the release of IL-2 and IFN- γ produced by Th1 and IL-4 produced by Th2 were altered. In the present study, IFN- γ levels in the CLP group were significantly elevated (P<0.01), IL-4 levels in the CLP group were significantly increased, levels of IFN- γ in CLP+PC61 group were much higher than that in CLP group, IL-4 level in CLP+PC61 group

The changes of cytokines levels during sepsis

showed decreased in comparison to CLP group. Further, IFN- γ levels in culture supernatants of CD4⁺CD25⁻ T cells was decreased after CLP, IL-4 levels was increased, thus treatment with anti-TGF- β antibody, the levels of IFN- γ were markedly increased, IL-4 levels were markedly decreased in comparison to without anti-TGF- β antibody treatment group. In this study, our data revealed that the polarization of splenic T cells could be affected by treatment with PC61 or anti-TGF- β antibody both in vivo and in vitro.

Immune suppressive cytokines including IL-10 and TGF- β are critically involved in the development of tolerance by Tregs [23-25]. In this study, both IL-10 and TGF- β levels in supernatants of naturally occurring CD4⁺CD25⁺ Tregs treated with anti-TGF- β antibody were significantly decreased as compared with those production under normal conditions. In vivo, IL-10, TGF- β in peripheral blood in CLP group after 24 h were significantly increased in comparison to normal group and sham group. Treated with PC61, IL-10, TGF- β in CLP group showed a different magnitude of decline in comparison to CLP group. Moreover, treatment with anti-TGF- β antibody, the levels of IL-10 and TGF- β in CLP group were markedly decreased in comparison to without anti-TGF- β antibody treatment group. Furthermore, the different time point after CLP would be studied.

In conclusion, based on our *in vivo* and *in vitro* study, it is demonstrated cytokine levels would be changed after decreasing regulatory T cells activity in mice after CLP induced sepsis, and signals mediated by TGF- β 1 might be critically involved in this process, thereby contributing to the development of immunosuppressive state following septic complications.

Disclosure of conflict of interest

None.

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