

## Original Article

# Adenosine promotes Foxp3 expression in Treg cells in sepsis model by activating JNK/AP-1 pathway

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Received February 29, 2016; Accepted April 1, 2016; Epub May 15, 2016; Published May 30, 2016

**Abstract:** Objectives: Forkhead/winged helix transcription factor p3 (Foxp3) increases in CD4<sup>+</sup>CD25<sup>+</sup>Treg cells during sepsis; however, related mechanisms are unclear. Our study aimed to explore the possible molecular mechanisms of high expression of Foxp3 in Treg cells during sepsis. Methods: Sepsis was induced by cecal ligation and puncture (CLP) method. CD4<sup>+</sup>CD25<sup>+</sup>Treg cells were isolated from peripheral blood and identified by flow cytometry (FCM). Treg cells were cultured with or without adenosine, adenosine agonist, adenosine antagonist, SMAD family member 3 (Smad3) agonist (transforming growth factor (TGF)-β1), or C-Jun N-Terminal Kinase (JNK) inhibitor. Expression levels of Foxp3 and activator protein 1 (AP-1) were determined. The binding of c-Fos or c-Jun to the Foxp3 promoter was then evaluated by the chromatin immunoprecipitation (ChIP) assay and quantified by quantitative real-time PCR (qRT-PCR). The mRNA and protein levels of Foxp3 were determined after transfection with siRNA against c-Fos, Fra-2, c-Jun or JunD. Results: Pharmacological inhibition of both adenosine and JNK reduced Foxp3 protein levels. JNK/AP-1 activation was involved in increased levels of Foxp3 protein in CD4<sup>+</sup>CD25<sup>+</sup>Treg cells. AP-1 regulated activity of Foxp3 promoter in Treg cells, and the induction of c-Fos or c-Jun activity leads to elevated transcription of Foxp3 gene. Knockdown of c-Fos, Fra-2, c-Jun, or JunD levels also reduced Foxp3 expression. Conclusion: We confirm that adenosine plays significant roles in the high expression of Foxp3. Adenosine promotes Foxp3 expression in Treg cells during sepsis via JNK/AP-1 pathway.

**Keywords:** Sepsis, adenosine, Foxp3, JNK/AP-1 pathway, regulatory T cells

## Introduction

Sepsis is defined as a systemic inflammatory response syndrome (SIRS) caused by bacterial, fungal or viral origin infection [1]. It is one of the most common reasons for death in critically ill-patients despite significant progress have been made in antibiotic and other supportive therapies [2]. Sepsis still represents a main challenge in treatment. It has been well demonstrated that sepsis progresses when the initial appropriate inflammatory response becomes extreme, leading to extensive and intense hyper inflammatory response [3, 4]. However, this shift often results in an immunosuppressive state, which has long been regarded as a key factor in late mortality in patients with sepsis [5].

In recent years, adaptive immune system has been paid a great attention on the develop-

ment of sepsis [6, 7]. Among all the T lymphocyte subpopulations, regulatory T cells (Tregs) CD4<sup>+</sup>CD25<sup>+</sup> have been shown to be critical in maintaining of immunologic homeostasis and tolerance [8, 9]. These cells exhibit a pronounced antiinflammatory activity via directly inhibiting other immune cells and producing high levels of soluble CD25 and other inflammatory factors [10-12]. But their development and function are regulated by forkhead/winged helix transcription factor p3 (Foxp3) that is specifically expressed on CD4<sup>+</sup>CD25<sup>+</sup>Treg cells. Foxp3 has been considered as the control gene for the differentiation and function of Treg cells, as well as regulation of intracellular molecules related to effector T cell [11, 13]. There is accumulating evidence indicating that markedly increased levels of Tregs in patients and animals during the early onset of sepsis [14-16]. Besides, expression levels of Foxp3<sup>+</sup> are also increased in CD4<sup>+</sup>CD25<sup>+</sup>Tregs cells in patients with sepsis

[17]. However, rare little information is available on the contribution of mechanism of high expression of Foxp3<sup>+</sup> in CD4<sup>+</sup>CD25<sup>+</sup>Treg cells in sepsis patients.

In this study, a murine cecal ligation and puncture (CLP) model was used to explore the possible mechanism of high expression of Foxp3 in CD4<sup>+</sup>CD25<sup>+</sup>Treg cells. Better and deeper understanding of knowledge and management processes in sepsis may contribute to tailored therapeutic approaches for sepsis.

### Materials and methods

#### *Experimental animals*

Twenty adult female Sprague-Dawley (SD) rats (8-12 weeks old, weighing 200-250 g) were purchased from the Shanghai SLAC Laboratory Animal Co., Ltd. (Chinese Academy of Sciences, Shanghai, China). The rats were kept in a conventional, temperature-controlled (22 ± 0.5°C) facility with a 14:10-hour light-dark cycle and fed with food and water *ad libitum*. This study was performed according to the Guide for the Care and Use of Medical Laboratory Animals (Ministry of Health, PR China, 1998) and the guidelines of the Laboratory Animal Ethical Commission of our university.

#### *Induction of sepsis model*

Polymicrobial sepsis model was induced using a CLP method according to a previous study [18], but with a minor modifications. All the rats were anesthetized by thiopental (25 mg/kg). The abdomen was shaven, and a midline laparotomy was performed. Thereafter, the cecum was ligated just below the ileocecal valve and punctured twice through the cecum distal to the point of ligation by using a 12 G needle. The cecum was put into the abdomen and the incision was closed. After 24 h of CLP, the rats were sacrificed by cervical dislocation under deep anesthesia and blood was collected. The samples were anticoagulated with ethylenediaminetetraacetic acid.

#### *Flow cytometry (FCM) analysis*

After the samples were harvested, lymphocytes were separated by centrifugation on a lymphocyte separation medium. The lymphocytes were calculated, and the cell concentration was

adjusted to 1×10<sup>6</sup>/mL. The α CD4 (RM4-5) fluochrome-labeled antibody (BD Biosciences, San Diego, CA), antibody directed against CD25 (clone 7D4) (eBioscience, San Diego, CA), and intranuclear phycoerythrin-conjugated α Foxp3 antibodies (3G3) (eBioscience, San Diego, CA) were separately added to the diluted lymphocytes according to the manufacturer's instructions. FCM was performed by a FACS Calibur flow cytometer (Becton Dickinson, San Jose, CA), and CellQuest software (Becton Dickinson, Bedford, MA) was employed for data analysis.

#### *Drugs*

Treg cells was cultured with or without the following inhibitor or agonist for 24 h: SMAD family member 3 (Smad3) agonist, transforming growth factor (TGF)-β1 (1 ng/ml) (R&D Systems); C-Jun N-Terminal Kinase (JNK) inhibitor (SP60-0125, 10 μM) (LC laboratories); Adenosine (10 μM) (R&D Systems); Selective A2A receptor adenosine agonist (CGS21680, 100 nM) (LC laboratories); Selective A2A receptor adenosine antagonist, 8-(3-chlorostyryl) caffeine (CSC, 500 nM) (Sigma-Aldrich).

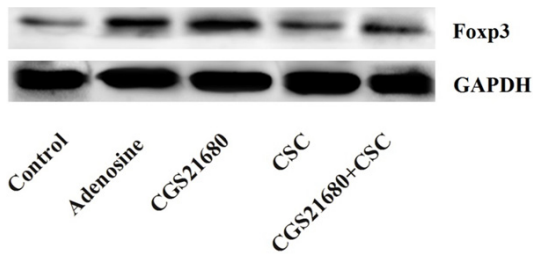
#### *Small interference RNA (siRNA)*

Cells were plated on 60-mm dish. After 24 hours of incubation, siRNA against c-Fos, Fra-2, c-Jun, JunD or control (nontargeting) siRNA were transfected into cells at a final density of 50 nM using Lipofectamine 2000 (Invitrogen, USA) according to the manufacturer's guidelines. The si-c-Fos, si-Fra-2, si-c-Jun and si-JunD were all purchased from GenaPharma Shanghai, China. At 48 h after the transfection, the cells were harvested for further protein expression.

#### *Western blotting*

Protein was extracted from the cells and determined using a BCA assay kit (Perbio, Cramlington, UK). The samples (20 μg per lane) were separated on a 10-12% sodium dodecyl sulfate (SDS)-polyacrylamide gel and blotted onto polyvinylidene difluoride (PVDF) membranes (Bedford, MA, USA), blocked in 5% defatted milk powder in phosphate buffer saline (PBS) and incubated overnight at 4°C with the following antibodies: anti-Foxp3 antibody, anti-pJNK antibody, anti-JNK antibody, anti-p-Smad3 antibody, anti-Smad3 antibody, anti-p-c-Fos antibody, anti-c-Fos antibody, anti-

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**Figure 1.** Foxp3 expression in the CD4<sup>+</sup>CD25<sup>+</sup>Treg cells after administration of adenosine, adenosine agonist (CGS21680) or adenosine antagonist (CSC). Treg, regulatory T cells; Foxp3, forkhead/winged helix transcription factor p3.

FosB antibody, anti-p-Fra-1 antibody, anti-Fra-1 antibody, anti-Fra-2 antibody, anti-p-c-Jun antibody, anti-c-Jun antibody, anti-p-JunB antibody, anti-JunB antibody, anti-JunD antibody, and GAPDH. All the antibodies were purchased from Cell Signaling Technologies (Beverly, MA). Thereafter, the membranes were incubated with the appropriate horseradish peroxidase-conjugated secondary antibodies for 2 h at room temperature. Enhanced chemiluminescence and densitometric analysis were finally performed.

### Chromatin immunoprecipitation (ChIP) assay

A chromatin immunoprecipitation (ChIP) assay for c-Fos and c-Jun binding to the Foxp3 site was undertaken using Treg cells and a Simple ChIP Enzymatic Chromatin IP Kit (Cell Signaling Technology) following the manufacturer's protocol. ChIP were performed with cross-linked chromatin starved overnight, and 10  $\mu$ l of c-Fos monoclonal antibody (mAb), c-Jun mAb or 2  $\mu$ l of normal rabbit IgG was performed using protein A-Sepharose beads. Precipitated DNA was quantified by quantitative real-time PCR (qRT-PCR) with Foxp3-specific primers using an ABI 7700Prism (Applied Biosystems) with the SYBR Green PCR core reagent kit. The amount of immunoprecipitate, expressed as the signal relative to the total amount of input chromatin, was determined.

### qRT-PCR

After 48 h of transfection with si-c-Fos, si-Fra-2, si-c-Jun or si-JunD, mRNA levels of Foxp3 were analyzed using qRT-PCR. Total RNA was extracted using a Trizol reagent (Invitrogen Corp., Carlsbad, CA) according to the manufacturer's

instructions. First strand complementary DNA (cDNA) was synthesized using the Superscript III reverse transcriptase (Invitrogen). The mRNAs levels were assessed using SYBR green-based qRT-PCR. GAPDH gene was used as a reference control. The primer sequences were as follows: 5'-primer-TTCCTGGCATTTC-CATCC-3' and reverse, 5'-primer-GCAGGCAGAG-ACACCATTCT-3'.

### Statistical analysis

All experiments were repeated in triplicate with similar results. The collected data were represented as the mean  $\pm$  standard deviation (SD). Statistical analyses were performed using statistical package for social science (SPSS, version 18.0, Chicago, IL) software. One-way analysis of variance (ANOVA) was used to calculate the *P*-values. A statistical significance was defined when *P* < 0.05.

## Results

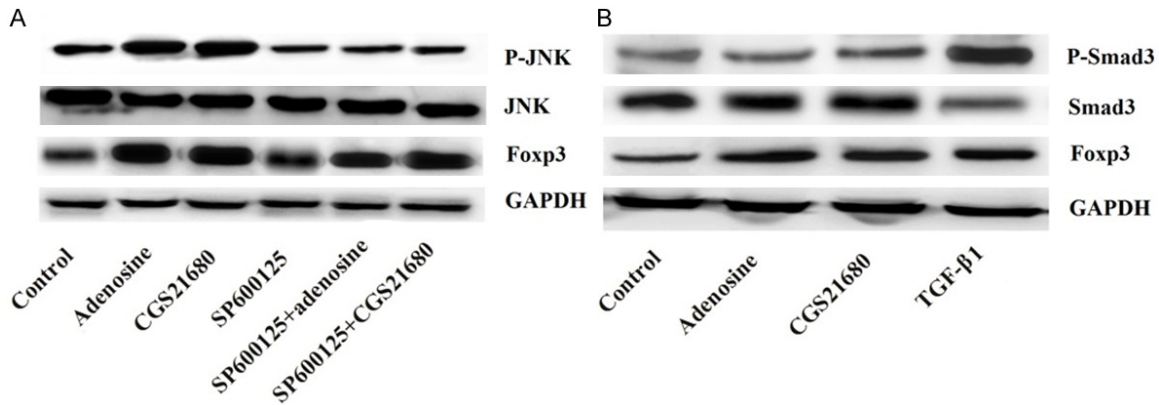
### Foxp3 was promoted by adenosine

To explore the effect of adenosine on the expression of Foxp3, we examined the expression of Foxp3 after administration of adenosine, selective A2A receptor adenosine agonist (CGS21680) or selective A2A receptor adenosine antagonist (CSC) to the CD4<sup>+</sup>CD25<sup>+</sup>Treg cells. As shown in **Figure 1**, the expression of Foxp3 was significantly increased by adenosine compared to the control group (*P* < 0.05). In addition, the levels of Foxp3 were higher in the CGS21680 group, but lower in the CSC group, indicating that adenosine promoted the expression of Foxp3. However, there were no significant differences between the control group and the CGS21680+CSC group, suggesting that the effect of adenosine on the expression of Foxp3 was offset by simultaneous application of agonist and antagonist.

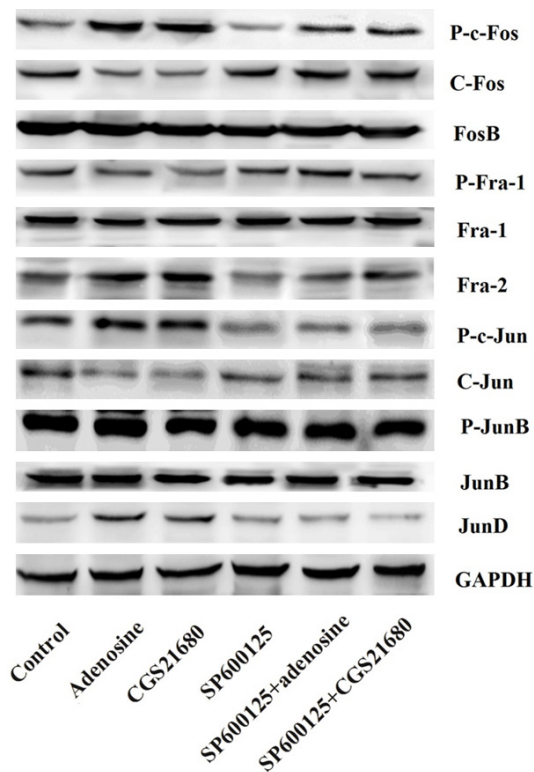
### Foxp3 was associated with JNK activation

To investigate the possible mechanism of adenosine on the expression of Foxp3, the relationships between the expression of Foxp3 and the activation of JNK or Smad3 were investigated. We found that both the levels of Foxp3 and p-JNK were markedly elevated by adenosine or agonist of adenosine (CGS21680), but were decreased by JNK inhibitor (SP600125) com-

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**Figure 2.** Expressions of Foxp3 are associated with JNK but not Smad3 activation. A. Expressions of Foxp3 are associated with JNK activation; B. Expressions of Foxp3 are not associated with Smad3 activation. Foxp3, forkhead/winged helix transcription factor p3; JNK, C-Jun N-Terminal Kinase; Smad3, SMAD family member 3.



**Figure 3.** Expressions of Foxp3 is involved in activation of the JNK/AP-1 pathway. Foxp3, forkhead/winged helix transcription factor p3; C-Jun N-Terminal Kinase; AP-1, activator protein 1 JNK.

pared with the control group ( $P < 0.05$ ). Moreover, simultaneous application of SP600125 with adenosine or agonist of adenosine had no effect on the levels of p-JNK compared with only application of SP600125, but could significantly increase the levels of Foxp3. But the lev-

els of Foxp3 were still lower than that only application of adenosine or agonist of adenosine (**Figure 2A**). Although the levels of Foxp3 and p-Smad3 were markedly increased by Smad3 agonist (TGF- $\beta$ 1) ( $P < 0.05$ ), the levels of p-Smad3 were not affected by adenosine or its agonist (**Figure 2B**). These results demonstrated that the expression of Foxp3 was associated with the activation of JNK.

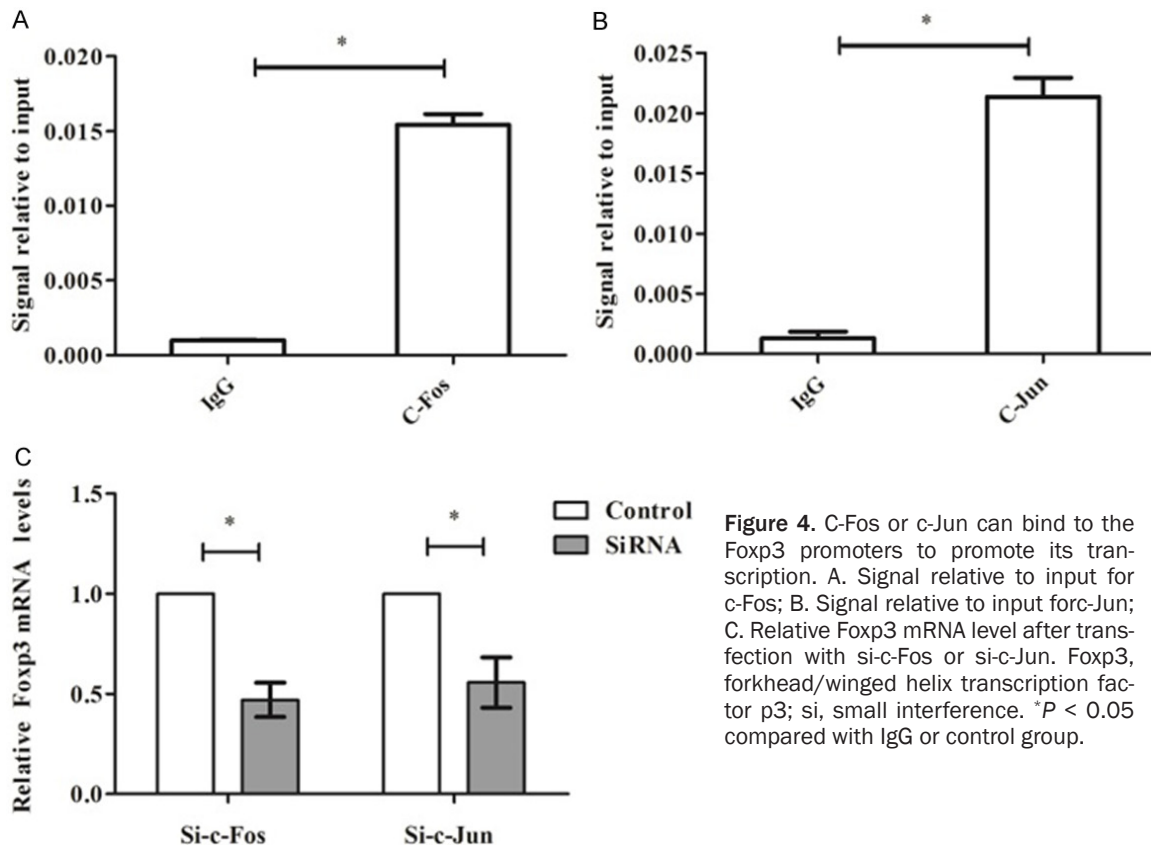
### *Foxp3 was involved in activation of the JNK/activator protein 1 (AP-1) pathway*

To characterize the involved signaling pathway of the effect of adenosine on the expression of Foxp3, we studied the effect of adenosine on activation of AP-1. Western blotting analysis revealed that no significant differences were found in the levels of FosB, Fra-1, p-JunB, and JunB among the different groups. However, SP600125 was capable of significantly increasing c-Fos, p-Fra-1, and c-Jun expression but suppressing p-c-Fos, Fra-2, p-c-Jun, and Jun-D expression ( $P < 0.05$ ) (**Figure 3**). In addition, these abilities were reversed by adenosine or its agonist. Overall, these results suggested that the JNK pathway and the transcription factor AP-1 contributed to the effect of adenosine on the expression of Foxp3.

### *Both c-Fos and c-Jun can bind to the Foxp3 promoters to promote its transcription*

The binding of c-Fos and c-Jun to the Foxp3 promoter was identified by the ChIP assay, as well as the effect of si-c-Fos or si-c-Jun on expression of Foxp3. Sonicated chromatin was pre-

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**Figure 4.** C-Fos or c-Jun can bind to the Foxp3 promoters to promote its transcription. A. Signal relative to input for c-Fos; B. Signal relative to input for c-Jun; C. Relative Foxp3 mRNA level after transfection with si-c-Fos or si-c-Jun. Foxp3, forkhead/winged helix transcription factor p3; si, small interference. \* $P < 0.05$  compared with IgG or control group.

precipitated with antibodies against c-Fos or c-Jun, with non-immune rabbit IgG as a control. The region of the Foxp3 promoter pulled down by immunoprecipitation was evaluated by qRT-PCR using Foxp3-specific primers. As shown in **Figure 4A** and **4B**, signal relative to input for both c-Fos and c-Jun was significantly higher than those in the IgG group ( $P < 0.05$ ). Besides, binding of c-Fos or c-Jun to the AP-1 region containing Foxp3 promoter was respectively decreased in cells treated with si-c-Fos or si-c-Jun ( $P < 0.05$ ) (**Figure 4C**). These results suggested that the binding of c-Fos or c-Jun to the Foxp3 promoter was necessary and could promote the expression of Foxp3 in CD4<sup>+</sup>CD25<sup>+</sup>Treg cells.

### Knockdown of c-Fos, Fra-2, c-Jun, or JunD decreased expression of Foxp3

We tested whether knockdown of c-Fos, Fra-2, c-Jun, or JunD could decrease expression of Foxp3. Pre-incubation of CD4<sup>+</sup>CD25<sup>+</sup>Treg cells with specific siRNA (si-c-Fos, si-Fra-2, si-c-jun, or si-JunD) significantly decreased protein levels of c-Fos, Fra-2, c-jun, or JunD Foxp3 compared with the control group ( $P < 0.05$ ) (**Figure**

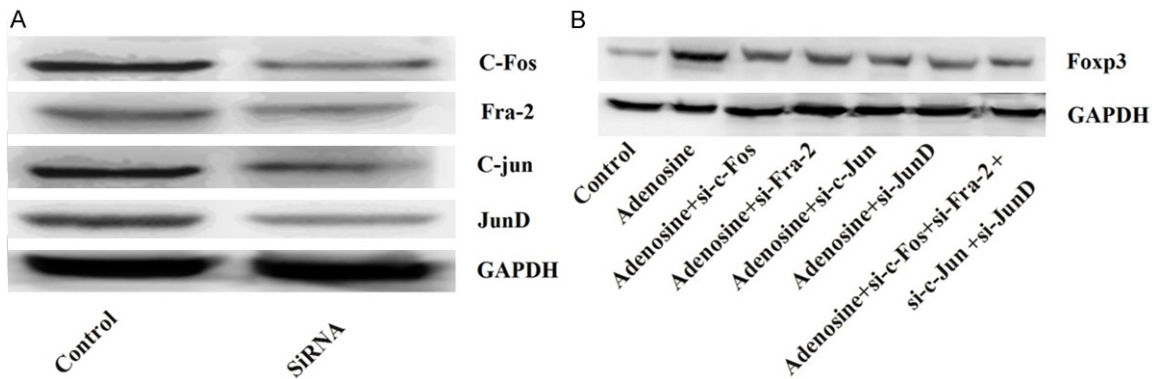
**5A**). Besides, knockdown of c-Fos, Fra-2, c-Jun, or JunD considerably reduced the protein levels of Foxp3. The levels were the lowest when co-transfection with the four kinds of siRNA (**Figure 5B**).

### Discussion

Researchers have proven the high expression of Foxp3 in Treg cells during the sepsis; however, the possible mechanism is still unclear. In the present study, we confirm that JNK/AP-1 activation is involved in the elevated levels of Foxp3 protein in CD4<sup>+</sup>CD25<sup>+</sup>Treg cells. We demonstrate that AP-1 regulates Foxp3 promoter activity in CD4<sup>+</sup>CD25<sup>+</sup>Treg cells and that the induction of c-Fos or c-Jun activity leads to increased transcription of the endogenous Foxp3 gene. Pharmacological inhibition of both adenosine and the JNK pathway reduced Foxp3 protein levels, and siRNA knockdown of c-Fos, Fra-2, c-Jun, or JunD levels also reduced Foxp3 expression.

It has been well demonstrated that severe sepsis and septic shock disrupt immune homeostasis by induction of an initial intense systemic

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**Figure 5.** Knockdown of c-Fos, Fra-2, c-Jun, or JunD decreases expression of Foxp3. A. Expression levels of c-Fos, Fra-2, c-Jun, or JunD after transfection with siRNA; B. Expression levels of Foxp3 after knockdown of c-Fos, Fra-2, c-Jun, or JunD. Foxp3, forkhead/winged helix transcription factor p3; siRNA, small interference RNA.

inflammatory response, and then followed by a negative feedback of anti-inflammatory process [3, 19]. Moreover, studies have confirmed that Tregs are associated with the pathogenesis of sepsis, which might be significantly contributed to the increased morbidity and mortality in patients with sepsis. In spite of CD4<sup>+</sup>CD25<sup>+</sup> Treg cells are only a small part of the T lymphocyte lineage in the immune system, these cells have shown potent regulatory properties on cellular activation which make them critical factors in the suppression of immune response during sepsis [20]. Clinical and experimental studies have suggested that sepsis exerts a relative and high elevation in Treg number and augmentation of its inhibitory function [21, 22]. Considering this reason, it is necessary to be explicit about a better and deeper understanding of Tregs. The physiological action of these Treg cells will be benefit to develop new and targeted therapeutic strategies for regulating the immune response in severe sepsis and septic shock. Yet, studies on how to modulate Tregs for the improvement of sepsis and survival rate has been paid only little attention, and limited to experimental researches.

Treg cells can be distinguished from other activated T cells by expression of the transcriptional regulator Foxp3 that belongs to the family of X chromosomes. Foxp3 has been acted as a master switch gene for the differentiation, development, and phenotype of Tregs [23] because its mRNA and encoded protein are exclusively expressed on Tregs. But Foxp3 gene stable [24] and high expression level [25] are essential to these processes, whereas, muta-

tions of Foxp3 gene caused by either spontaneous or experimental reasons may result in a series of autoimmune/inflammatory diseases [24, 26-29]. In addition, the levels of Foxp3 might be involved in the extent of Treg suppressive activity [30]. It has been reported that Foxp3 regulates expression and function of interleukin (IL) 2, CD25, cytotoxic T lymphocyte antigen (CTLA) 4, and glucocorticoid-induced TNF receptor (GITR) which is critical for Treg [31]. Despite significant progresses have been made to reveal the mechanistic details of its contribution to Treg cells, the mechanism of Foxp3-mediated transcriptional regulation is still largely unknown.

It has been reported that the induction of *Foxp3* gene is modulated by several regulatory regions including a promoter, two enhancers (Enhancer 1 and Enhancer 2), and conserved noncoding sequence (CNS) 1-3 [32]. Enhancer 1 is located in CNS1, Enhancer 2 is located in CNS2 in the intron of the *Foxp3* gene, and while CNS3 is located downstream of exon1. Nuclear factor of activated T cells (NFAT) and Smad3 could interact with CNS1 to induce the expression of Foxp3 [33]. Activation of signal transducer and activator of transcription (STAT) 5 directly binds the promoter of Foxp3 to CNS2 to maintain Foxp3 expression [34]. Also, c-Relin conjunction with Foxp3 promoter or CNS3 plays a unique role in *Foxp3* gene expression, leading to the open up of the Foxp3 locus in natural Treg (nTreg) precursor cells [32]. Additionally, several transcription factors such as NFAT and acute leukemia-1 (AML1)/runt-related transcription factor 1 (Runx1) have shown to bind to

Foxp3 promoter and potentially interacts with AP-1 and nuclear factor  $\kappa$ B (NF- $\kappa$ B) [35, 36]. Both T cell receptor (TCR) and TGF- $\beta$  signals have been reported to induce the activation of AP-1 through mitogen-activated protein kinase (MAPK) pathway, transiently inducing the expression of Foxp3 [37, 38]. To better elucidate the functional role of the Foxp3 gene in Treg development and differentiation, we further explore the molecular mechanisms of AP-1 on Foxp3 gene expression. In the present study, a sepsis mouse model was induced by using a CLP method. We found that a significant elevation in Treg cells following polymicrobial sepsis in the blood, which was in line with previous studies. Additionally, adenosine and pharmacological stimulation and inhibition of adenosine were performed to the purified CD4<sup>+</sup>CD25<sup>+</sup>Treg cells. In line with previous studies [39-41], the levels of Foxp3 were significantly increased by adenosine and its selective A2A receptor agonist CGS21680, but were decreased by adenosine antagonist CSC. The results implied that the existence of an AP-1 binding site in the Foxp3 promoter region was necessary to induce the expression of Foxp3. Then we explored the possible mechanism of adenosine on the expression of Foxp3 by investigation the relationships between the expression of Foxp3 and the activation of JNK or Smad3. The results confirmed that the expression of Foxp3 was associated with the activation of JNK but not associated with Smad3. To characterize the possible signaling pathway, we studied the levels of the transcription factor AP-1 after administration of different drugs. These results confirmed that the JNK pathway and AP-1 were involved in the effect of adenosine on the expression of Foxp3. Our study confirmed with previous studies. Mantel *et al.* identified the AP-1 binding sites in the FOXP3 promoter [37]. Xu *et al.* has found that AP-1 site located in a Foxp3 enhancer region plays a significant role in TCR/TGF- $\beta$  induced Foxp3 gene expression [41]. Furthermore, we identified that c-Fos and c-Jun could bind to the Foxp3 promoter by using ChIP assay. Besides, the activity of c-Fos, Fra-2, c-Jun, or JunD promoted the expression of Foxp3 in CD4<sup>+</sup>CD25<sup>+</sup>Treg cells.

In conclusion, adenosine enhanced the high expression of Foxp3 by activation of JNK/AP-1, and followed by c-fos and c-Jun activation in the Foxp3 enhancer region.

### Acknowledgements

This research was supported by Grant No. 81471895 and No. 81401575 from National Natural Science Foundation of China to Tao Yang and Rui Bao (Beijing, China).

### Disclosure of conflict of interests

None.

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