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Astragaloside IV reduces lung injury in lethal sepsis via promoting treg cells expansion and inhibiting inflammatory responses

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Abstract: Sepsis is a systemic inflammatory response syndrome caused by an infection progressing to sepsis-associated organ failure (such as lung injury). Our previous review revealed that Astragaloside IV (ASI-IV), one of the primary bioactive ingredients in *Astragalus membranaceus* (Fisch) Bge (Huang-Qi), had been shown to exert anti-inflammatory and immunomodulatory effects. Nevertheless, it is still unclear whether ASI-IV could attenuate septic lung injury via activating regulatory T-cells (Tregs). This study was designed to evaluate the therapeutic potential of ASI-IV on sepsis-induced lung injury and to further explore its underlying mechanism. In the murine models of cecal ligation and puncture (CLP) and lipopolysaccharide (LPS) induced sepsis, ASI-IV can markedly improve the survival rate and reduce inflammatory lung injury, protect mice against exacerbated inflammatory responses by decreasing myeloid cell infiltration and down-regulating IL-6 and TNF-α in lung tissue. Meanwhile, Treg cell-related gene expression, including Foxp3 and IL-10, significantly increased after ASI-IV treatment. Furthermore, ASI-IV notably promoted the differentiation of naïve $CD4^+$ T cells into T regulatory cells without obviously affecting Th1 and Th17 differentiation. Our results indicated that ASI-IV could attenuate septic lung injury by promoting Treg cell expansion and inhibiting inflammatory responses. It represents a promising agent for the treatment of sepsis.

Keywords: Sepsis, astragaloside IV, t regulatory cells, cecal ligation and puncture, lung injury.

INTRODUCTION

Sepsis is characterized by the massive release of cytokines and other mediators, leading to a dysregulated immune response, organ damage and even death (van der Poll T. *et al*., 2021). In sepsis, an immune imbalance that is triggered by microbial infection failing to return to normal homeostasis can result in uncontrolled inflammatory responses and subsequent multiple organ failure (Van Der Poll *et al.*, 2017). One of the most damaged organs is the lung and acute lung injury (ALI) is always a complication of sepsis associated with respiratory dysfunction (Herridge *et al.*, 2003). However, effective treatments have yet to be developed against sepsis-induced lung injury. Astragaloside IV (ASI-IV) is one of the major bioactive components of Astragalus *membranaceus (Fisch) Bge*. It has nourishing properties and has been widely used to treat cancer and other immune diseases in China and Southeast Asia for thousands of years. More and more evidence has shown that ASI-IV has many medicinal properties, including anti-inflammatory (Zhang and Frei, 2015; Zhang *et al*., 2003), antiviral (Indu *et al*., 2021) and immunomodulatory effects (Wan *et al.*, 2013; Yao J. *et al*., 2023).

ASI-IV has been proven to significantly inhibit the production of nitric oxide (NO) and pro-inflammatory cytokines (IL-1β and TNF-α) in RAW 264.7 cells stimulated by LPS (lipopolysaccharide) and primaryperitoneal macrophages (Wang *et al.*, 2002). In addition, ASI-IV significantly inhibited the levels of MCP-1 and TNF-α in serum and the expression of MCP-1, TNF-α, IL-6 and TLR4 mRNA in lung tissue in LPS-induced acute inflammatory response. In addition, ASI-IV can also reduce the infiltration and activation of pulmonary neutrophils (Zhang and Frei, 2015). In the mouse model of sepsis induced by cecal ligation and puncture (CLP), ASI-IV protects mice from microbial sepsis by inhibiting inflammatory reactions and lymphocyte apoptosis (Liu *et al.*, 2016). In a word, ASI-IV is a promising treatment for experimental sepsis. However, the potential immunomodulatory mechanism of ASI-IV has not been fully clarified.

In recent years, it has been well demonstrated that adaptive immune response is critical for the pathogenesis of sepsis. Although Treg cells are only a tiny part of the T lymphocyte subpopulation, they are needed to limit immune pathology and maintain immune tolerance (Fabienne Venet *et al.*, 2008). Immunological or genetic inhibition of Tregs function will increase mortality risk during sepsis due to exaggerated inflammatory responses (Fattahi and Ward, 2017; Fabienne Venet *et al*., 2008). In contrast, clinical and pre-clinical studies have shown that elevated expression of Tregs can markedly suppress inflammatory responses in patients following septic shock (Taylor and Llewelyn, 2010; F. Venet *et al.*, 2004).

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Besides, the expression of Foxp3 and gene/protein expression of inhibitory cytokines (IL-10 and TGF-β) were increased (Huang *et al.*, 2010).

A recent study has demonstrated that depletion of Tregs was associated with increased IL-6 production and mortality in the early phase of sepsis, implying that Tregs can inhibit uncontrolled acute inflammation and help patients survive the initial hyper-inflammatory phase of sepsis (Delano and Ward, 2016; Jensen *et al.*, 2018). It is well known that Tregs are critical to maintaining immune cell homeostasis and function. While in patients with severe sepsis or septic shock, this balance is disturbed and the suppressive activity of Tregs may reduce host resistance to secondary infection, which leads to disease deterioration or poor prognosis. Therefore, manipulating the expression of Tregs is critical to improving patient outcomes. In the present study, the immunomodulatory properties of ASI-IV were investigated in two murine models, including LPS-induced endotoxemia and CLP-induced polymicrobial sepsis. Our results revealed that ASI-IV could protect mice against lung injury by inducing Treg cell expansion and suppressing uncontrolled inflammation.

MATERIALS AND METHODS

Antibodies and Reagents

ASI-IV was purchased from Dalian Meilun Biology Co., Ltd (Dalian, China), with a more than 99% purity. IL-1β enzyme-linked immunosorbent assay (ELISA) kit was purchased from Thermo Fisher Scientific Inc (CA, USA). LPS, PMA, Brefeldin A and ionomycin were obtained from Sigma-Aldrich and a Reverse transcription kit and SYBR Premix Ex Taq II were purchased from TaKaRa (Dalian, China). The primers were all synthesized by Shanghai Bioengineering Co., Ltd (Shanghai, China). The RNA extraction kit was from Qiagen (CA, USA). PE-Cy7 anti-mouse CD4, APC anti-mouse CD3(145-2C11), FITC anti-mouse IFN-γ (XMG1.2), PE-anti-mouse CD11b (M1/7), PE anti-mouse CD45R/B220 (RA3-6B2), Alexa Fluor 488 anti-mouse/rat/human Foxp3 (150D), APC anti-mouse IL-17A (TC11-18H10.1) were purchased from Biolegend (San Diego, CA, USA). Rabbit anti-mouse-CD11 b antibody was purchased from Abcam (Cambridge, UK). All these reagents and chemicals were of analytical grade.

Experimental animals

Female C57BL/6 mice (8-10 weeks old) were obtained from the Shanghai Experimental Animal Center of the Chinese Academy of Sciences and placed under specific pathogen-free conditions (12h light/12h dark, 22±1°C, 55 ± 5% relative humidity). All mice were allowed to adapt to the environment in this facility for four weeks before the experiment. The experiment was conducted following the National Guidelines for the Use of Experimental Animal Health Care. It was approved by the Animal Care Use Committee of the First Affiliated Hospital of Yunnan University of Traditional Chinese Medicine (S2021-133).

Experimental design

Sepsis models

CLP was performed as previously described (Toscano *et al.*, 2011). Briefly, C57BL/6 mice were anesthetized with isoflurane to prepare the abdominal skin. The skin was first disinfected with betadine solution and then wiped with a 70% alcohol cotton swab. Under sterile conditions, open the midline 1-2cm, expose the cecum and tightly ligate the bottom of the ileocecal valve with 6.0 silk thread. Each mouse was punctured 1-2 times on the same side of the cecum with needle 19. Then gently squeezed the cecum and a small amount of feces was extruded from the perforated area. Restore the cecum to its normal intra-abdominal position and suture the peritoneum with 6.0 silk thread. Then, the abdominal incision was closed in two layers and 1ml of preheated 0.9% saline was subcutaneously injected to resuscitate the mice. Pseudo-surgical animals undergo laparotomy and intestinal procedures without ligation or puncture. Throughout the entire experimental process, animals can freely obtain food and water. LPS-induced endotoxemia model C57BL/6 mice with LPS (15mg/kg, ig). Immediately inject ASI-IV (10mg/kg or 20mg) or LPS into mice 1 hour after surgery. After 24hours, blood samples were collected and centrifuged (3000rpm, 10 minutes, 4°C) to obtain serum samples, which were stored at -80°C for testing. Simultaneously lung tissue was separated and stored in 10% formalin buffer or -80°C until detection.

H&E and immunohistochemical staining

After CLP surgery or LPS injection, lung tissue samples were separated for histological analysis. The separated samples were immersed in a 10% (v/v) formaldehyde solution for 2 days, then decalcified, embedded, sliced (with a thickness of 4mm each) and subjected to blind histological evaluation using hematoxylin-eosin (H&E) staining.

Regarding immunohistochemical staining, lung tissues were deparaffinized using xylene before being, hydrated in a serial dilution of alcohol and microwaved in the buffer to obtain the antigens, after this, the endogenous peroxidases were blocked using 3% H_2O_2. The sections were incubated with primary antibodies against CD11b (Abcam, 1:1000) overnight at 4°C and immunoreactions were visualized using HRP anti-rabbit DAB detection kit (Maixin). The percentage of CD11b-positive cells was analyzed using specified software.

Determination of cytokines

Blood samples were collected after CLP surgery or LPS injection and centrifuged (3000 rpm, 10 minutes, 4°C) to obtain serum samples. The concentration of IL-1β in serum was quantified using an ELISA kit (Thermo Fisher Scientific Inc) according to the manufacturer's instructions.

qPCR analysis

The qPCR analysis was performed as previously described (Cao *et al.*, 2019; Ying *et al*., 2023). Briefly, total RNA from lung tissue was isolated using RNeasy Kit (Qiagen). 1μg of total RNA was used to synthesize cDNA using the PrimeScript RT Master Mix Perfect Real-Time kit (TaKaRa) and the relative mRNA expressions were measured. Samples were assayed on Stratagene MX3000P Real-Time PCR machine (Agilent). Then, the relative quantitation of mRNA expression was calculated as the fold increase in expression by using the DDCt method and the housekeeping gene is β-actin. The primer sequences of specific genes were described as follows:

Foxp3: (Forward) 5′-CCCATCCCCAGGAGTCTTG-3′;
(Reverse) 5′-ACCATGACTAGGGGCACTGTA-3′;
IL-10: (Forward) 5′-CTTACTGACTGGCATGAGGATCA-3′;
(Reverse) 5′-GCAGCTCTAGGAGCATGTGG-3′;
TGF−β: (Forward) 5′-CCACCTGCAAGACCATCGAC-3′;
(Reverse) 5′-CTGGCGAGCCTTAGTTTGGAC-3′;
IL-6: (Forward) 5′- CGGAGAGGAGACTTCACAGAG -3′;
(Reverse) 5′- CATTTCCACGATTTCCCAGA -3′;
IL-1β: (Forward) 5′- GCAACTGTTCCTGAACTCAACT-3′;
(Reverse) 5′- ATCTTTTGGGGTCCGTCAACT-3′;
TNF-α: (Forward) 5′- CCCTCACACTCAGATCATCTTCT-3′;
(Reverse) 5′- CTTTGAGATCCATGCCGTTG-3′;
β-actin: (Forward) 5′- GGCTGTATTCCCCTCCATCG-3′;
(Reverse) 5′- CCAGTTGGTAACAATGCCATGT-3′;

Flow cytometric analysis

Murine splenocytes were harvested and blocked with rat-anti-mouse CD16/CD32 (2.4G2, BD Pharmingen). Then, these cells were fluorescently stained for 15 min at 4℃ with the following mAbs diluted in PBS with 0.2% BSA: PE-conjugated anti-mCD11b (M1/7, Biolegend) and APC-anti-mCD45 (30-F11, Biolegend). Intracellular cytokines were stored according to the previously described method (Cao *et al*., 2019; Hou *et al*., 2015)**.** Splenocytes from septic mice were restimulated for four h with PMA (50ng/ml) and ionomycin (750ng/ml) (Sigma-Aldrich) in the presence of Brefeldin A. Then, the cells were collected and blocked with rat-anti-mouse CD16/CD32. The cells were stained with fluorescence-labeled surface antibodies and then fixed, permeabilized and stained with fluorochrome-conjugated anti-mIL-17A (TC11-18H10.1, Biolegend) and anti-mIFN-γ (XMG1.2, Biolegend). The above stimulations were not required for staining Foxp3. Samples were acquired on a flow cytometer (FACs Canto ™ II, BD Biosciences) and analyzed using the Flowjo software (Tree star).

T helper cell differentiation in vitro

Cells were cultured in a 24-well plate coated with anti-mouse CD3 (clone 154-2C11, 5mg/ml, Biolegend) and anti-mouse CD28 (clone 37.51, 2.5mg/ml, Biolegend) and incubated at 37°C for 2 hours or 4°C overnight. Aseptically decant antibody solution from the plate and then wash the plate 3 times with sterile PBS. Purified CD4 T cells were isolated by using immunomagnetic negative selection (Miltenyi Biotec) according to the manufacturer's instructions. Then, the purity of CD4 T cells was checked by staining PE-Cy7-conjugated anti-mCD4. For Th1 differentiation, cultures were added with mIL-12 (10 ng/ml, Biolegend) and anti-mIL-4 (10 μg/ml, Biolegend). In order to induce Treg differentiation, cultures were added with hTGF-b1 (10 ng/ml, Biolegend) and mIL-2 (100 U/ml, Biolegend) plus anti-mIFN-γ (10 μg/ml). For Th17 differentiation, we used a cocktail containing recombinant mouse IL-6 (10 ng/mL, Biolegend), recombinant human TGF-b1 (2 ng/ml, Biolegend), anti-mouse IL-4 (10 μg/ml, Biolegend) and anti-mouse IFN-γ (10 μg/mL, Biolegend). Cells stimulated in 'neutral' conditions (anti-mIL-4 plus anti-mIFN-γ but no additional cytokines) were considered as naïve cells. After 72 h of incubation at 37℃, differentiated cells were restimulated with phorbol myristate acetate and ionomycin in the presence of Brefeldin A for 4 h and then fixed, permeabilized and stained with the related antibody. The above stimulations were not required for staining Foxp3. Samples were acquired on a flow cytometer (FACs Canto TM II, BD Biosciences) and analyzed using the Flowjo software (Tree star).

STATISTICAL ANALYSIS

Statistical analyses were performed using SPSS software (version 26.0 Chicago, IL,USA). Data are expressed as mean ± SEM. Student's t-tests were used to determine differences between the two groups. $P<0.05$ was considered as statistically significant.

RESULTS

ASI-IV ameliorated LPS-induced lung injury

Histopathological examination showed that the lung tissues of mice in the sham group showed no abnormalities. However, the mice from the vehicle group developed typical pathological changes, including blood congestion, alveolar wall swelling, debris, erythrocyte and monocyte aggregation in the alveolar space. However, ASI-IV treatment significantly reduced the infiltration process of large numbers of erythrocytes and monocytes into the alveolar space (fig. 1B). Immunohistochemical staining showed that ASI-IV administration decreased the infiltration of $CD11b^+$ cells in the lung section compared with the vehicle group, which was consistent with CLP results (fig. 1C). ASI-IV treatment also protected mice from liver injury with lower serum concentration of ALT and AST. Furthermore, compared with the vehicle group, the level of inflammatory cytokine IL-1β in the blood was significantly down-regulated in the ASI-IV-treated group at 24h after LPS treatment (fig. 1D). These results suggested that ASI-IV could ameliorate LPS-induced lung injury.

ASI-IV protected mice from exacerbated inflammatory responses

To explore the impacts of ASI-IV with respect to inflammation-associated cytokines profiles in LPS-

induced sepsis, we detected mRNA expressions of inflammation-associated cytokines in the lungs of septic mice using q-PCR assay. As shown in fig. 2, administration of ASI-IV markedly decreased the mRNA expression of IL-6 (fig. 2A) and TNF-α (fig. 2B). However, no influence on IL-1β mRNA expression was observed when we detected IL-1β mRNA expression in the same samples as IL-6 and TNF-α (fig. 2C). CD11b/CD18 is critical for the transendothelial migration of monocytes and neutrophils and is also involved in granulocyte adhesion, phagocytosis and neutrophil activation. Therefore, we further detected CD11b expression in splenocytes of septic mice using flow cytometric analysis. As shown in fig. 2D, ASI-IV treatment significantly decreased the expression of $CD45^+CD11b^+$ cells in splenocytes compared to the vehicle group. These findings further proved that ASI-IV could protect mice against exacerbated inflammatory responses.

ASI-IV improved the survival of septic mice and attenuated lung injury

Cecal ligation and puncture in mice have become the most widely used and clinically relevant experimental model for sepsis. ASI-IV markedly decreased LPS-induced acute lung injury, as described above. In the present study, the CLP model was developed to assess the therapeutic potential of ASI-IV on sepsis. As shown in fig. 3B, mice from the sham group survived 7d. However, the CLP group's survival rate was 20% on the first day. The survival rate in septic mice receiving DEX (1mg/kg) and ASI-IV at 10mg/kg or 20mg/kg after CLP were increased compared with mice receiving PBS. In order to further assess the effects of ASI-IV on acute lung injury, the pathological changes in the lungs of septic mice were analyzed by H&E staining. Photomicrographs of H&E-stained sections of lung tissue of the mice from the sham, vehicle and ASI-IV (10mg/kg)-treated group are presented. The sham mice exhibited normal lung histology. In contrast, mice from the vehicle group showed changes such as infiltration of inflammatory cells into the lungs, congestion and septal thickening, which are generally considered to be typical pathological changes. The pathological changes were rescued by administration of 10 mg/kg of ASI-IV (fig. 3C). Immunohistochemical staining indicated that the percentage of CD11b-positive cells in the lung section was significantly increased in the CLP group compared with the sham group. Whereas the percentage of CD11b-positive cells in the ASI-IV group was lower than that of the vehicle group (fig. 3D). These observations demonstrated that ASI-IV might represent a promising agent for treating sepsis, especially for acute lung injury.

ASI-IV markedly regulated Th1 cell response and γδ TCR cell expression in CLP

Although the etiological mechanism of sepsis remains unclear, accumulating evidence indicates that T cells are crucial for the pathogenesis of sepsis and other inflammatory disorders. Thus, the subset constitution of mouse spleens obtained from ASI-IV-treated and vehicle groups was analyzed using flow cytometry (fig. 4). Surface marker staining showed that ASI-IV significantly reduced the percent of γδ TCR cells. However, no significant changes were observed in the percent of $CD3^+$T cells, $CD4^+$T cells and $CD8^+$T cells (fig. 4A). Further, intracellular cytokines staining revealed that the expression of IFN-γ^+ producing $CD4^+$ T cells (Th1 cells) was decreased in the ASI-IV-treated group compared with the vehicle group. Nevertheless, there was no significant difference in the expression of IL-17^+-producing $CD4^+$ T cells (Th17 cells) between the vehicle and ASI-IV treated group (fig. 4B).

ASI-IV induced regulatory T-cell expansion in CLP

$CD4^+$ helper T (Th) cells play crucial roles in the induction and persistence of sepsis by producing pro-inflammatory cytokines. Naïve T cells can differentiate into pathogenic Th1 cells, pro-inflammatory T helper cells (Th17), or regulatory T cells (Treg) under different conditions (Saravia *et al.*, 2019). To further validate whether ASI-IV could affect the differentiation of Treg cells in septic mice, we used flow cytometry to measure the expression of Foxp3 in $CD4^+$T cells. As depicted in fig.5A, the percentage of $Foxp3^+$ in the $CD4^+$T cells of the septic mice receiving ASI-IV was increased compared with the septic mice receiving only saline.

The activities of $CD4^+CD25^+$ Tregs are either cell contact-dependent or independent, which is dependent on the expression of Foxp3 or cytokines. When Foxp3 is expressed, it is cell contact-dependent. It is independent when anti-inflammatory cytokines, such as IL-10 and TGF-β, are produced. Thus, we determined whether ASI-IV influenced the mRNA expression of Treg-related genes in lung tissue using q-PCR. As expected, administration of ASI-IV markedly up-regulated the transcription of Foxp3 and IL-10 (fig.5B). These results suggested that the expansion of Treg cells may be due to the protective effects of ASI-IV on sepsis.

ASI-IV induced Treg cells differentiation

Recent evidence has shown that the function of Treg cells was tightly associated with the pathogenesis of sepsis. The current study also found that ASI-IV treatment significantly induced Treg cell expansion. In order to further determine whether ASI-IV could specifically promote the differentiation of Tregs from naïve CD4 cells *in vitro*, we performed differentiation experiments. The result presented in fig. 6 showed that compared with the sham group, the percentage of $Foxp3^+$ in the $CD4^+$T cells in the ASI-IV-treated group was markedly increased. However, ASI-IV treatment did not affect the differentiation of Th1 and Th17 cells.

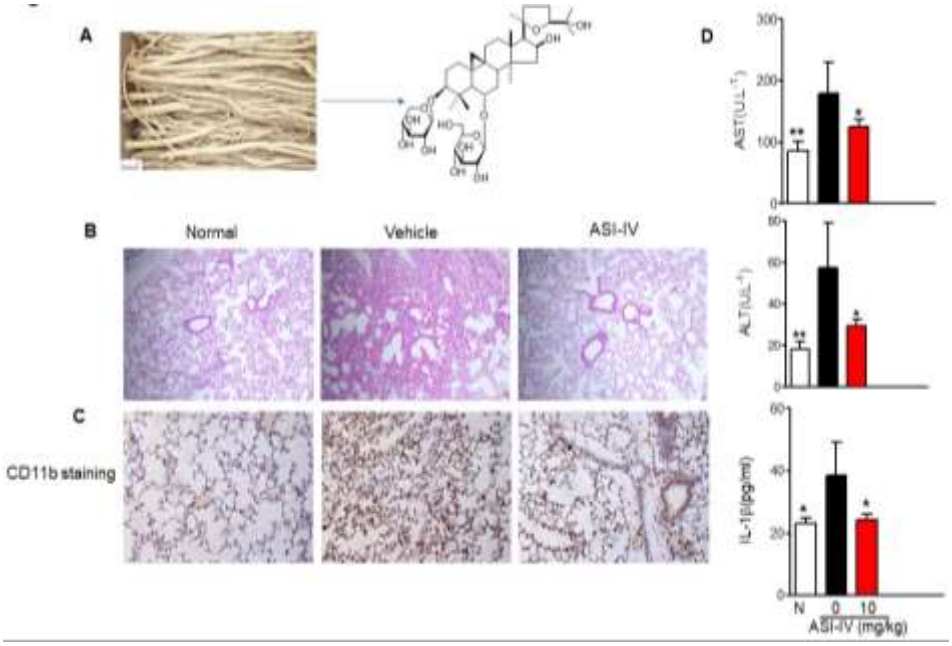

Fig. 1: ASI-IV inhibited LPS-induced lung injury. Image of Astragalus membranaceus and chemical structure of Astragalosides IV from the roots of Astragalus membranaceus (A). LPS-induced lung injury was established by the injection of LPS (15mg/kg). The vehicle, 2mg/kg of methotrexate, 10 and 20mg/kg of ASI-IV were injected for 24h. Sepsis-induced lung injury. and expression of CD11b were respectively evaluated by the use of H&E(B) and immunohistochemical staining (C) . The cobasc311 automatic biochemical analyzer determined the serum concentration of ALT and AST. ELISA detected the level of IL-1β (D). Results are presented as mean ± s.e.m., n=6 *P<0.05, ***P*<0.01 versus the vehicle group.

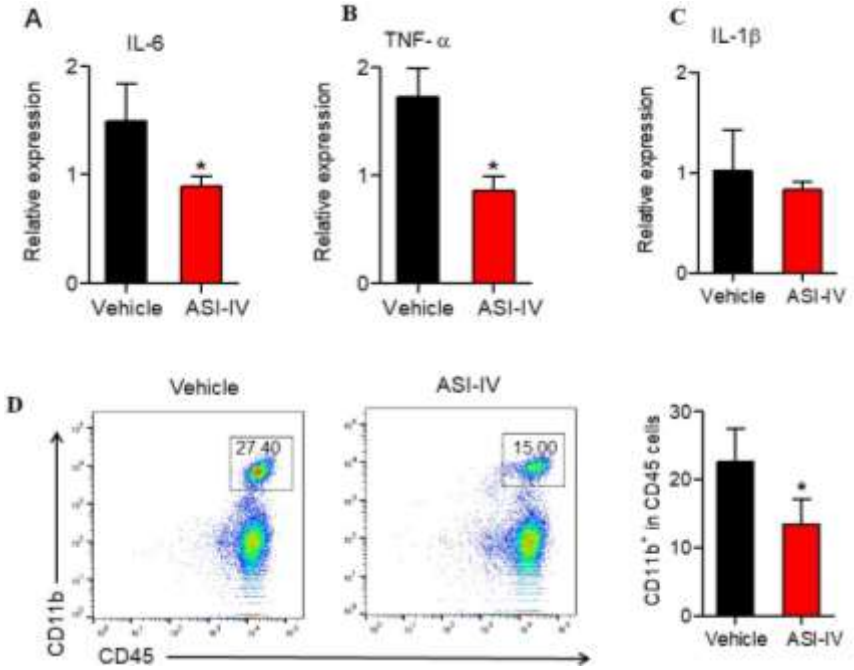

Fig. 2: ASI-IV protected mice against exacerbated inflammatory responses. Lung tissue from LPS-treated mice were prepared and total RNA was isolated using RNeasy kits. Then, 1μg of total RNA was used to synthesize cDNA. q-PCR assay was performed using SYBR Premix Ex Taq II ki. Relative mRNA expression was calculated as the fold increase using $2^{-\Delta\Delta CT}$ method. The housekeeping gene was β-action (A-C). The expression of $CD45^{+}CD11b^{+}$ cells in splenocytes of septic mice was measured using flow cytometric analysis (D). Results are presented as mean ± s.e.m., *n*=4. **P*<0.05, ***P*<0.01 versus the vehicle group.

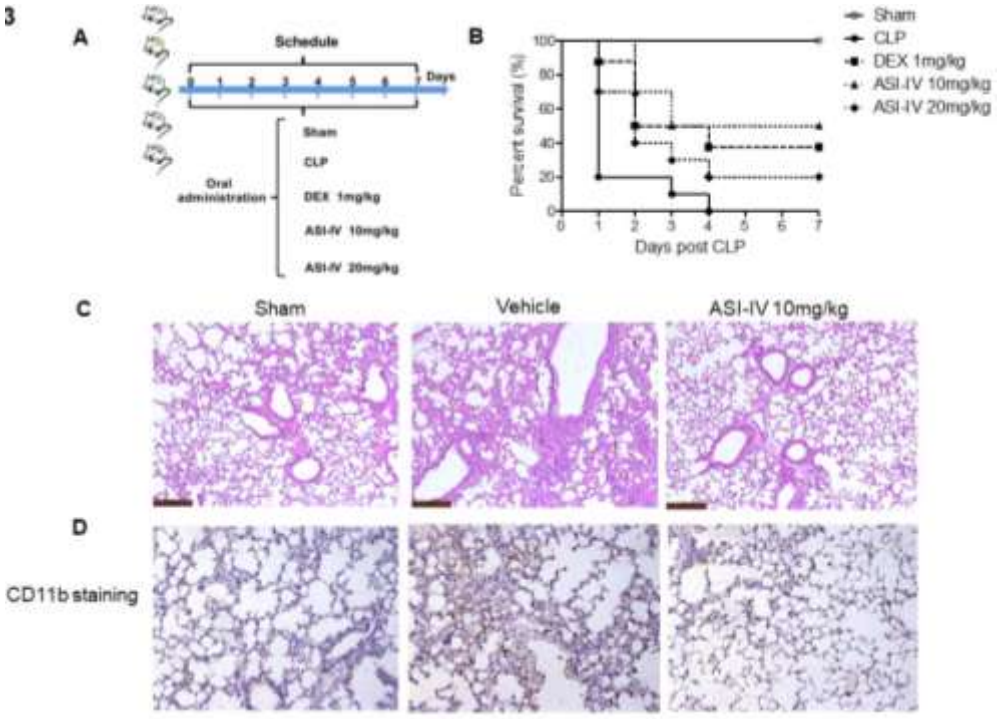

Fig. 3: ASI-IV improved the survival of septic mice and attenuated lung injury in CLP. Scheme of the CLP and animal treatments (A). The CLP model was developed and the survival rate was assessed during 7d (B). The lung injury (C) and expression of CD11b (D) from lung tissues were respectively evaluated using H&E and immunohistochemical staining. Results are presented as mean ± s.e.m., n=10. $^{*}P$<0.05, $^{**}P$<0.01 versus the vehicle group.

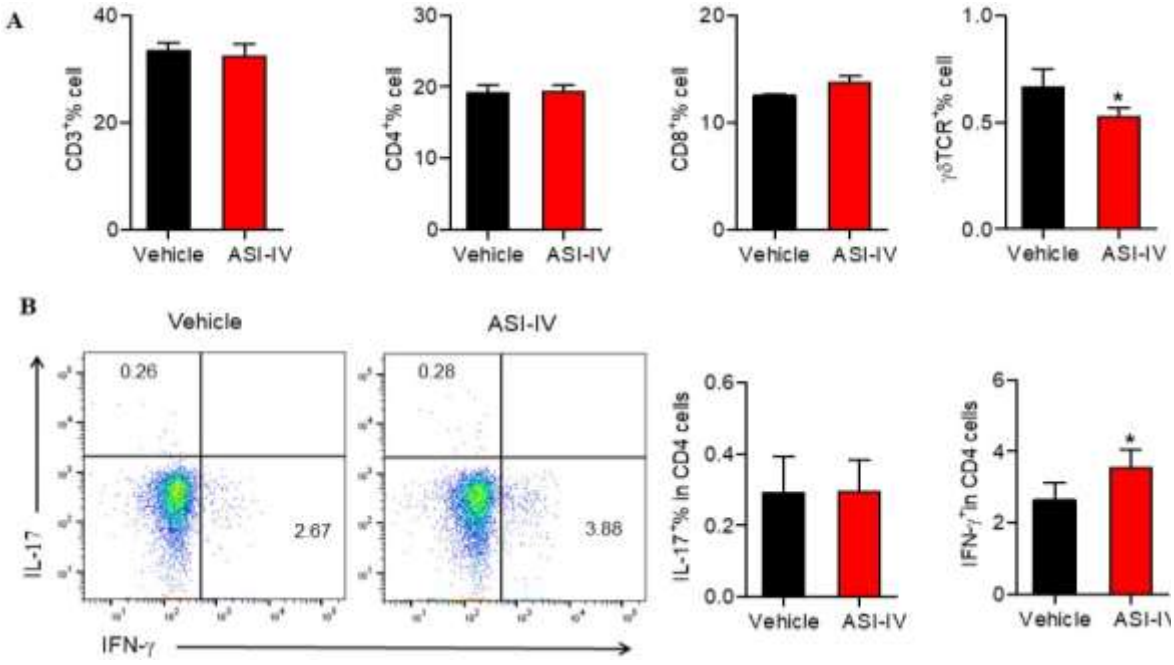

Fig. 4: ASI-IV markedly regulated Th1 cell response and TCR cell expression in CLP. Splenocytes from the mice with CLP were harvested and blocked with rat anti-mouse CD16/CD32 and then the cells were fluorescently labeled by incubation for 15 min at 4°C with the related antibody. Samples were acquired on a flow cytometer and analyzed using FlowJo software (A). The expression of IFN-γ^{+} and IL-17^{+} producing $CD4^{+}$ T cells were detected by intracellular cytokines staining and then were harvested on a flow cytometer (B). Results are presented as mean±s.e.m., n=4. $^{*}P$<0.05, $^{**}P$<0.01 versus the vehicle group.

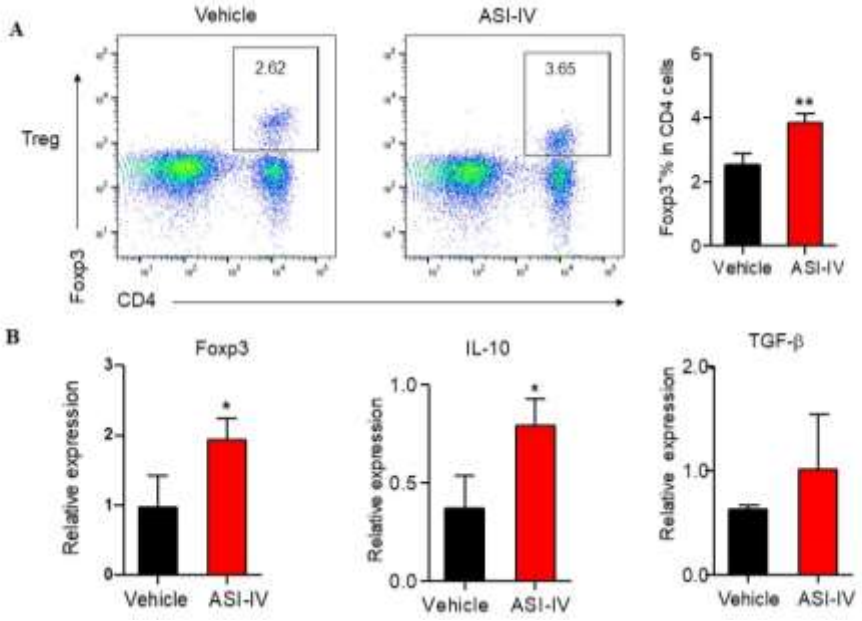

Fig. 5: ASI-IV induced regulatory T cell expansion in CLP. Splenocytes from the mice with CLP were harvested and then these cells were blocked with rat anti-mouse CD16/CD32. The surface marker was stained with PE-Cy7-anti-mCD4 antibodies. The cells were fixed, permeabilized and stained intracellularly with fluorochrome-conjugated m Foxp3. Samples were acquired on a flow cytometer and analyzed using FlowJo software (A). Expressions of Treg cell-related genes (Foxp3, TGF-β and IL-10) were estimated by real-time PCR (B). Results are presented as mean±s.e.m., n=4. *P<0.05, ** P<0.01 versus the vehicle group.

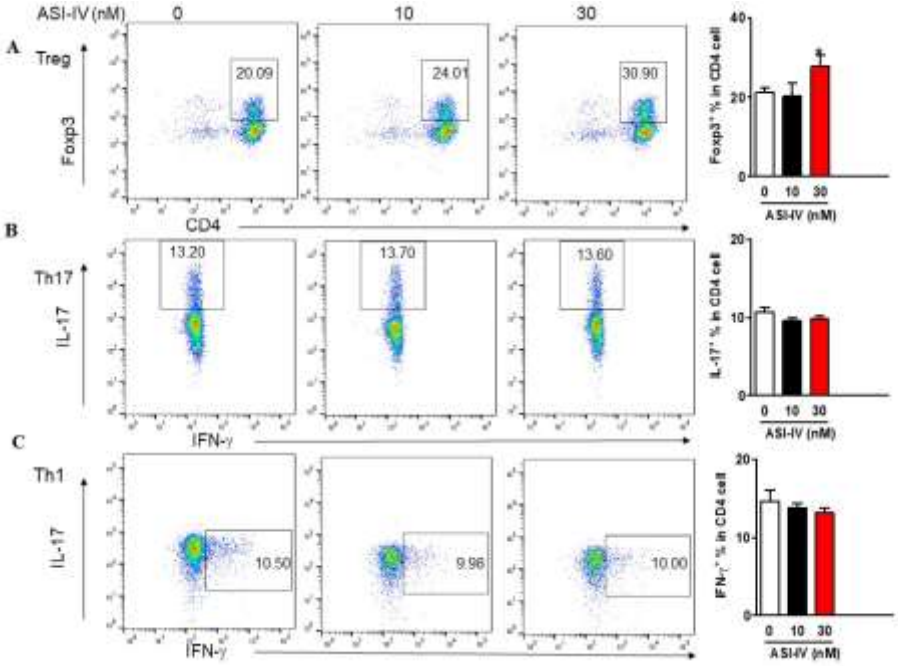

Fig. 6: ASI-IV-induced Treg cells differentiation. Naïve CD4 T cells (4×10^5) in 24-well plates precoated with anti-CD3 and anti-CD28, were cultured in RPMI containing 10% FBS in the presence of Treg, Th17 and Th1 polarizing cytokines at various concentrations of ASI-IV. The resulting cells were harvested for three days and the percent of Tregs (A), Th17(B) and Th1 (C) were evaluated by flow cytometry. Results are presented as mean±s.e.m., n=3. *P<0.05, **P<0.01 versus the control group.

DISCUSSION

Sepsis is the most common cause of death in hospitalized patients. That results in millions of deaths yearly (Australian *et al.*, 2008). Sepsis and sepsis-associated multiorgan failure remain a significant challenge for scientists and clinicians, but the underlying mechanisms have not yet been fully elucidated. In order to reveal underlying mechanisms and develop promising interventional strategies, various experimental animal models have been established, all of which try to mimic pathophysiological characteristics typically seen in patients with sepsis. The most commonly studied animal models of sepsis are cecal ligation and puncture (CLP)-induced polymicrobial sepsis and LPS-induced endotoxemia models (Liu *et al.*, 2018; Toscano *et al*., 2011). In the CLP model, sepsis arises from polymicrobial infections within the abdominal cavity, accompanied by bacterial translon cation into the blood compartment, triggering systematic inflammatory responses (Toscano *et al*., 2011). The current study showed that administration of ASI-IV prominently improved the survival of septic mice and attenuated lung injury induced by CLP. LPS is another widely-used stimulus for inducing lung injury in mice. Substantial evidence indicated that intraperitoneal injection of LPS caused successive waves of cytokine release into the circulation as well as transient lung injury and dysfunction (Matsuda *et al.*, 2004). Furthermore, ASI-IV significantly suppressed LPS-induced lung inflammatory responses, reflected by less lung damage and decreases of CD11b-positive cells as well as down-regulated expression of IL-6 and TNF-α. Compared with the vehicle group, the secretion of IL-1β and the proportion of $CD45^+CD11b^+$cells were significantly decreased in the ASI-IV-treated group. This observation indicated that ASI-IV may be a promising agent for treating septic-induced lung injury.

Dysregulation of various immune cells induced by sepsis is associated with increased morbidity and mortality rates. Evidence has demonstrated that Tregs are crucial in regulating immune homeostasis and tolerance, including several pathological conditions such as allergic lung inflammation, autoimmune disease and transplant rejection. During infectious processes, Treg cells can inhibit the activation of naïve autoreactive CD4 helper and CD8 cytotoxic T cells, which can potentially attack the body's healthy tissues (Yao and Huang, 2011). In addition, recent studies have indicated that Treg cells can also repress neutrophil cells, B lymphocytes, monocytes/macrophages and dendritic cells. Furthermore, Treg cell exert their effects on the maintenance of immune tolerance through producing various potent anti-inflammatory cytokines, such as IL-10 and TGF-β, which can cause immunosuppression of effector T cells, suppression of antigen-presenting cells function, induction of cytolysis and metabolic disruption of effector cells (Vignali *et al.*, 2008). It is well known that Forkhead box P3 (Foxp3) is essential for Treg cells development and function (Williams and Rudensky, 2007). Mice or humans that lack Foxp3 usually display defective functions of Treg cells and suffer from severe lymphoproliferative disorders and immune diseases (Bennett *et al.*, 2001; Brunkow *et al.*, 2001). Adoptive transfer of *in vitro*-stimulated Tregs significantly improved the survival of mice after 6 hours of CLP and enhanced the peritoneal bacterial clearance (Venet *et al.*, 2018). In contrast, depletion of Tregs through genetic or immunological inhibition caused acute death in LPS-treated mice because of uncontrolled inflammatory responses (Venet *et al*., 2008).

Thus, Treg cells may serve as an important target for treating sepsis. In the present study, ASI-IV has been shown to elevate the expression of $Foxp3^+$ in $CD4^+$T cells significantly but failed to affect the percentage of $IL\text{-}17^+$ producing $CD4^+$ T (Th17 cells) and IFN-γ^+ producing $CD4^+$ T cells (Th1 cells). Importantly, ASI-IV also up-regulated the transcriptional expression of IL-10 and Foxp3, specific transcriptional factors for the development, differentiation, and function of Tregs. Combined with the finding that Tregs acted on monocytes to increase the survival of LPS-treated mice, we speculated that ASI-IV treatment suppressed exaggerated inflammatory responses induced by sepsis via Treg-mediated immunosuppression.

In mice, differentiation of Tregs mainly depends on TGF-β1 and IL-2, consistent with *in vivo* results. ASI-IV treatment significantly enhanced the differentiation of naïve $CD4^+$T cells into Treg cells under Treg-polarizing conditions. However, the differentiation of Th1 and Th17 cells was not affected in the presence of ASI-IV. ASI-IV-induced Treg cell expansion may improve host immune dysfunction and sepsis-induced lung injury.

CONCLUSION

Our findings revealed that ASI-IV could exert anti-sepsis effects by triggering the expansion of Tregs and inhibiting inflammatory response. It may represent a promising therapeutic agent for treating sepsis and septic lung injury.

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