Propofol regulates imbalanced Th17/Treg responses in lipopolysaccharide-induced septic shock rats

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Abstract: Propofol (PPF) has previously been shown to inhibit the inflammatory response to septic shock. The main purpose of the present study was to investigate the effects of PPF on the levels of regulatory T cells (Treg) and Th17 in septic shock. Septic shock in rats was induced by intraperitoneal injection of lipopolysaccharide (LPS), and PPF (100 mg/kg) was administered. Mortality, the mean arterial pressure (MAP) and heart rates (HR) were recorded for 24 h after LPS injection. The Treg and Th17 ratios were analysed by flow cytometry. Moreover, the expression of p-STAT3, p-STAT5, STAT3, and STAT5 in PBMCs was measured by western blotting. The results showed that the MAP and HR of the PPF group were more stable than those of the LPS group. Mortality at 24 h after LPS injection was much lower in the PPF group compared to that in the LPS group. PPF significantly reduced the levels of IL-17, TNF-α and IL-6 but increased the IL-10 concentration. Moreover, PPF-treated rats exhibited a higher level of circulating Treg cells and a lower level of circulating Th17 cells in comparison to untreated rats. PPF decreased the level of phosphorylated STAT3 (p-STAT3), increased the level of p-STAT5, but did not change the levels of STAT3 and STAT5. Our data suggest that PPF regulates the imbalanced level of Th17/Treg in septic rats, possibly through modulating the expression of p-STAT3 and p-STAT5.

Keywords: Propofol, septic shock, lipopolysaccharide, Treg, Th17.

INTRODUCTION

Septic shock is a serious medical condition that occurs when sepsis, which is organ injury or damage in response to infection, leads to dangerously low blood pressure and abnormalities in cellular metabolism. Septic shock can cause multiple organ dysfunction syndrome (known as multiple organ failure) and death. Therefore, septic shock is a common problem in intensive care units (ICU) with a very high mortality (Singer *et al*., 2016; Busani *et al*., 2017; Esposito *et al*., 2017). Currently, the pathophysiological mechanism of septic shock is poorly understood, but a widespread consensus has been reached for the significant inflammatory responses in response to infection (Cinel and Opal, 2009).

Propofol (PPF) is an intravenous general anaesthetic that is widely used in the ICU because it can be easily titrated and offers the prospect of rapid recovery (Chidambaran *et al*., 2015). Recently, several reports (Li *et al*., 2010; Li *et al*., 2016; Ma *et al*., 2013) have shown that PPF exhibits significant anti-inflammatory activity, decreases the mortality rate and alleviates the multiple organ dysfunctions (lung, liver) induced by LPS in septic rats. In septic animal models, PPF reduces the inflammatory cytokine levels, such as tumour necrosis factor-alpha (TNF-a), interleukin-6 (IL-6) and IL-1β and down-regulates the expression of CD14 and Toll-like receptor-4 (TLR4), two well-known targets of LPS.

$CD4^+$ T cells are the main effect or cells of T cell-mediated inflammatory and immune responses. Currently, $CD4^+$ cells can be differentiated into different subsets, T-helper ((Th)1, Th2, Th9, Th17, Th22) and regulatory T (Treg) cells, based on their different cytokine profiles (Golubovskaya and Wu, 2016). Among these subsets, Treg and Th17 have received wide attention and have been well studied in inflammatory diseases. Tregs secrete the anti-inflammatory cytokine IL-10 and induce immunological self-tolerance to the relive inflammatory response, causing tissue damage and preventing the development of various autoimmune diseases. By contrast, Th17 and its produced IL-17 induce inflammatory responses and enhance host defence against infection caused by bacteria and fungi (Golubovskaya and Wu, 2016; Fasching *et al*., 2017). It has recently been confirmed that the absolute counts of Th17 and Treg lymphocytes in survivors of severe sepsis are higher than in those in non-survivors and an imbalanced level of Th17/ Treg in sepsis is related to the occurrence and prognosis of multiple organ dysfunction syndromes (Wu *et al*., 2013; Guo *et al*., 2017). Therefore, in this paper, we hypothesized that PPF could modulate Treg/Th17 equilibrium, which in turn, prevents LPS-induced inflammatory responses. To test this hypothesis, we examined the effects of PPF on LPS-induced sepsis in a rat model.

MATERIALS AND METHODS

Animals

Male Sprague Dawley rats (10-12 weeks old, 280-300 g)

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were purchased from the animal department of Anhui Medical University. All animal cares and experimental protocols in this study were approved by the Animal Care and Use Committee of Maternal and Child Care Hospital of Anhui Province (Hefei, China). All rats were housed under a 12 h light-dark cycle in a room with an ambient temperature of 22 ± 1°C and humidity of 50 ± 5%.

Septic shock model and groups

Septic shock in rats was induced via slow intravenous infusion of lipopolysaccharide (LPS, Klebsiella pneumoniae; Sigma Chemical, St Louis, MO, USA) dissolved in 0.5 mL normal saline at a rate of 10 mg/kg over 20 min. A total of 30 rats were randomly divided into three groups, namely, the normal, model and PPF groups. All rats were anesthetized by inhaling ether. Rats in the normal group were treated with saline through the left femoral vein cannula 1h before intravenous administration of 0.9% NaCl into the tail vein (7.5 mg/kg). Rats in the PPF group received a PPF infusion (10 mg/kg·h, AstraZeneca, China) continuously through the left femoral vein cannula 1h before intravenous administration of LPS (7.5mg/kg) into the tail vein, and rats in the LPS group received an intravenous injection of 0.9% NaCl through the left femoral vein cannula 1h before injection of LPS (7.5mg/kg) into the tail vein. Finally, the right femoral artery was cannulated to monitor the mean arterial pressure (MAP) and heart rates (HR).

Survival curve analysis

The mortality of rats in each group was observed and recorded at 24 h after LPS injection.

Plasma cytokine levels

At 24h after LPS administration, plasma was prepared from the right femoral artery, and then, serum was collected by centrifugation at 1,800 g for 15 min at 4°C. The levels of cytokines (IL-6, TNF-α, IL-10 and IL-17) in plasma were quantified using enzyme-linked immunosorbent assays kits (R&D Systems, Inc., Minneapolis, USA).

Flow cytometric analysis of Th17 and Treg

Peripheral mononuclear blood (PBMCs) cells were prepared by a routine method as previously reported (Zhang *et al*., 2014). Th17 and Tregs were surface-stained with anti-CD3 APC, anti-CD4 FITC and/or anti-CD25 PE antibodies (all from eBioscience, San Diego, CA, USA). For intracellular staining, cells were fixed and permeabilized using a Fixation/ Permeabilization Kit (eBioscience, San Diego, CA, USA), followed by staining with anti-rat/mouse IL-17A (eBioscience, San Diego, CA, USA) and/or anti-rat Foxp3 antibodies (BioLegend, San Diego, CA, USA).

Western blot analysis

PBMCs were lysed and homogenized in lysis buffer for 30 min on ice and then centrifuged at 14,000 rpm for 15 min at 4°C to separate intracellular protein. A total of 20 μg protein was loaded on a 10% sodium dodecylsulfate polyacrylamide gel and then separated and blotted on a nitrocellulose membrane. The transfers were blocked overnight with 5% skim milk at 4°C. The membranes were then incubated with p-STAT3, p-STAT5, STAT3 and STAT5 antibodies (Santa Cruz Biotechnology, USA) at 1:500 dilutions for 2h at 37°C. After being washed 3 times in PBST, the membranes were incubated with a second antibody (Pierce, USA) for 1h at room temperature. All of the blots were developed using an enhanced chemiluminescence detection system. The relative band density was determined by using a scion imaging system (Scion Corporation, USA). Gel loadings were normalized to ß-actin levels.

STATISTICAL ANALYSIS

All data are shown as the mean ± SD and were analysed by SPSS13.0 (SPSS, Inc., Chicago, IL, USA). Differences between the two groups were investigated by two-tailed Student's t-test (two groups at the 5% significance level).

RESULTS

Hemodynamic parameters and mortality rates

As seen in fig. 1, no significant differences in the baseline HR and MAP were observed among the groups. LPS stimulation significantly induced a decrease in the MAP value in rats in the model and PPF groups compared with those in the normal group (fig. 1A, $P<0.01$). PPF treatment had no effects on the MAP. Moreover, we found that there were no significant differences in HR among the groups before LPS injection. However, HR in the LPS group significantly increased at 6h and then decreased at 12h compared with that in the control group (fig. 1B, $P<0.01$). Notably, PPF treatment could significantly eliminate the variable HR trend induced by LPS (fig. 1B, $P<0.01$). Finally, we noted that the mortality in the PPF group was significantly lower than that in the LPS group (fig. 1C, $P<0.01$).

Plasma cytokine concentrations

At 24 h after LPS administration, the cytokine profiles of the different groups were investigated. As shown in fig. 2, the levels of IL-17, TNF-a and IL-6 were significantly higher in the LPS group than those in the normal group, while IL-10 was significantly lower ($P<0.01$). Moreover, treatment with PPF significantly reduced the levels of IL-17, TNF-a, and IL-6 and increased the level of IL-10 in LPS-treated rats ($P<0.01$).

Treg and Th17 ratios in peripheral blood

At 24 h after LPS administration, PBMCs were collected from rats and the Th17 and Treg ratios were analysed by flow cytometry analysis. As shown in fig. 3, the Th17

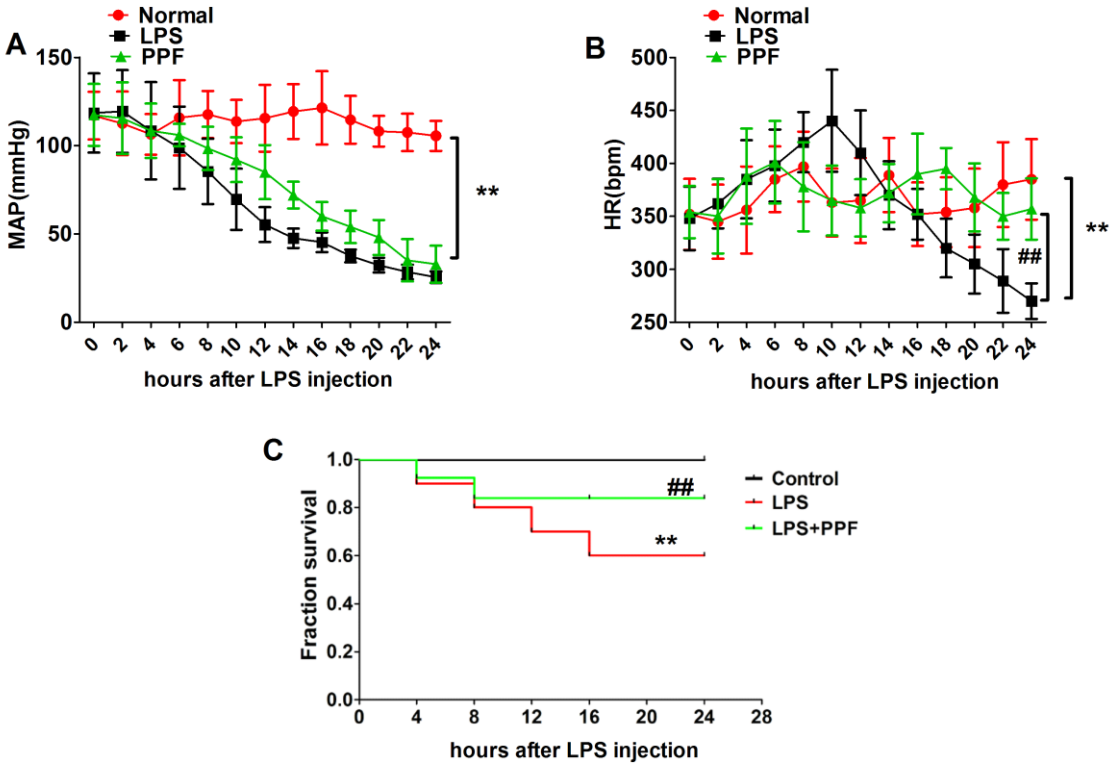

Fig. 1: MAP, HR and survival curves after LPS injection. (A) MAP: mean arterial pressure; (B) HR: heart rates; (C) survival curves; LPS: lipopolysaccharide, PPF: propofol. Data are shown as the mean ± SD, n=6-10; $^{\#\#}P<0.01$, compared to the control group; $^{**}P<0.01$, compared to the LPS group.

ratio was significantly higher and the Treg ratio was markedly lower in the LPS group compared with those in the normal group ($P<0.01$). Interestingly, in PPF-treated septic shock rats, the ratio of Th17 was significantly decreased, while that of Treg was obviously increased ($P<0.01$).

JAK-STAT signalling pathway

As shown in fig. 4, PBMCs were collected, and intracellular protein was extracted. Compared to the control group, the ratio of p-STAT5/t-STAT5 in the LPS group was significantly decreased, while that of p-STAT3/t-STAT3 was significantly increased ($P<0.01$). However, compared to the LPS group, the ratio of p-STAT3/t-STAT3 was significantly decreased and the ratio of p-STAT5/t-STAT5 was significantly increased ($P<0.01$).

DISCUSSION

Septic shock is a complicated syndrome that causes systemic infection-induced multiple organ dysfunctions. It is well known that septic shock causes severe pathophysiological changes, including uncontrolled inflammation and circulation dysfunction (Singer *et al*., 2016; Busani *et al*., 2017; Esposito *et al*., 2017; Cinel and Opal, 2009). LPS, as the main component of the cell wall of Gram-negative bacteria, is an effective trigger of the inflammatory response during infection of Gram-negative bacteria. The binding of LPS to its targets (such as CD14 and TLR4) on the cell surface activates inflammation-related intracellular signalling pathways, including the NF-κβ and MAPK pathways, resulting in the activation of monocytes, macrophages, neutrophils and lymphocytes. As a consequence, LPS increases the secretion of pro-inflammatory mediators, including TNF-a, IL-1 and IL-6 (Peri *et al*., 2010; Zanoni and Granucci, 2013). Therefore, LPS has been widely used to induce sepsis or septic shock in laboratory animals to evaluate the anti-septic activity of drugs and study pathologic mechanisms.

PPF is an intravenous hypnotic agent and is extensively used in the initiation and maintenance of general anaesthesia, sedation of mechanically ventilated adults, and procedural sedation. As previously reported, PPF can alleviate multiple organ dysfunctions and decrease the mortality of septic animals induced by LPS or endotoxin. In 2010, Li S *et al* (Li *et al*., 2010) reported that pretreatment with PPF improved HR and reduced the mortality rate of rats in an endotoxin shock model; in 2013, Ma L *et al* (Ma *et al*., 2013) reported that PPF exerted anti-protective effects on acute lung injury in rats treated with LPS. Moreover, Li J *et al*. (Li *et al*., 2016) recently confirmed that PPF could attenuate LPS-triggered liver dysfunction in rats via inhibition of TNF-α production. In the present study, we found that PPF could reduce mortality and improve HR in septic rats 24 h after

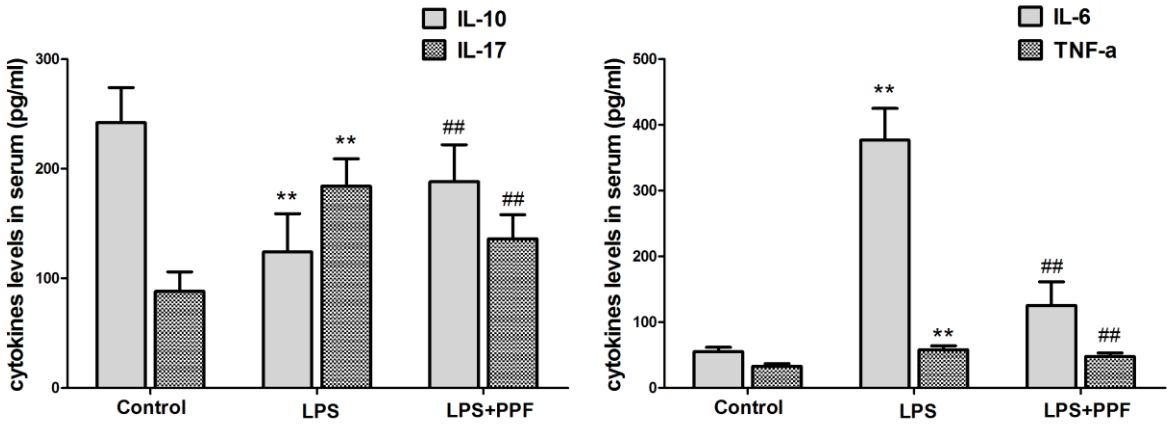

Fig. 2: Serum cytokine profiles at 24 h after LPS injection. Data are shown as the mean ± SD, n=6-10; $^{**}P<0.01$, compared to the LPS group; $^{\#\#}P<0.01$, compared to the control group.

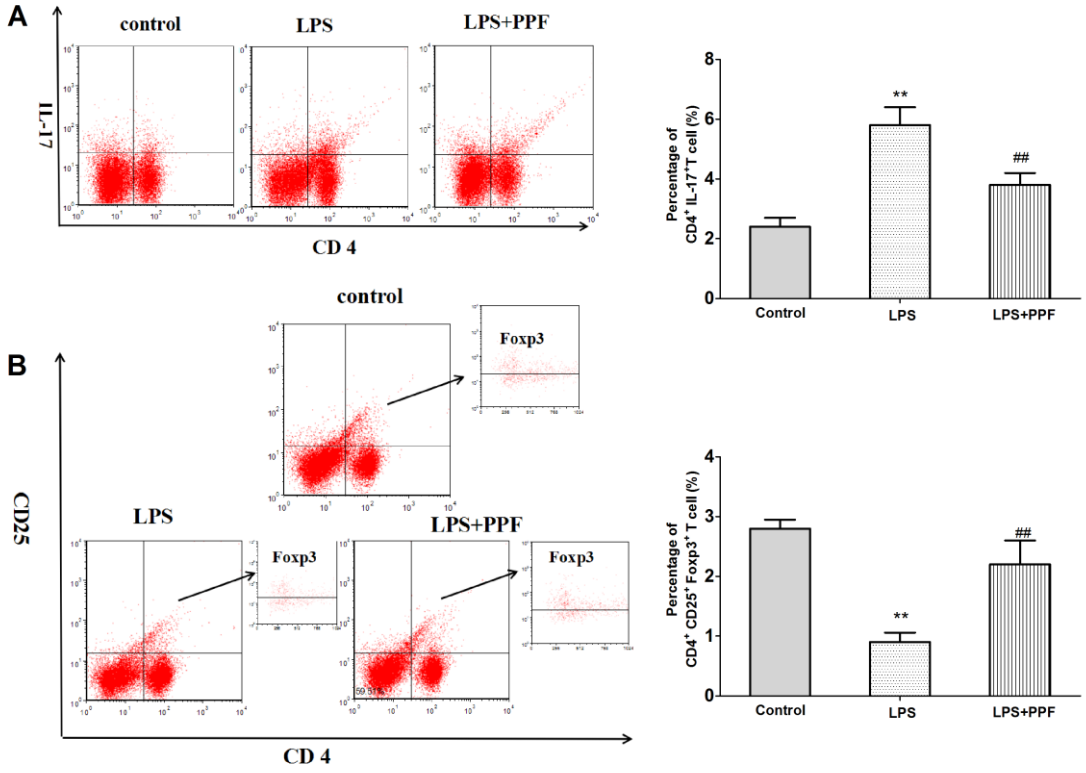

Fig. 3: Th17 and Treg ratios at 24 h after LPS injection. (A): Th17 cells are shown as $CD4^+IL\text{-}17^+$ positive cells; (B): Tregs are shown as $CD4^+CD25^+Foxp3^+$ positive cells; data are shown as the mean ± SD, n=6-10; $^{\#\#}P<0.01$, compared to the control group. $^{**}P<0.01$, compared to the LPS group.

injection of an endotoxin, similar to previously published reports.

Inflammatory and immune reactions are the main pathological foundations of septic shock. In response to LPS or endotoxaemia, pro-inflammatory cytokines are released from activated monocytes, macrophages, neutrophils and other immune cells into surrounding tissues, which results in tissue damage and organ failure (Ayala *et al*., 2003). Therefore, inhibition of the release of pro-inflammatory cytokines has beneficial effects on sepsis. It has previously been shown that PPF has good anti-inflammatory activity against LPS stimulation. In a septic animal model (Singer *et al*., 2016; Busani *et al*., 2017; Esposito *et al*., 2017; Hsu *et al*., 2005), PPF reduces the levels of the inflammatory cytokines TNF-a, IL-6 and IL-1β and down-regulates the expression of CD14 and TLR4, two well-known targets of LPS. In *vitro* investigations (Chiu *et al*., 2009; Li *et al*., 2015; Peng *et al*., 2014; Wei *et al*., 2013; Zhou *et al*., 2015) have shown

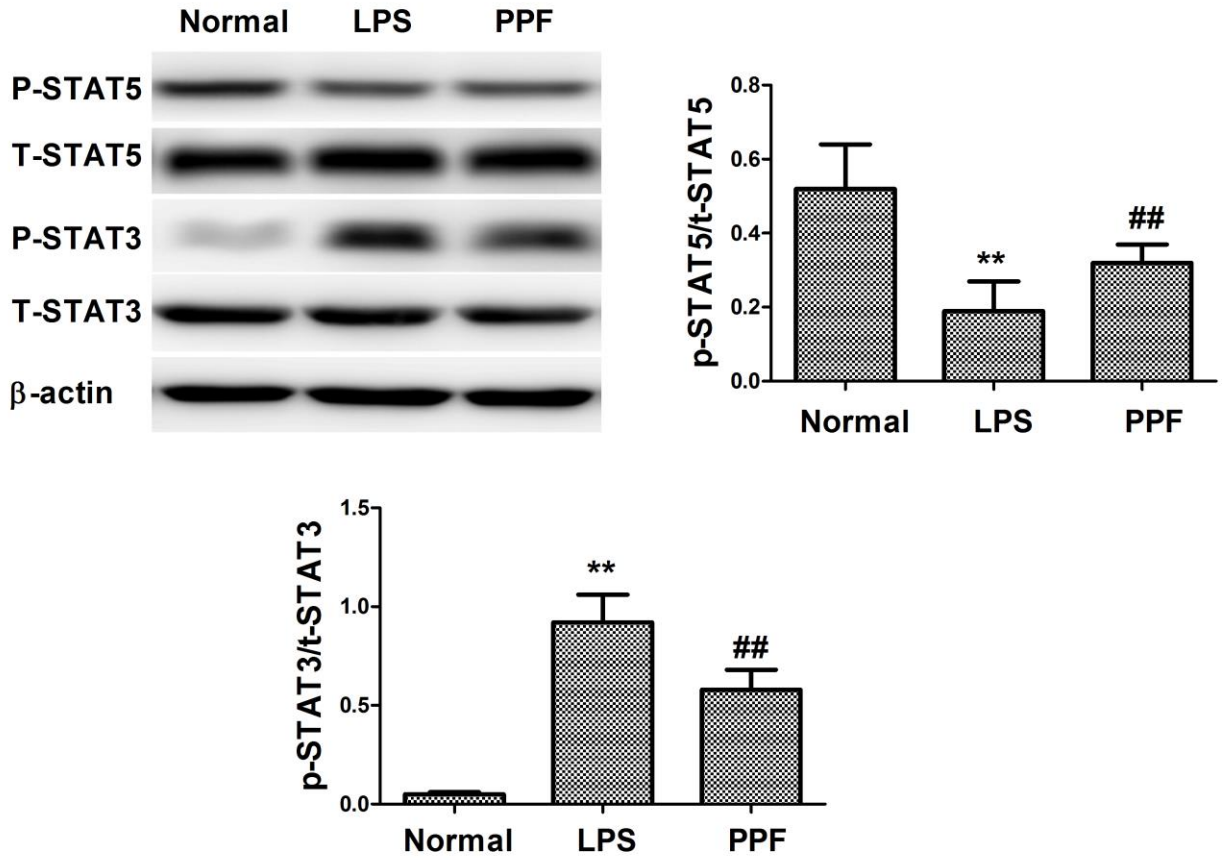

Fig. 4: PPF regulates p-STAT3 and p-STAT5 expression in PBMCs from septic shock rats. Western blots were performed with p-STAT3, p-STAT5, STAT3 and STAT5. Data are shown as the mean ± SD, n=6-10; $^{**}P<0.01$, compared to the normal group; $^{\#\#}P<0.01$, compared to the LPS group.

that PPF decreases the levels of inflammatory mediators, including TNF-a, IL-6, IL-1β, cyclooxygenase-2, prostaglandin E2, NO and MCP-1, in several LPS-treated cells, including macrophages, primary microglia, liver Kupffer cells, alveolar epithelial cells and spinal astrocytes and down-regulates CD14 and TLR4 expression in alveolar type II epithelial cells (Ma *et al*., 2010). In the present study, we found that PPF treatment reduced the TNF-α and IL-6 levels in LPS-induced septic rats, consistent with previously published results.

Th17 and Treg cells are a subset of CD4+ T cells and mainly produce IL-17 and IL-10, respectively. Th17 plays a key role in host defence against extracellular bacteria and fungi. IL-17 is a powerful pro-inflammatory cytokine that can induce the expression of intercellular adhesion molecule 1 and vascular cell adhesion molecule-1, chemokines, TNF-α and IL-6 (Krasimirova *et al*., 2017). By contrast, Tregs induce immune tolerance and produce the anti-inflammatory cytokine IL-10 to inhibit tissue inflammation (Golubovskaya and Wu, 2016; Fasching *et al*., 2017). In recent studies (Wu *et al*., 2013; Guo *et al*., 2017), an imbalanced level of Th17/Treg has been implicated in the development of sepsis. The absolute counts of Th17 and Treg lymphocytes in survivors of severe sepsis were higher than those in non-survivors, and the imbalanced level of Th17/Treg in sepsis is related to the occurrence and prognosis of multiple organ dysfunction syndromes. Therefore, it is suggested that normalization of Th17/Treg is a potential target for preventing sepsis. In previous studies, the mechanism of the inhibitory effects of PPF on the inflammatory response in sepsis was shown to inhibit the expression of LPS-targeted CD14 and TLR4 and subsequently block the MAPK and NF-κß pathways (Ma *et al*., 2013; Chiu *et al*., 2009; Wei *et al*., 2013; Zhou *et al*., 2015). However, the anti-inflammatory mechanism of PPF still needs to be more precisely defined. In the present study, we hypothesized that PPF affects unbalanced Th17/Treg mediated responses. To test our hypothesis, we first focused on the effects of PPF on the ratios of Th17 and Treg in sepsis shock. The results showed that PPF treatment significantly regulated the imbalanced levels of Th17/Treg in the peripheral blood of septic shock rats. It is well known that Th17 and Treg differentiation are regulated by the JAK/STAT signalling pathway. As published previously, IL-6, IL-23 and IL-21 signalling through JAK-mediated phosphorylation of STAT3 are required for Th17 cell generation (Zheng *et al*., 2015). Over expression of STAT3 in Treg cells promotes their

transformation into Th17-like cells (Takahashi *et al*., 2011; Yang *et al*., 2007). Moreover, STAT5-dependent Treg cells enhance Foxp3 expression, while STAT3 is an important inhibitor of Foxp3 (Gardner *et al*., 2013; Murawski *et al*., 2006; Vogtenhuber *et al*., 2010). Therefore, to further investigate the possible mechanism of PPF-regulated Th17 and Treg differentiation, we collected PBMCs from septic shock rats and analysed the expression of p-STAT3, p-STAT5, STAT3 and STAT5. Our data confirmed that PPF therapy significantly increased the expression of p-STAT5, decreased the level of p-STAT5 and had no effects on STAT3 and STAT5. Accordingly, our results suggest that PPF may regulate the Treg/Th17 balance in septic shock, which appears to be caused by the down-regulation of p-STAT3 and up-regulation of p-STAT5.

CONCLUSION

In conclusion, the results of our study clearly show that PPF can regulate the imbalanced level of Treg/Th17 in septic shock rats. Moreover, the modulation of PPF on Treg and Th17 differentiation may occur through regulation of the phosphorylation of STAT3 and STAT5. Our findings suggest that the anti-inflammatory molecular mechanism of PPF might involve increasing the Treg/Th17 ratios and targeting the JAK-STAT signalling pathway.

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