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# Ouabain Attenuates Sepsis-Induced Immunosuppression in Mice by Activation and Anti-Apoptosis of T Cells

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Data Interpretation D  
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**Background:** Sepsis is known to trigger impaired T cell function, which relates to immunosuppression, contributing to refractory infection and high mortality. The mechanisms of T cell recovery remain to be elucidated, and novel and effective therapeutics for sepsis are needed. Ouabain, a small molecule of cardiac glycosides, can reverse immunoparalysis in many settings.

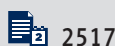
**Material/Methods:** Our study was designed to determine if ouabain can relieve sepsis by modulating T cell response and related pathways. The "two-hit" model of sepsis was applied, established by intraperitoneally LPS injection 3 days after cecal ligation puncture (CLP-LPS). Ouabain was administered to mice intravenously (0.1 mg/kg) after *in vivo* LPS stimulation every day for 4 days. The survival rate of mice, level of serum cytokines, percentage of activated T cells, apoptosis of T cells, and possibly related genes were assessed.

**Results:** The results suggest that ouabain administration after establishment of the CLP-LPS model improved survival rates, elevated pro-inflammatory cytokines, and decreased anti-inflammatory cytokines in serum. More activated T cells and fewer apoptotic T cells were detected in the spleens after treatment with ouabain. Such changes might correlate with the genes of Bcl-2, PUMA, IRAK-M, and SOCS1.

**Conclusions:** Taken together, our data show ouabain is a T cell mediator during sepsis recovery.

**MeSH Keywords:** **Immunosuppression • Ouabain • Sepsis**

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## Background

Sepsis represents the host's systemic inflammatory response to severe infection, trauma, or surgery. The statistical data suggests global estimates of 31.5 million sepsis cases, with 5.3 million deaths annually [1]. At the early stage, sepsis is characterized by the extensive release of pro-inflammatory cytokines and other mediators, resulting in a dysregulated immune response, leading to organ damage. The "cytokines storm" gradually transitions into a state of immunosuppression featuring loss of leukocytes, especially apoptosis of T cells, at the later stage in sepsis survivors [2–4]. It has been reported that the over-activated "cytokine storm" triggered by uncontrolled inflammatory responses is the early sign of septic immune dysfunction, and further apoptosis of lymphocytes eventually leads to irresponsiveness to stimuli as immunosuppression, followed by refractory infection and death [4–6]. Previous studies demonstrated that anti-inflammatory therapies such as TNF-alpha antibodies or TLR4 receptor antagonists have no effect on patient survivals, even worsening the mortality [7,8]. However, the activation of immune response, such as by application of pro-inflammatory cytokines such as IL-7 or IFN-gamma [9–12], shows promising effects on patient survival rate [10,11,13].

Ouabain, a small molecule of cardiac glycosides, antagonizes the activity of  $\text{Na}^+, \text{K}^+$ -ATPase. The family of  $\text{Na}^+, \text{K}^+$ -ATPase inhibitors, including digoxin, have been often administered to treat heart failure and atrial arrhythmia. However, ouabain can act as an inflammatory mediator and is involved in a great number of inflammation-related diseases, such as sepsis [14,15]. Sepsis-induced immunosuppression is mainly characterized by T cell anergy. As mentioned above, IL-7 and IFN- $\gamma$ , which affect patient survival rate, stimulate proliferation of all cells in the lymphoid lineage, especially T cells. Ouabain as a potential inflammatory regulator might have effects on T cells, contributing to amelioration of immunosuppression and mortality in sepsis survivors.

Thus, we hypothesized that ouabain protects against sepsis-induced immunosuppression by activation of T cells, possibly via signaling. To investigate the underlying mechanisms of immunomodulation in treatment using ouabain, we established an animal septic model by a combination of CLP and LPS injection (CLP-LPS) and then evaluated immunological parameters in the absence and presence of ouabain.

## Material and Methods

### Animals

We used healthy adult male C57BL/6 mice that weighed between 20 and 25 g. The mice were obtained from the Laboratory

Animal Center of Fudan University and were kept in specific-pathogen-free conditions at around 22°C on a 12-h light-dark cycle with free access to food and water. All experiments were approved by the Institutional Animal Care and Use Committee of Huashan Hospital, Fudan University.

### Cecal ligation and puncture (CLP) model

C57BL/6 mice, 8–10 weeks old, were used for CLP surgery to induce polymicrobial sepsis. Mice were anesthetized with intraperitoneal injection of chloral hydrate (1 g/kg) and immobilized on an aseptic table. In a sterile operation environment, a 1–2 cm abdominal midline incision was made to expose the cecum, which was mobilized, ligated, and then punctured once with a 22-gauge needle. The bowel was then put back into the abdomen and the incision was sutured with sterile 6-0 silk. The sham-operated mice underwent similar procedures without ligation and puncture. After the surgery, 1 ml of normal saline was injected subcutaneously for fluid resuscitation.

### Experimental protocol, LPS injection, and ouabain treatment

Forty-eight mice were randomly assigned to 4 groups: the sham group received the sham operation without any other treatment; the CLP group received the CLP surgery; the CLP-LPS group intraperitoneally received 5 mg/kg of purified *E. coli* LPS (serotype O111: B4, Sigma-Aldrich, Steinheim, Germany) 3 days after CLP surgery; and the Oua group received 0.1 mg/kg of ouabain (Sigma-Aldrich, St. Louis, MO, USA) intravenously via tail vein every day for 4 days after LPS challenge.

### Enzyme-linked immunosorbent assays (ELISAs)

Mouse heart blood was harvested, heparinized, and centrifuged at 12 000 rpm for 15 min, by which serum were collected. Mouse TNF- $\alpha$ , IL-6, and IL-10 levels were detected from serum according to the manufacturer's instructions (R&D Systems, USA). The results were measured using the Microtiter Plate Reader (TECAN, Switzerland) at 450 nm, in triplicate.

### Flow cytometry and apoptosis analysis

Mouse cells were stained following the manufacturers' protocols with optimal concentrations of mAb with the following antibodies: anti-CD44-PE (eBioscience, USA), anti-CD4-APC (R&D Systems, USA) and anti-CD8a-APC (R&D Systems, USA). Briefly, splenocytes from the mice were obtained by dissecting and mincing the spleens, which were prepared in PBS and stained with the antibodies at 4°C for 15 min. The cells were washed 3 times and resuspended in 200  $\mu\text{L}$  PBS for flow cytometry. Fluorescent data for 10 000 lymphocyte events per sample were acquired on a FACS LSR II (BD Bioscience, USA)

and were analyzed using FlowJo software (Tomy Digital Biology Co., Ltd., Japan). The 2 types of activated T cells were marked as CD4<sup>+</sup>CD44<sup>+</sup> or CD8<sup>+</sup>CD44<sup>+</sup>. Alexa Fluor 488 Annexin V-FITC (BD, USA) was used for analysis of apoptosis, by which apoptotic T cells were defined as CD4<sup>+</sup>AnnexinV<sup>+</sup> or CD8<sup>+</sup>AnnexinV<sup>+</sup>. All of the measurements were performed at least in triplicate.

### Real-time RT-PCR

Total RNA was isolated from splenocytes using the Total RNA Purification Kit (Sigma, USA) and reverse-transcribed to cDNA according to the manufacturer's instructions. Gene transcription was quantified on the 7900HT Fast Real-time PCR system (Life Technology Corporation, USA) using SYBR green dye and normalized with GAPDH. Primer sets used were:

IRAK-M (Interleukin-1 receptor-associated kinase M):

F: 5'-ACGCTACTGAAGGCACTGAG-3',

R: 5'-TTTGACTGAGAGGCGGTAC-3';

SOCS1 (Suppressor of cytokine signaling 1):

F: 5'-TAACCCGGTACTCCGTGACT-3',

R: 5'-CTCCACGTGTTCCAGAAA-3';

Bcl-2 (B-cell lymphoma 2):

F: 5'-TCCGCTGAAGGCTTGAGATGT-3',

R: 5'-TCCCTTTCTAGACCCAG-3';

PUMA (p53 upregulated modulator of apoptosis):

F-5'-AGATATTGGCGGAAGCCACC-3',

R-5'-CCAGATGCTGTGCTACTGGT-3';

GAPDH (Glyceraldehyde 3-phosphate dehydrogenase):

F-5'-GAGAGTGTTCCTCGTCCCGTAG-3',

R-5'-GCCTCACCCATTTGATGTTAGT-3'.

All measurements were performed in triplicate.

### Western blot analysis

Splenocytes were collected and lysed with RIPA lysis buffer. After sonication, the lysates were centrifuged; the proteins were separated using SDS-PAGE and then transferred to PVDF membranes. After being blocked with 10% skim milk in Tris-buffered saline at room temperature for 1 h, the membrane was incubated with primary antibodies against IRAK-M, SOCS1, Bcl-2, PUMA, and beta-actin (1: 1000) overnight at 4°C. The membranes were washed 3 times with TBST, and then incubated with HRP-conjugated secondary antibody for 1 h at room temperature. The blots were imaged using the Bio-Rad imaging system and quantified by ImageJ.

### Statistical analysis

Data are presented in the figures as the mean ±SD. For each figure, statistical tests are justified, as appropriate. The significant difference between 2 groups was determined with the two-tailed Student's *t* test. Multiple comparisons were made using analysis of variance (ANOVA). The discrepancy in

survival rates was analyzed by Gehan-Breslow-Wilcoxon test. SPSS software, version 16.0, was used for all of the statistical analyses, and differences with a *P* value less than 0.05 were considered statistically significant (\* *P*<0.05).

## Results

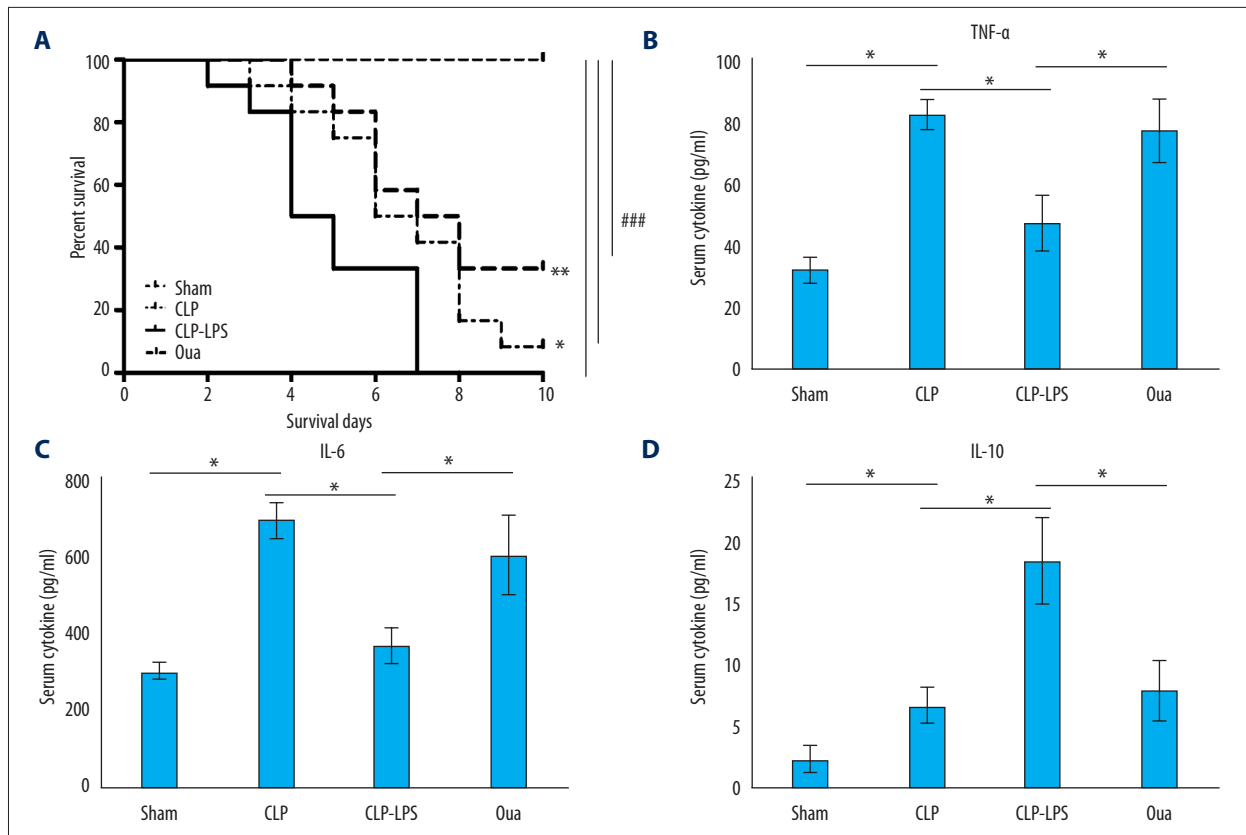
### Ouabain improved survival rate in septic mice in parallel with increased inflammatory response

We first investigated if ouabain increased survival rate in the CLP-LPS mice. The survival rate is shown in Figure 1A. The overall survival rate in the sham group was 100%. However, there was a dramatic decrease in the survival rate for the CLP or CLP-LPS or CLP-LPS+Oua groups (8.3% or 0% or 33.3%, versus sham group, *P*<0.001) at day 10. In this "two-hit" model, mice with LPS challenge (5 mg/kg, i.p.) after 3 days of CLP displayed a significant increase in mortality compared with mice subjected to CLP alone (*P*<0.05). Ouabain improved survival when intravenously administered at a dose of 0.1 mg/kg at 0, 24, 48, and 72 h after LPS injection (*P*<0.01).

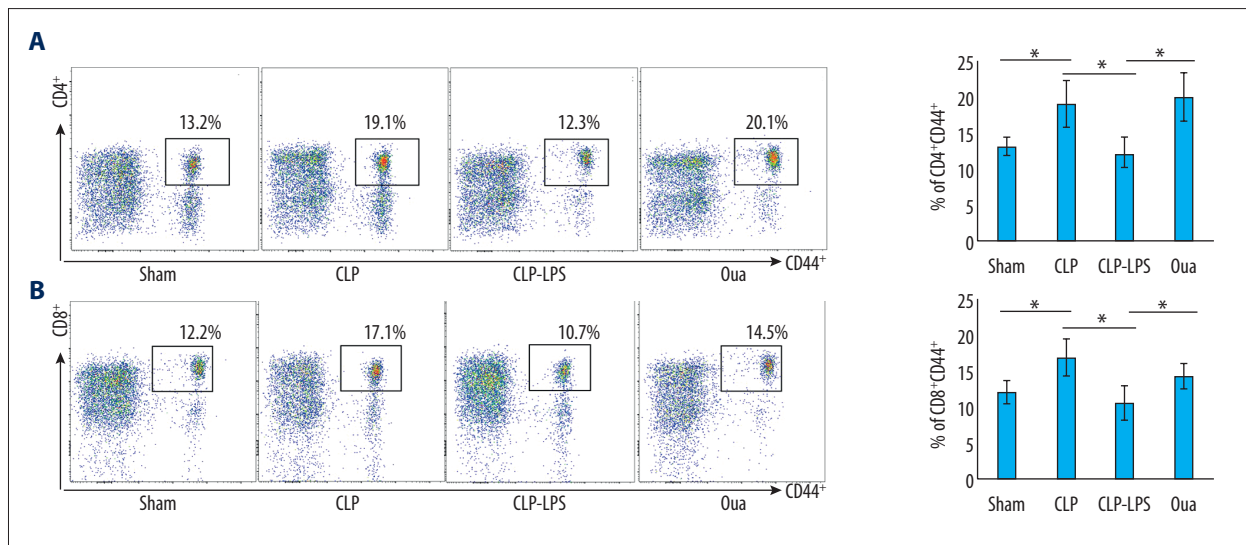
As shown in Figure 1B–1D, the serum cytokine level in the sham animals was kept at the lowest stage. The CLP procedure alone enhanced the cytokine level (*P*<0.05), while the immunoparalysis caused by CLP-LPS process was associated with a decrease in pro-inflammatory cytokine level (TNF-α and IL-6) and an increase in anti-inflammatory cytokine level (IL-10) in comparison with the CLP group (*P*<0.05), as ouabain ameliorated these changes by immunoparalysis (*P*<0.05). These results indicate that ouabain treatment improved the survival rate of septic mice and restored pro-inflammatory cytokine responses in septic immunosuppression mice.

### Ouabain elevated activated T cells in septic immunosuppression mice

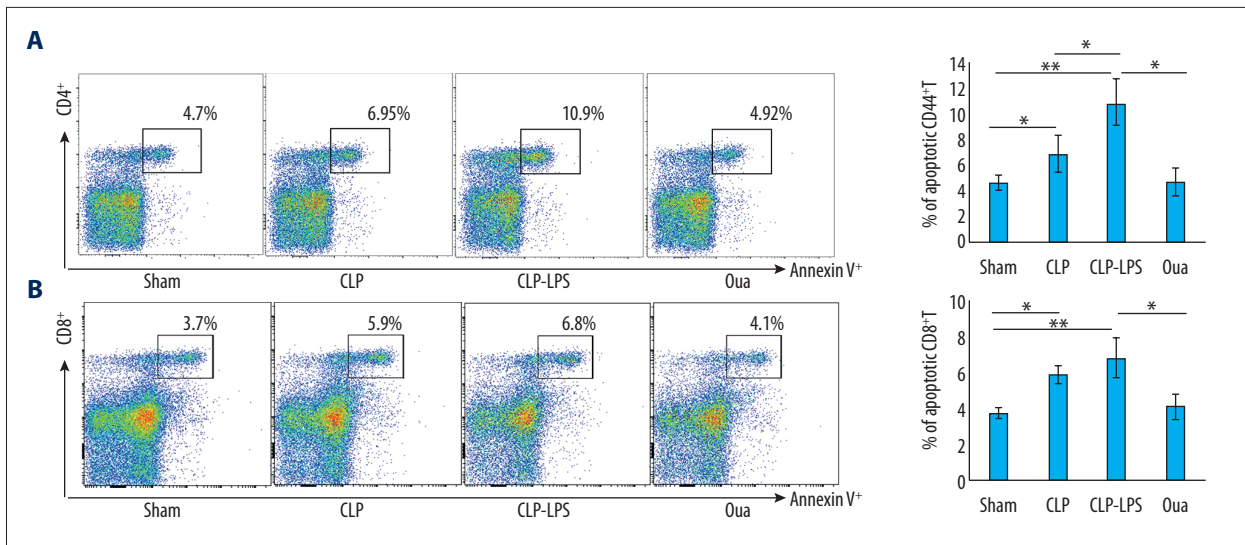
As ouabain might exert protective effects on septic immunosuppression characterized by T cell anergy and loss in mice, we explored whether ouabain positively regulated T cells in the CLP-LPS model. Thus, we mainly evaluated the percentage of activated CD4<sup>+</sup>CD44<sup>+</sup> and CD8<sup>+</sup>CD44<sup>+</sup> activated T lymphocytes in the absence or presence of ouabain at day 7. An increase in both activated CD4<sup>+</sup> and CD8<sup>+</sup>T cells was observed in the spleen 7 days after CLP induction when compared with the sham group (*P*<0.05), while additional LPS stimulation diminished percentage of the T cells (*P*<0.05). Ouabain significantly rescued the decline of the activated CD4<sup>+</sup> and CD8<sup>+</sup>T cells by CLP plus LPS (Figure 2A, 2B).



**Figure 1.** Protective effect of ouabain on the survival rate after CLP-LPS treatment and enhanced serum inflammatory cytokine level. (A) Mice were treated with ouabain after CLP-LPS, and ouabain was given every day for 4 days after LPS challenge. Values are expressed as survival percentage (n=12 for each group). \* P<0.05 and \*\* P<0.01 in comparison to the CLP-LPS group, and ### P<0.001 in comparison to the sham group. (B-D) TNF-alpha, IL-6, and IL-10 cytokine levels in serum were analyzed at day 7 using ELISA assay. Data are mean ±SD of 6 mice per group. \* P<0.05, ANOVA.



**Figure 2.** Increased percentage of activated T cells in the spleens by ouabain injection. (A, B) Representative pseudocolor plots show the percentage of activated T cells (CD4<sup>+</sup>CD44<sup>+</sup> and CD8<sup>+</sup>CD44<sup>+</sup> cell populations) in the spleens within 4 different groups at day 7. Activated T cells enhanced by CLP surgery and decreased by additional LPS strike. The reversal effects of ouabain on activated T cells was found in the CLP-LPS + ouabain group. Data are shown as mean ±SD of 6 mice per group. \* P<0.05, ANOVA.



**Figure 3.** Diminished percentage of apoptotic T cells in the spleens by ouabain treatment. **(A, B)** Representative pseudocolor plots show the apoptotic CD4<sup>+</sup> (CD4<sup>+</sup>Annexin V<sup>+</sup>) and CD8<sup>+</sup> (CD8<sup>+</sup> Annexin V<sup>+</sup>) T cells in 4 different groups at day 7. An increase in apoptotic T cells was observed in the CLP-LPS group compared with the sham and CLP groups, while application of ouabain attenuated apoptosis of T cells. Data are shown as mean  $\pm$ SD of 6 mice per group. \*  $P < 0.05$ , ANOVA.

### Ouabain ameliorated apoptosis of T cells in septic immunosuppression mice

After we proved the elevated level of splenic activated T lymphocytes by ouabain injection, we tried to further identify whether this elevation was associated with decreased apoptosis of T cells. Thus, we analyzed apoptosis in splenic CD4<sup>+</sup> and CD8<sup>+</sup> T lymphocytes. An increased apoptotic rate in both CD4<sup>+</sup> and CD8<sup>+</sup> T cells was detected in the spleen 7 days after CLP induction ( $P < 0.05$ ), and additional LPS administration exacerbated apoptosis of the T cells ( $P < 0.05$ ). Ouabain significantly reversed the increase of apoptotic T cells by CLP plus LPS (Figure 3A, 3B).

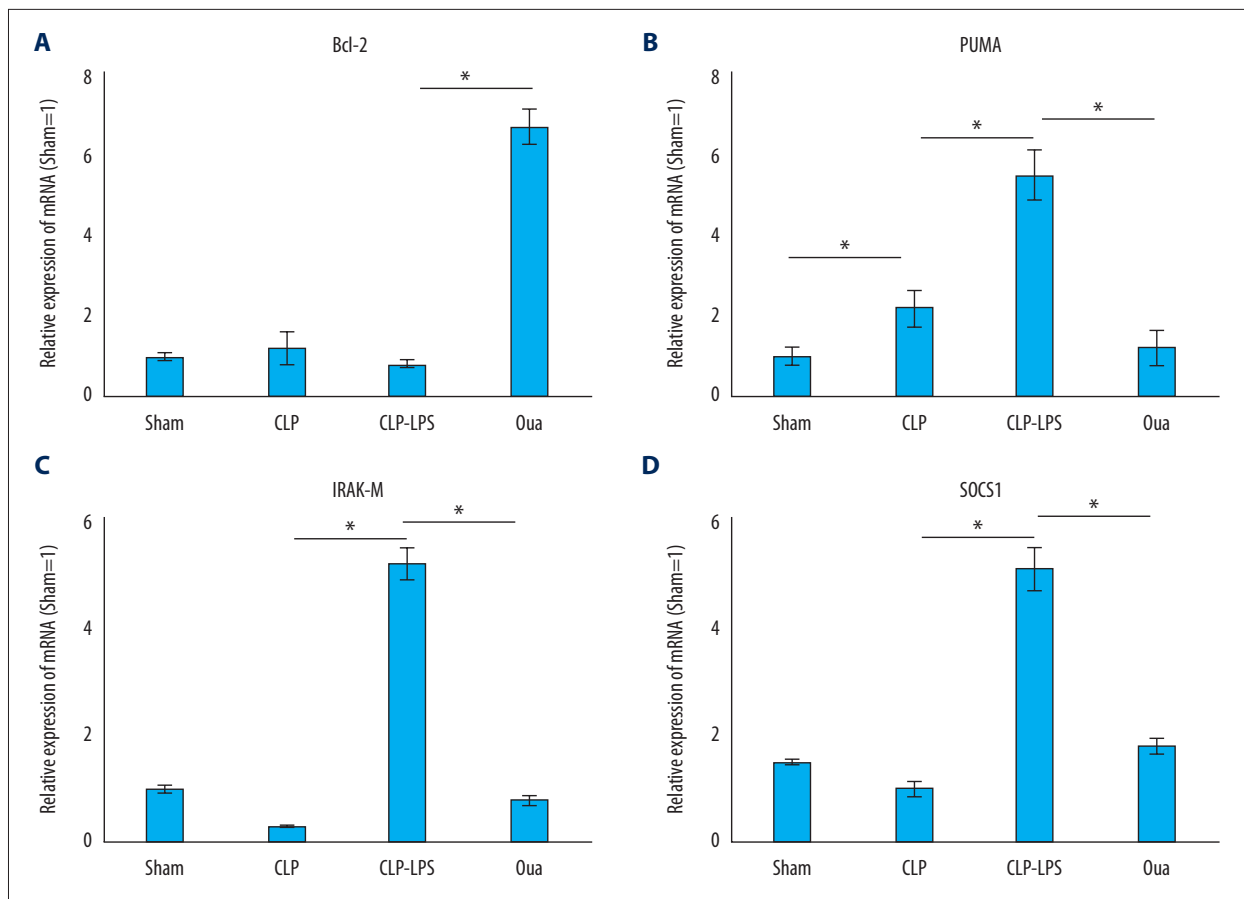
### Ouabain mediated the molecules with respect to apoptosis and inflammation

After discovering the protective effects of ouabain on septic immunosuppression mice, we next studied its underlying molecular mechanisms. Owing to abatement of apoptosis by ouabain treatment, we focussed on analyzing Bcl-2 (anti-apoptotic molecule) and PUMA (p53 upregulated modulator of apoptosis, PUMA, pro-apoptotic molecule). We found significantly higher mRNA and protein expression of PUMA in the CLP group than at the basal level ( $P < 0.05$ ). Upon stimulation with LPS, the mRNA and protein levels of PUMA in murine splenocytes rose ( $P < 0.05$ ). On the contrary, the transcriptional and translational level of Bcl-2 tended to decline after LPS challenge. In comparison with the CLP-LPS group, ouabain upregulated expression of Bcl-2 and downregulated expression of PUMA, as expected, in both RT-PCR and Western blot analysis (Figures 4A, 4B, 5A, 5B).

TLR signaling is pivotal in immunoregulation of T cells; therefore, we sought to determine if the negative regulators of TLR signaling, such as IRAK-M and SOCS1, play a role in ouabain-induced immunomodulation. A decrease in mRNA and protein levels of IRAK-M was observed in the CLP group compared to baseline. With additional LPS stimulation, the mRNA ( $P < 0.05$ ) and protein ( $P < 0.001$ ) levels of IRAK-M, and the mRNA ( $P < 0.05$ ) level of SOCS1, were enhanced in splenocytes. Compared with the CLP-LPS group, ouabain abolished the up-regulation of IRAK-M and SOCS1 by CLP-LPS treatment, as shown by analysis of RT-PCR and Western blot results (Figures 4C, 4D, 5A, 5B). Our results show that ouabain treatment can regulate molecules involved in apoptosis, such as Bcl-2 and PUMA, and promote immune response via down-regulating the negative regulators in TLR signaling, such as IRAK-M and SOCS1, thereby protecting lymphocytes and leading to mitigation of septic immunosuppression in mice.

## Discussion

Sepsis is the leading cause of death in non-cardiac ICUs worldwide [1], resulting from a systematic immune response to an infection, which leads to an early hyper-inflammatory state, followed by a state of immunosuppression [4–6], mainly characterized by T cell anergy and apoptosis. Much evidence has indicated that the approaches to overcome the early phase of over-activated inflammatory responses fail to improve the high mortality of sepsis [7,8], with additional serious adverse effects. Nonetheless, the approaches to address the later phase of septic immunosuppression managed to alleviate mortality by improving immune dysfunction [10–12].



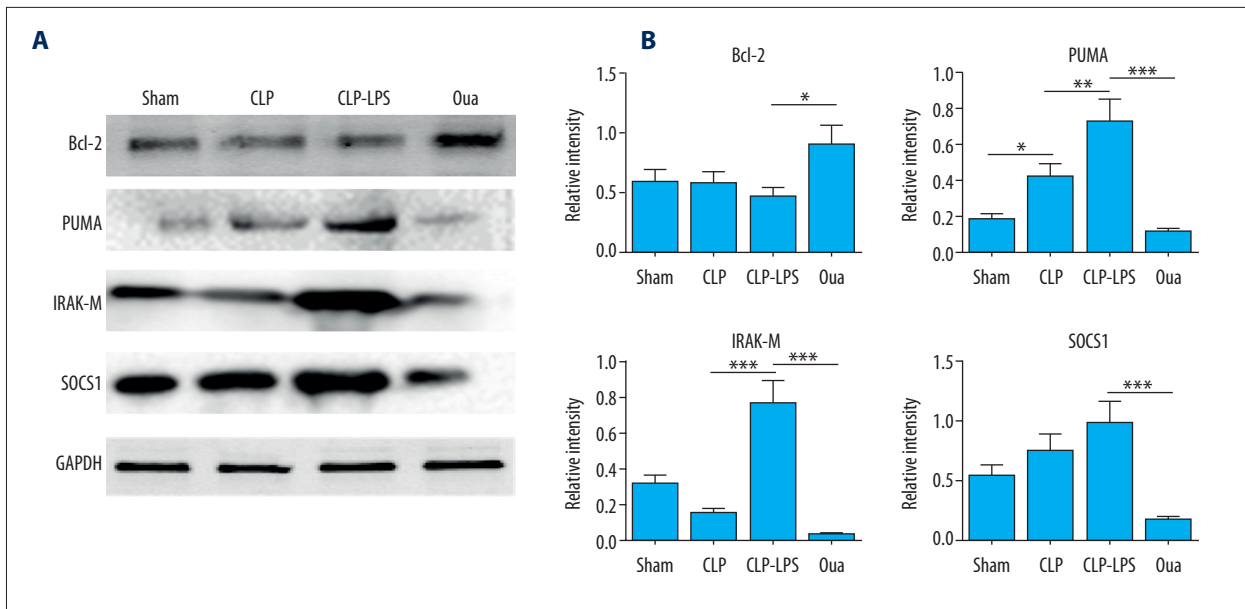
**Figure 4.** Change in inflammation mediatory genes by ouabain in the splenocytes. (**A, B**) For mRNA expression analysis, anti-apoptotic gene Bcl-2 and pro-apoptotic gene PUMA were determined by Q-PCR in 4 different groups. (**C, D**) Negative regulators IRAK-M and SOCS1 mRNA expression was quantified by Q-PCR in different groups. The mRNA expression was normalized to GAPDH and relative expression levels are depicted. The level in the sham group is regarded as 1. Data are expressed as the mean  $\pm$ SD of 3 mice per group. \* P<0.05, ANOVA.

Ouabain is a cardiac glycoside secreted by the adrenal glands. Most relevant studies have investigated its function in inhibiting the activity of Na<sup>+</sup>-K<sup>+</sup> ATPase. Recently, more evidence has shown that ouabain exerts its effects on immune response. Quastel et al. [15] proposed that ouabain acts as a suppressor of lymphocyte transformation, and a study by Yin et al. [14] demonstrated that ouabain can regulate the expression of Th1 cytokines in monocytes and thus mediate pro-inflammatory responses. However, so far, little is known about its direct influence on T cell activation and apoptosis or its regulatory roles in immune dysfunction during sepsis.

In our study, we used a CLP model combined with LPS injection (CLP-LPS) to induce septic immunosuppression, by which Yin's immunosuppressive model [14] was modified, as proved and validated by our cytokine and T cell measurement data, with adequate reliability and reproductivity. By intravenously injecting ouabain into septic mice, we found that ouabain improved the survival rate, with an increase in pro-inflammatory

mediators and a decrease in anti-inflammatory factors, in which activated T cells possibly play a role.

The viability of T cells is balanced by activation and apoptosis. Consequently, we selected IRAK-M and SOCS1 as activation targets and Bcl-2 and PUMA as apoptosis targets. IRAK-M is a negative regulator of TLR signaling and its expression is restricted to monocytes/macrophages. However, a recent study showed that IRAK-M<sup>-/-</sup> naïve CD4<sup>+</sup> T cells were more prone to differentiate to pro-inflammatory Th17 subset, which indicates that IRAK-M is related to immunosuppression in T cell-mediated immune response, supported by our results that ouabain decreased the level of IRAK-M. SOCS1 is a protein that functions downstream of cytokine receptors, taking part in attenuating cytokine signaling. It contributes to peripheral T cell tolerance by slowing aberrant T cell activation driven by inflammatory cytokines, which might be related to our results showing the potential of ouabain to downregulate SOCS1 and thus initiate T cell response. The expression alterations of Bcl-2



**Figure 5.** Alteration of translational profiles by ouabain in the splenocytes. **(A)** Representative images of the Western blot results are shown. **(B)** Ouabain increased expression of Bcl-2 and abated expression of PUMA, IRAK-M, and SOCS1. The protein relative intensity is the ratio of target proteins to GAPDH. Data are expressed as the mean  $\pm$  SD of 3 mice per group. \*  $P < 0.05$ , ANOVA.

and PUMA in splenocytes might show an anti-apoptotic effect of ouabain on T cells. While some changes have been detected in many genes involved in immune response, the cause-effect relations between ouabain and IRAK-M, SOCS1, Bcl-2, and PUMA on T cells still remain to be verified by cell sorting, silencing, and more *ex vivo* experiments.

Overall, our study suggests that ouabain confers a significant protective effect against immunosuppression induced by sepsis via T cell response, yet the underlying mechanisms by which ouabain mediates all types of immune cells are still poorly investigated. Therefore, further research is needed to discover the molecular mechanisms by which ouabain affects different leukocytes during sepsis, including a comprehensive examination of clinical use of ouabain in the treatment of sepsis. In addition, it should be noted that although the immunomodulation of ouabain was partly investigated in our research, the exact mechanisms through which it improves sepsis outcomes may be multifactorial. Future research might also focus on the possible reversal effects on other pathogenesis of sepsis, such as apoptosis of vascular endothelial cells mediating

septic disseminated intravascular coagulation (DIC), or secretion of glucocorticoid and its profound effects on anti-inflammatory immune response.

## Conclusions

Our results demonstrate the possible protective effects of ouabain in septic immunosuppression mice by improving survival rate, associated with elevated immune response and diminished vulnerability of T cells. The optimal T cell response achieved by ouabain might be correlated with apoptotic and TLR signaling. This study provides evidence for use of ouabain in immunoregulatory therapy during sepsis.

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