Therapeutic efficacy of Schistosoma japonicum cystatin on sepsis-induced cardiomyopathy in a mouse model

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Abstract

Background

Myocardial dysfunction is one of the most common complications of multiple organ failure in septic shock and significantly increases mortality in patients with sepsis. In spite of many studies have confirmed that helminth-derived proteins have strong immunomodulatory functions and could be used to treat inflammatory diseases, there is no report on the therapeutic effect of *Schistosoma japonicum*-produced cystatin (*Sj*-Cys) on the sepsis-induced cardiac dysfunction.

Methods

A model of sepsis-induced myocardial injury was established by cecal ligation and puncture (CLP) in mouse. Upon CLP operation, each mouse was intraperitoneally treated with 10 μg of recombinant *Sj*-Cys (r*Sj*-Cys). Twelve hours after CLP operation, the systolic and diastolic functions of left ventricular were examined by echocardiography. The levels of myoglobin (Mb), cardiac troponin I (cTnI), N-terminal pro-Brain Natriuretic peptide (NT-proBNP) in sera and the activity of myeloperoxidase (MPO) in cardiac tissues were examined as biomarkers for heart injury. The heart tissue was collected for checking pathological changes and pro-inflammatory cytokine levels. To address the signaling pathway involved in the anti-inflammatory effects of r*Sj*-Cys, myeloid differentiation factor 88 (MyD88) was determined by Western blot in heart tissue of mice with sepsis and LPS-stimulated H9C2 cardiomyocyte cells. In addition, the therapeutic effects of r*Sj*-Cys on LPS-induced cardiomyocyte apoptosis were also detected in H9C2 cells. The pro-inflammatory cytokines TNF-α and IL-6 and regulatory cytokines IL-10 and TGF-β were measured in sera and their mRNA levels in heart tissue of r*Sj*-Cys-treated mice.

Results

After being treated with r*Sj*-Cys, the sepsis-induced heart malfunction was largely improved. The inflammation and injury of heart tissue were also significantly alleviated characterized by significantly decreased infiltration of inflammatory cells in cardiac tissues and fiber swelling, reduced levels of Mb, cTnI and NT-proBNP in sera and MPO activity in heart tissue. The therapeutic efficacy of r*Sj*-Cys is associated with down-regulated pro-inflammatory cytokines (TNF-α, IL-6) and up-regulated regulatory inflammatory cytokines (IL-10, TGF-β), possibly through inhibiting LPS-TLR4- MyD88 signal pathway.

Conclusions

Recombinant *Sj*-Cys significantly reduced sepsis-induced cardiomyopathy and could be considered as be a potential therapeutic agent for the prevention and treatment of sepsis associated cardiac dysfunction.

Background

Sepsis is life-threatening organ dysfunction caused by serious infection, affecting the lives of millions of people around the world [1, 2]. Myocardial dysfunction is a common complication of hospitalized sepsis

patients, and myocardial depression occurs in 40%-50% of patients with sepsis [3, 4]. Septic myocardial dysfunction is associated with overproduction of pro-inflammatory cytokines, including IL-6 and TNF-α, which play pivotal roles in myocyte apoptosis and injury [3, 5]. In recent years, studies have suggested that sepsis-induced cardiac dysfunction, the major cause of sepsis mortality (70-90%) [6], is caused by myocardial apoptosis mediated by MyD88 signal pathway that activates and over-expresses a variety of pro-inflammatory cytokines, including TNF-α and IL-6 [7]. At present, the control of sepsis-induced cardiac failure depends on drug therapies. The most commonly used drugs for the treatment of sepsis-induced cardiac dysfunction are glucocorticoid, norepinephrine, low molecular weight heparin and antibiotics. Although these drugs are capable of preventing the development of inflammation, activating the anti-coagulation system, enhancing anti-inflammatory function, or suppressing the bacterial proliferation, there is still a part of patients who cannot survive from the severe sepsis. Other alternative approach to better control sepsis and reduce sepsis related myocardial dysfunction is largely needed.

Parasitic helminths co-evolve with mammalian hosts to develop some strategies to survive within hosts. These strategies include immune-modulating host immune system to down-regulate their immune response to helminths (parasite-specific immunomodulation) [8], characterized by a dominant Th2-mediated immune response and activated egulatory T (Treg) cell or monocyte responses [9, 10, 11]. The helminth-induced regulatory responses not only facilitate the survival of worms in the host, but also benefit host to reach immune homeostasis between the resistance and tolerance and reduce immunopathology [12]. Helminth infection induced alternately activated macrophages (AAM) [13] and Tregs play important roles in the control of inflammation and tissue repair [13, 14, 15]. More evidences showed that helminth infection induced host immunomodulation through secreted proteins as mediators between parasites and hosts [16, 17]. Due to their potent immunomodulatory functions, helminth infections or helminth-derived or secreted proteins have been used as therapeutic regents to treat some immunno-inflammatory diseases such as allergies and autoimmune disorders [18, 19, 20]. In particular, cystatin derived from various parasitic helminthes receives the most attention because they have been identified as strong immunomodulatory proteins [14] and successfully used as potential therapeutic agents for inflammatory and autoimmune diseases [21, 22, 23, 24, 25]. Parasitic helminth cystatins have been demonstrated to ameliorate arthritis, asthma and colitis [14, 22, 24, 25]. *Sj*-Cys is a cysteine protease inhibitor (cystatin) derived from a blood-feeding trematode *Schistosoma japonicum* [26]. Treatment with r*Sj*-Cys protein significantly stimulated Treg cells and inhibited the antigen-presenting functions of DCs [27]. It also inhibited the release of pro-inflammatory factors (TNF-α, IL-6) in LPS-stimulated macrophages [23]. Recombinant *Sj*-Cys protein has been used as therapeutic agent to alleviate the severity of DSS-induced colitis in mice [21] and murine collagen-induced arthritis [22]. Our previous study has identified that r*Sj*-Cys displayed the therapeutic effect on cecal ligation and puncture (CLP)-induced bacterial sepsis characterized by the increased survival rates, alleviated overall disease severity and tissue injury of liver, kidney and lung [12]. These therapeutic effects are associated with downregulation of pro-inflammatory cytokines and upregulation of regulatory cytokines [12]. In this study, we explore the therapeutic effect of r*Sj*-Cys on sepsis-triggered cardiac dysfunction and we found that treatment with r*Sj*-Cys protein

significantly reduced the sepsis-induced cardiomyopathy and heart injury in a mouse model, providing an alternative approach to control sepsis-induced heart failure and death.

Materials And Methods

Production of recombinant r*Sj*-Cys

DNA coding for *Sj*-Cys was cloned in-frame into pET28a and the sequencing confirmed recombinant plasmid DNA with correct insert was transformed into *E. coli* BL21 by calcium transfection method. The recombinant *Sj*-Cys (r*Sj*-Cys) with His-tag at N-terminus was induced with 1 mM isopropylthio-β-galactoside (IPTG, Sigma-Aldrich, Steinheim, Germany) at 37℃ for 5 hours, and purified from the induced bacteria soluble fraction using a HisPur™ Ni-NTA Spin Column (Thermo Fisher Scientific Inc., USA). The contaminated endotoxin was removed from the purified recombinant protein using a ToxOut™ High Capacity Endotoxin Removal Kit (BioVision, Palo Alto, California, USA) and the residual endotoxin level was measured by ToxinSensor™ Chromogenic Limulus Amebocyte Lysate (LAL) Endotoxin Assay Kit (GenScript Biotechnology, Nanjing, China) following the manufacturer's protocol. The concentration of recombinant *Sj*-Cys was measured using a Bicinchoninic Acid Protein Assay Kit (Beyotime Biotechnology, Shanghai, China) and the recombinant protein stored at -80 °C until use.

Animals

Male BALB/c mice with 6-8 weeks old (body weight: 20~22g) and specific pathogen free (SPF) were purchased from Anhui Medical University Experimental Animal Facility (animal ethics approval number: AMU26-08061). The mice were housed in a climate-controlled facility maintained at 23 ± 1° C and 55 ± 5% humidity with a 12 h light/dark cycle and free access to food and water ad libitum. The animal experiment was performed based on an IACUC approved protocol of AMU26-08061.

Sepsis-induced cardiomyopath

The mice were subjected to CLP surgery according to the method described previously [4]. Briefly, mice were fasted for 12 h with drinking water only and then anesthetized by intraperitoneal injection of 0.2 ml/20 g of 4% chloral hydrate. Following a 1-2 cm midline laparotomy incision, 66% of the cecum was ligated with a 4-0 silk tie (Syneture, Norwalk, CT). A through-and-through puncture was made on the anti-mesenteric side with an 18-gauge needle and a small amount of feces was extruded through the puncture holes to ensure perforation. The cecum was placed back in its original location and the abdomen was closed in two layers with 4-0 silk. Following CLP, sterile normal saline (300 μl) was injected subdermally for fluid resuscitation. Sham mice underwent the above process except for CLP.

Treatment of sepsis-induced cardiomyopathy with r*Sj*-Cys

Total 6 CLP-operated mice were treated intraperitoneally with 10 μg of r*Sj*-Cys in a total volume of 200 μl 30 min after surgery. The same number of CLP-operated mice were given with 200 μl of PBS only as control. As normal controls, 12 mice with sham surgery were divided into two groups and 6 received the

same amount of r*Sj*-Cys and 6 received PBS only. Twelve hours later, all mice were measured for echocardiography. Blood was collected from each mouse under anesthesia and sera were separated by centrifuged and stored at -80°C until use. All mice were euthanized and hearts were collected for histopathological staining and measurement.

Echocardiography

After the mice were treated in different ways, echocardiographic evaluation was performed using a high-resolution echocardiograph (Vevo 2100, VisualSonics, Canada). Briefly, a mixture of 1% isoflurane and oxygen was inhaled via a nose cone, and each mouse was carefully kept under mild anesthesia and then subjected to M-mode and Doppler echocardiography according to the method described [28]. The ejection fraction (EF %) and fractional shortening (FS %) of left ventricle were calculated from M-mode tracing to reflect left systolic function. Peak early-diastolic transmitral velocities (E wave) and peak late-diastolic transmitral velocities (A wave) across mitral valve inflow were examined on Doppler flow tracings and were used to calculate E/A ratios, a commonly used parameter of left ventricular diastolic function. All echocardiographic procedures are performed by the same skilled operator and the data are averaged form at least three consecutive cardiac cycles.

Histological examination of myocardium

Mouse hearts collected from different experimental groups were fixed in 4% buffered paraformaldehyde for 12 hours. Fixed heart left ventricles were sectioned and stained with hematoxylin and eosin (H&E) stain. H&E stained sections were observed under light microscopy (200 ×) (Nikon, Tokyo, Japan) for pathological changes.

Biochemical Analysis

The heart-released myoglobin (Mb), cardiac troponin I (cTnI) and N-terminal pro-Brain Natriuretic peptide (NT-proBNP) in sera and myeloperoxidase (MPO) in heart tissue were measured as biochemical markers for heart injury. The levels of cTnI and NT-proBNP in sera were detected using an enzyme-linked immunosorbent assay (ELISA) kit (Elabscience Biotechnology Co., Ltd, Wuhan, China). The concentration of Mb was measured in the mouse sera by a Fully Automated Biochemistry Analyzer (Beckman Coulter, Brea, California, USA). The heart tissue was weighed and homogenized, the MPO activity in the homogenate was determined using a MPO test kit (Bioenginering Institute, Nanjing, China).

Detection of IL-6, TNF-α, TGF-β and IL-10 in sera and cell supernatants

The concentration of pro-inflammatory (TNF-α, IL-6) and regulatory (IL-10, TGF-β) cytokines in cell culture supernatants and experimental mouse sera were detected by ELISA in accordance with the manufacturer's instructions (ABclonal Biotechnology Co., Ltd. Wuhan, China).

Detection of cardiac TNF-α, IL-6, IL-10 and TGF-β mRNA expression by quantitative real time PCR (qRT-PCR)

Total RNA from left ventricular myocardium was extracted with QIAzol reagent (Ambion, USA). Then cDNAs were reverse-transcribed from 2 μg total RNA with reverse transcription kit (Thermo Electron, Waltham, MA, USA). The cDNA was used as templates for qRT-PCR using SYBR Green Super mix kit (Takara Bio Inc., Japan). All samples were duplicated and the qPCR signal of the target transcript in the treated group was compared with the control housekeeper gene (GAPDH) signal by relative quantification. The $2^{-\Delta\Delta CT}$ method was used to analyze the relative changes in gene expression. The forward and reverse primers of target genes are listed in Table 1.

Cell culture and treatment

H9C2 rat embryo cardiomyocytes were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA) and cultured in Dulbecco' s modified Eagle' s medium (DMEM) containing 10% fetal bovine serum (Biowest S.A.S, USA) and 1% penicillin/streptomycin (Gibco, Grand Island, NY) at 37°C, 5% CO_2. Cultured H9C2 cells were treated with r*Sj*-Cys (0.5ug/ml) for 0.5 h, and then exposed to 1 μg /mL of LPS (Solaibao, Beijing, China) for 24 h. Cells incubated with LPS without r*Sj*-Cys treatment, or cells incubated with r*Sj*-Cys or medium only were used for controls. After 24-h incubation, the culture was centrifuged at 1000 rpm for 15 minutes at 4°C, the supernatants were stored at −80°C until use, and the cells were used for flow cytometry assays.

Detection of myocardial cell apoptosis by flow cytometry (FCM)

The LPS-induced myocardial cell apotosis was measured by annexin v-fitc and propidium iodide (pi) staining in accordance with the manufacturer's instructions (Invitrogen Thermo Fisher Scientific, USA). Flow cytometric analysis was performed on a CYTEK DxP AthenaTM Analyzer (CyTeK Biosciences, USA). The results were analyzed with FlowJo V7.6.5 software.

Detection of MyD 88 by Western blotting

MyD88 expression level in treated H2C9 cells and myocardial tissue was determined by Western blotting. Briefly, cells or left ventricular myocardium were collected and homogenized in ice-cold RIPA buffer containing 0.1% phenylmethylsulfonyl fluoride. The homogenates werecentrifuged at 12,000 rpm for 15 min at 4°C. Supernatants were collected and protein concentration was quantified using BCA assay kit (Pierce, Rockford, IL, United States). Equal amounts of cell extracts or heart homogenates were separated by 12% SDS-PAGE and electroblotted onto PVDF membranes. After blocking with 5% non-fat milk for 2 h at room temperature, the membranes were incubated with rabbit anti-MyD88 antibody (1:800) (Cell Signaling Technology, Danvers, Massachusetts, USA) overnight at 4°C, followed by HRP-conjugated goat anti-rabbit IgG (1:4000) (Merck Millipore, Basilica, Massachusetts, USA) for 1 h at 37°C. Immunoreactive protein bands were visualized using a Tanon 5200 Chemiluminescence imaging system (Tanon, Shanghai, China).

Statistical analysis

All data are expressed as the mean ± SME (standard error of the mean), and the statistical analyses were performed by using GraphPad Prism 5.0 software (GraphPad Inc., La Jolla, CA, USA). One-way ANOVA followed by the Student-Newman-Keuls test was used for multigroup comparisons. $P < 0.05$ was considered as statistically significant.

Results

Treatment with r*Sj*-Cys alleviated sepsis-induced myocardial malfunction

Left ventricular function was examined by echocardiography 12 hours after CLP surgery. As shown in Fig. 1 and 2, CLP-induced sepsis caused significant reduction of left ventricular systolic function in micecharacterized by reduced EF and FS compared with sham operation group (ANOVA: $F_{(3,23)} = 63.07$, $P < 0.0001$; $F_{(3,23)} = 21.08$, $P < 0.0001$). In contrast, r*Sj*-Cys treatment dramatically reversed the sepsis-induced decrease in the left ventricular EF and FS to the similar level of mice with sham surgery control (ANOVA: $F_{(3,23)} = 63.07$, $P < 0.0001$; $F_{(3,23)} = 21.08$, $P < 0.0001$), indicating that treatment with r*Sj*-Cys reduced the sepsis-induced myocardial systolic malfunction in mice. In addition, administration of r*Sj*-Cys in sham surgery mice did not markedly alter left ventricular EF and FS compared with sham group without treatment.

To assess the left ventricular diastolic function, E/A ratio was calculated from Doppler-derived mitral valve inflow measurements. The results showed that sepsis mice displayed a significant decline in E/A ratio values compared to sham control mice (ANOVA: $F_{(3,23)} = 10.99$, $P < 0.0002$). Treatment with r*Sj*-Cys significantly recovered the E/A ratio to the similar level of mice with sham surgery or sham + r*Sj*-Cys (ANOVA: $F_{(3,23)} = 10.99$, $P < 0.0002$) (Fig. 3). These results indicate that treatment with r*Sj*-Cys also improves sepsis-induced diastolic malfunction in mice. No significant difference was observed between sham group and sham with r*Sj*-Cys treated group in terms of E/A ratio (Fig. 3E).

Treatment of r*Sj*-Cys reduced sepsis-induced heart pathological abnormality

The morphological structures of the myocardial tissues of mice 12 hours after CLP surgery were determined by H&E staining. The results showed that the sham surgery group and sham with r*Sj*-Cys group had no significant inflammatory cell infiltration with normal appearance of the myofibrillar structure (Fig. 4). However, the heart tissue in mice with CLP showed significant swollenness, myocardial fiber arrangement disorder and highly recruited inflammatory cell infiltration (Fig. 4). Of note, tissue sections from the CLP + r*Sj*-Cys group mice showed significantly improved muscle fiber structure with reduced inflammatory cell infiltration compared with CLP group without r*Sj*-Cys treatment (Fig. 4). Therefore, r*Sj*-Cys effectively alleviates CLP-induced cardiac lesion and inflammation in mice.

Administration of r*Sj*-Cys reduced sepsis-induced heart injury

In myocardial injury, the level of Mb, cTnI, NT-proBNP and MPO in sera or heart tissue are usually used as biomarkers to evaluate the ischemic severity of heart injury [29, 30, 31]. Compared with the sham group,

the CLP group showed markedly increased MPO activity in the myocardial tissue homogenate and elevated levels of cTnI, Mb, NT-proBNP in sera (ANOVA: $F_{(3,23)}$ =32.10, P < 0.0001; $F_{(3,23)}$ = 61.36, P < 0.0001; $F_{(3,23)}$ = 10.42, P < 0.0002; $F_{(3,23)}$ = 42.79, P < 0.0001) (Fig. 5). After being treated with r*Sj*-Cys, the MPO activity in the myocardial homogenate and cTnI, Mb, NT-proBNP in sera were significantly reduced in mice with sepsis compared with CLP mice without treatment (ANOVA: F(3,23) =32.10, P < 0.0001; F(3,23) = 61.36, P < 0.0001; F(3,23) = 10.42, P < 0.0002; F(3,23) = 42.79, P < 0.0001) (Fig. 5), while the level of cTnI, NT-proBNP, Mb and MPO remained at low levels in mice with sham surgery and there was no significant difference between sham surgery groups and sham with treatment of r*Sj*-Cys. The increased MPO activity in heart tissue of CLP-induced sepsis mice is correlated with the increased inflammatory cells, especially neutrophils, infiltration in the tissue (Fig. 4).

The reduced sepsis-caused heart injury in mice treated with r*Sj*-Cys was associated with reduced pro-inflammatory cytokines and increased regulatory cytokines.

To understand the mechanism underlying the improvement of CLP-induced sepsis caused cardiac dysfunction with treatment of r*Sj*-Cys, the levels of pro-inflammatory cytokines (TNF-α, IL-6) and regulatory cytokines (IL-10, TGF-β) were measured in sera, and the mRNA levels measured in heart tissue of experimental mice. The results showed that the inflammatory cytokines (TNF-α, IL-6) were dramatically increased in the sera of CLP-induced sepsis mice compared to that in mice with sham surgery only or sham + r*Sj*-Cys (ANOVA: $F_{(3,23)}$ =18.39, P < 0.0001; $F_{(3,23)}$ =361.3, P < 0.0001). Treatment with r*Sj*-Cys significantly reduced the production of TNF-α and IL-6 in CLP-induced sepsis mice compared with CLP mice without treatment (ANOVA: $F_{(3,23)}$ =18.39, P < 0.0001; $F_{(3,23)}$ =361.3, P < 0.0001) (Fig. 6A and B). However, there was no significant difference of TNF-α and IL-6 levels in sera of mice between the sham group and sham + r*Sj*-Cys group (ANOVA: $F_{(3,23)}$ =18.39, P < 0.0001; $F_{(3,23)}$ =361.3, P < 0.0001) (Fig. 6A and B). The reduced TNF-α and IL-6 levels were correlated with the increased IL-10 and TGF-β levels in sera of CLP-induced sepsis mice treated with r*Sj*-Cys compared with CLP group without treatment (Fig. 6C and D). Interestingly, the IL-10 and TGF-β levels were significantly lower in CLP-induced sepsis mice than that in sham surgery or sham + r*Sj*-Cys mice (ANOVA: $F_{(3,23)}$ = 9.032, P < 0.0006; $F_{(3,23)}$ = 9.789, P < 0.0004). The results suggested that CLP-induced sepsis mice stimulated the secretion of pro-inflammatory cytokines (TNF-α and IL-6) (ANOVA: $F_{(3,23)}$ =18.39, P < 0.0001; $F_{(3,23)}$ =361.3, P < 0.0001), but inhibited the regulatory immune pathway (lower IL-10 and TGF-β) (ANOVA: $F_{(3,23)}$ = 9.032, P < 0.0006; $F_{(3,23)}$ = 9.789, P < 0.0004). Treatment of r*Sj*-Cys was able to significantly inhibit the activation of pro-inflammatory pathway possibly through activating regulatory immune pathway.

The mRNA transcription levels of TNF-α, IL-6, IL-10 and TGF-β in heart tissue of experimental mice also showed the similar pattern. As shown in Figure 7A and B, the mRNA levels of TNF-α and IL-6 were significantly stimulated in heart tissue of mice with CLP-induced sepsis (ANOVA: $F_{(3,11)}$ =37.27, P < 0.0001; $F_{(3,11)}$ =47.19, P < 0.0001). The treatment with r*Sj*-Cys significantly inhibited the mRNA expression of these two pro-inflammatory cytokines with more significance for TNF-α which was reduced to the similar level as sham control group, at the meanwhile, significantly stimulated the mRNA expression of IL-

10 and TGF-β was observed in r*Sj*-Cys-treated hearts (ANOVA: $F_{(3,11)}$ =66.91, P < 0.0001; $F_{(3,11)}$ =6.302, P < 0.0168) (Fig. 7C and D). The results are consistent with the measured protein levels of the cytokines in the experimental mice.

The inhibitory effect of r*Sj*-Cys on LPS-induced inflammatory response of H9C2 cardiomyocytes

LPS is blamed to be the major component to cause cardiac dysfunction in sepsis through inducing the innate immune inflammatory response [32]. In this study, we identified that LPS significantly induced H9C2 cardiomyocytes to release pro-inflammatory cytokines IL-6 and TNF-α (ANOVA: F(3,11) =20.78, P < 0.0004; F(3,11) =18.53, P < 0.0006), but inhibited the release of regulatory cytokines TGF-β and IL-10 (ANOVA: F(3,11) =25.67, P < 0.0002; F(3,11) =14.41, P < 0.0014). After being treated with r*Sj*-Cys, the LPS-induced IL-6 and TNF-α were reduced to the level of medium control (ANOVA: $F_{(3,11)}$ =20.78, P < 0.0004; $F_{(3,11)}$ =18.53, P < 0.0006), and TGF-β and IL-10 were significantly boosted compared to cells without r*Sj*-Cys treatment (ANOVA: $F_{(3,11)}$ =25.67, P < 0.0002; $F_{(3,11)}$ =14.41, P < 0.0014) (Fig. 8). The r*Sj*-Cys alone has no significant effect on the innate immune response of normal cardiomyocytes.

r*Sj*-Cys reduced LPS-induced cardiomyocte apoptosis.

To further determine whether r*Sj*-Cys reduced LPS-induced cardiomyocyte apoptosis, H9C2 cells were incubated with LPS alone or with r*Sj*-Cys. The flow cytometry results revealed that incubation with LPS induced 14.4% H9C2 cells apoptosis, whereas co-incubation with r*Sj*-Cys significantly reduced LPS-induced apoptosis to the similar level without LPS (ANOVA: $F_{(3,11)}$ =22.68, P < 0.0003) (Fig. 9). There was no remarkable difference in apoptotic rate between r*Sj*-Cys alone group and blank control group (Fig. 9).

r*Sj*-Cys suppressed the activation of MyD88 in LPS-stimulated H9C2 cells *in vitro* and CLP-induced cardiac tissues *in vivo*

MyD88 is a crucial molecule involved in the inflammatory TLR signaling pathway. To determine if MyD88 is involved in the therapeutic effect of r*Sj*-Cys on the sepsis-induced inflammation and damage of cardiomyocytes, we detected the expression of MyD88 in heart tissue of mice with CLP-induced sepsis treated with r*Sj*-Cys *in vivo*, and in LPS-stimulated H9C2 cells co-incubated with r*Sj*-Cys *in vitro*. The elevated level of MyD88 was observed in cardiac tissue 12 h after CLP surgery compared with sham surgery control (ANOVA: $F_{(3,11)}$ =8.823, P < 0.0064) (Fig. 10A). Treatment with r*Sj*-Cys significantly reduced the expression of MyD88 in cardiac tissue of sepsis mice (ANOVA: $F_{(3,11)}$ =8.823, P < 0.0064) (Fig. 10A). There was no effect of r*Sj*-Cys on the expression of MyD88 in normal mice (sham control). At the similar pattern, the expression of MyD88 was increased in cardiomyocytes incubated with LPS for 24h. Co-incubation with r*Sj*-Cys significantly suppressed the expression of MyD88 in LPS-stimulated cardiomyocytes (ANOVA: $F_{(3,11)}$ =8.550, P < 0.0071) (Fig. 10B).

Discussion

Myocardial dysfunction is a fatal complication of patients with sepsis [18]. Researches have found that 50% of patients with sepsis have systolic and diastolic dysfunction in left and right ventricular, possibly caused by endotoxin-induced myocardial injury [33] which has been confirmed both in animal and clinical observations [34]. In this study, we also confirm that CLP-induced sepsis caused serious myocardial damage characterized by significant reduction of left ventricular systolic and diastolic functions in mice, which closely mimicked the pathological features of myocardial infarction observed in clinical patients [35]. We further confirm that LPS released by Gram-negative bacteria could cause apoptosis of H9C2 cardiomyocytes when co-incubation *in vitro*.

Cysteine proteases have been regarded as key molecules in regulating inflammation, cell apoptosis, cancer progression, protein degradation and antigen presentation [36, 37, 38, 39, 40]. Since cysteine proteases are largely involved in the inflammation and immune responses, their inhibitor, cystatin, could be a potential modulator for immunological reaction. Actually, the cystatins secreted by several helminths have been proven to play important roles in modulating host immune responses [11, 21]. Previous studies demonstrated that *S. japonicum* cystatin (*Sj*-Cys) contained conserved domains of type II family cystatins with inhibitory activity on bovine cathepsin B [23]. *Sj*-Cys also inhibited LPS-stimulated macrophages to release nitric oxide, TNF-α and IL-6 cytokines and induced M2 macrophage polarization [23]. Treatment with r*Sj*-Cys significantly reduced TNBS-induced experimental colitis in mice through up-regulation of Treg cells and related cytokines IL-10 and TGF-β and down-regulation of pro-inflammatory cytokines TNF-α and IL-6 in the colon tissues of mice [41].

In an effort to reduce sepsis-induced cardiomyopathy, the life-threatening complication and consequence of systemic infection, we established the mouse model of CLP-induced sepsis and sepsis-induced cardiomyopathy. We demonstrated that 12 hours after CLP, the ventricular systolic and diastolic functions of affected mouse heart were seriously impaired associated with myocardial structural damage and inflammatory cell infiltration. Physiological changes in cardiac dysfunction include ventricular dilatation, decreased ejection fraction, systemic or regional left ventricular wall dysfunction and systolic and diastolic dysfunction [42]. EF%, FS% and E/A ratio of left ventricle (LV) are important indicators reflecting cardiac function [43, 44]. Echocardiographic results demonstrated that the CLP-induced sepsis resulted in significant decrease in left ventricular EF, FS and E/A ratio, indicating the serious damage on heart systolic and diastolic functions. In addition, the levels of cTnI, NT-proBNP and Mb in sera and MPO level in heart tissue are the important biochemical markers of cardiac damage and injury in early septic shock [29, 30, 45, 46]. The CLP-induced sepsis model established in this study demonstrated significant increase levels of cTnI, NT-proBNP and Mb in sera and high level of MPO in heart tissue, indicating that the heart tissue and cells were seriously damaged caused by the CLP-induced sepsis. The significantly increased inflammatory cell infiltration in heart tissue also demonstrated the serious inflammation occurred in the heart.

After being treated with r*Sj*-Cys, the sepsis-induced malfunction of heart has been significantly improved, showing recovered left ventricular EF, FS and E/A ratio. The inflammation of heart tissue was also significantly reduced characterized by the significantly decreased infiltration of inflammatory cells in

cardiac tissues and fiber swelling. The levels of Mb, cTnI and NT-proBNP in sera were significantly reduced upon the treatment, indicating that r*Sj*-Cys attenuated endotoxin-induced myocardial damage and dysfunction. The MPO level was also reduced in heart tissue. Neutrophils play a significant role in the development of inflammation [47], and the activated neutrophils and monocytes are the main sources of MPO [48]. MPO is released into the blood as an inflammatory mediator, and promotes the activation of neutrophils, leading to further increased MPO and inflammation. Studies have also shown that neutrophil recruitment mediated myocardial injury and cardiac dysfunction induced by ischemia-reperfusion [49]. The significantly reduced levels of these inflammatory biomarkers upon treatment of r*Sj*-Cys in this study which was correlated to the reduced inflammatory cell infiltration in heart and reduced level of pro-inflammatory cytokines IL-6, TNF-α in the sera of septic mice.

Pro-inflammatory cytokines IL-6 and TNF-α play essential roles in the onset and progression of sepsis [50], and their over-expressions were seen as early signals suppressing myocardial contraction and the major cause of progressive systolic dysfunction [33, 51]. As a triggering factor of inflammation, TNF-α is also recognized as a main mediator of septic shock, and involved in the induction of IL-1 production, the latter induces the secretion of secondary inflammatory factors such as IL-6, resulting in an inflammatory cascade [52, 53, 54]. The further evidence suggested that over-expressed IL-6 and TNF-α by systematic immune responses might support a vital role in the development of myocardial malfunction in sepsis [55]. At the same time, the septic cardiomyocytes were able to produce TNF-α and IL-6 themselves, indirectly leading to deteriorative damage to myocardial tissues [3]. Our present study showed that treatment with r*Sj*-Cys significantly reduced the level of TNF-α and IL-6 in sera (systematic) and their mRNAs in cardiac homogenate (local), suggesting the regulatory effects of r*Sj*-Cys on local (cardiac) and systematic (sepsis) immune system.

More evidences have showed that immunomodulatory functions of helminth infection or helminth-derived products are mediated by stimulating host regulatory T cell (Treg) response [16, 17, 20]. Treg cells are key factors in the induction of immune tolerance [56], mainly through the secretion of IL-10 and TGF-β to exert regulatory influence on immune system [16]. IL-10 played a counter-regulatory effect in the inflammatory response and was an endogenous inhibitor of inflammatory cytokines production [57]. The levels of IL-10 and TGF-β were dramatically increased upon the treatment of r*Sj*-Cys in this study, indicating that r*Sj*-Cys acted as an inhibitory immunomodulator in the case of excessive inflammation infection possibly through stimulating Treg and Treg cell-secreted IL-10 and TGF-β. The r*Sj*-Cys itself had little effect on the cytokines change compared to the control mice without r*Sj*-Cys treatment, suggesting that r*Sj*-Cys mainly plays the immunomodulatory role when the inflammation is activated. Incubation of r*Sj*-Cys with H9C2 cardiomyocytes *in vitro* inhibited LPS-induced heart cell apoptosis and induced similar cytokine changes in the culture supernatant to that in septic cardiomyopathy *in vivo*, further indicating that LPS-induced hyper-inflammatory responses are involved in cardiomyocyte apoptosis, and treatment with r*Sj*-Cys could inhibit LPS-triggered excessive inflammation partially through directly inhibiting the inflammatory cytokine IL-6 and TNF-α and stimulating the regulatory cytokines IL-10 and TGF-β in LPS-shocked cardiomyocytes.

As we know, LPS associates with its receptor toll-like receptor 4 (TLR4) through the help of LPS-binding protein CD14, subsequently resulting in the production of inflammatory cytokines, such as TNF-α, IL-1β, and IL-18, which might directly harm cardiac function [58]. Although the mechanism leading to sepsis-induced cardiac arrest remains controversial, there are increasing evidences supporting that TLR-mediated innate immunity and inflammatory responses play a key role in cardiac dysfunction caused by sepsis or septic shock [59, 60, 61].

Activation of the TLR4 signaling pathway may directly lead to myocardial cells' dysfunction. The invaded bacteria or other external stimulus firstly trigger innate immunity and then induce TLR4 expression by up-regulating MyD88-mediated pathway and activating the transcription of nuclear factor-κB (NF-κB), resulting in the productions of various inflammatory mediators, such as cytokines, chemokines and antimicrobial peptides [62]. The occurrence of sepsis is related to the TLR4/MyD88 signaling pathway, which activates the secretion of cytokines associated with cardiac dysfunction in adult mammalian hearts [7, 63] and in mice [64]. To investigate whether r*Sj*-Cys-involved anti-inflammatory and anti-apoptosis effects through inhibiting MyD88-dependent signaling pathway, the level of MyD88 in CLP-induced septic heart tissue and in LPS-stimulated H9C2 cells was measured. Our studies found that the expression of MyD88 was increased in CLP-induced myocardial tissue or LPS-stimulated H9C2 cells, and treatment with r*Sj*-Cys significantly reduced the expression of MyD88 in these cardiomycytes.

Our results suggest the possible mechanism of r*Sj*-Cys involved in the alleviation of septic cardiomyopathy is that treatment of r*Sj*-Cys stimulates Tregs and/or cardiomyocytes to produce regulatory cytokines such as IL-10 and TGF-β, the latter suppress the production of pro-inflammatory cytokines via inhibiting TLR4/MyD88 activation signal pathway as other helminth derived proteins did [65, 66, 67, 68].

Conclusions

The presented data have shown that r*Sj*-Cys strongly alleviated excessive inflammation and protected against sepsis-induced cardiac dysfunction. Therefore, it could be considered as a potential therapeutic agent for the prevention and treatment of sepsis associated cardiac dysfunction.

Abbreviations

CLP: Cecal ligation and puncture; LPS: lipopolysaccharides; Mb: myoglobin; cTnI: cardiac troponin I; NT-proBNP: N-terminal pro-Brain Natriuretic peptide; MPO: myeloperoxidase; MyD88: myeloid differentiation factor 88; EF: ejection fraction; FS: fractional shortening; E wave: peak early-diastolic transmitral velocities; A wave: peak late-diastolic transmitral velocities; LV: left ventricle; ELISA: Enzyme-linked immunosorbent assay; TNF-α: Tumor necrosis factor alpha; IL-6: Interleukin 6; IL-10: Interleukin 10; TGF-β: transforming growth factor-β; IL-1β: Interleukin 1β; DCs: dendritic cell; Tregs: Regulatory T cells; TNBS: trinitrobenzene sulfonic acid; DSS: dextran sulfate sodium; TLR4: toll-like receptor 4; NF-κB: nuclear

factor-κB; SPF: specific pathogen free; SDS-PAGE: sodium dodecyl sulfate polyacrylamide gel electrophoresis; PVDF: polyvinylidene fluoride.

Declarations

Acknowledgments

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Ethics approval and consent to participate

All procedures concerning to laboratory animals were in strict accordance with the Chinese National Institute of Health Guide for the Care and Use of Laboratory Animals, and approved by the Animal Care and Use Committee of Anhui Medical University (approval#: AMU26-08061).

Consent for publication

Not applicable.

Availability of data and materials

The datasets generated and/or analysed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

Xiaodi Yang, Hu Cui and Shifang Gao conceived and designed the study; Shifang Gao, HHL, Hong Xie, Siying Sun, Huijuan Yang, Lingqin Wu, Yongsheng Bai, Qiao Zhou, and Xin Wang performed the experiments; Yuan Yuan, Shili Wu and Liang Chu analyzed the data; Shifang Gao wrote the manuscript.

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Table

Table 1. The primers of qRT-PCR

Primer	Forward	Reverse
TNF-α	AACCTCCTCTCTGCCGTCAA	AAAGTAGACCTGCCCGGACTC
IL-6	TGGAGTCACAGAAGGAGTGGCTAA	TCTGACCACAGTGAGGAATGTCCA
TGF-β	CTACAATGAGCTGCGTGTG	TGGGGTGTTGAAGGTCTC
IL-10	CCAAGCCTTATCGGAAATGA	TTTTCACAGGGGAGAAATCG
GAPDH	GGTTGTCTCCTGCGACTTCA	TGGTCCAGGGTTTCTTACTCC

Figures

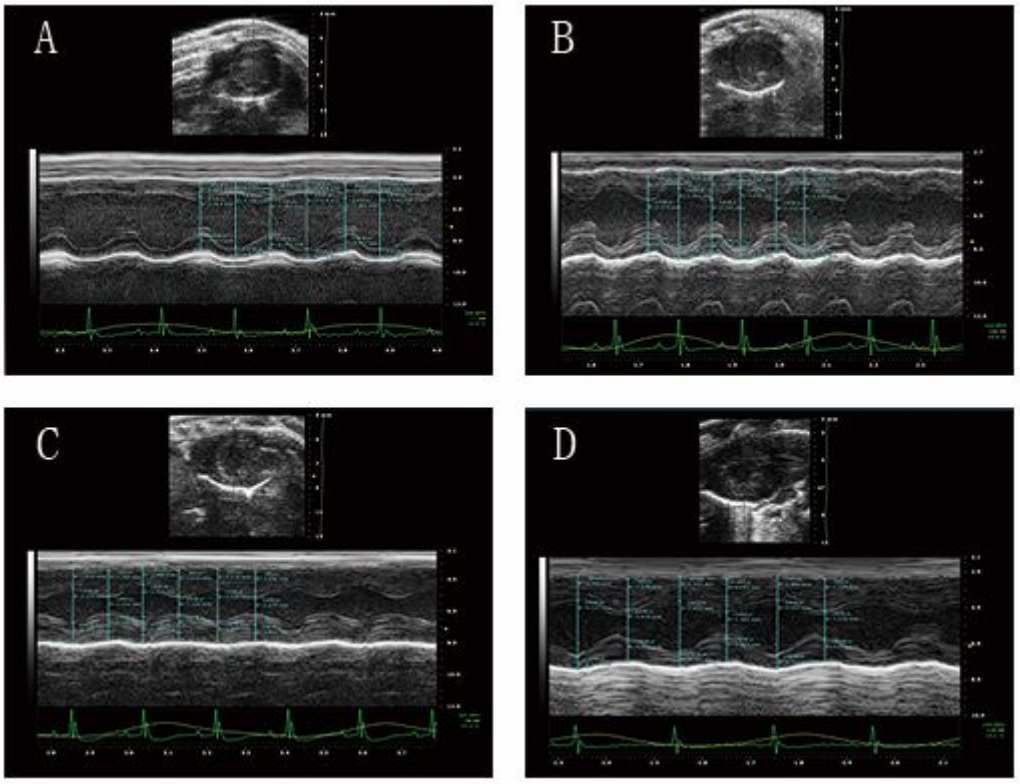

Figure 1

Representative M-mode echocardiograms obtained from mice 12 h after treatment of sham-operation (A), sham + rSj-Cys (B), CLP (C) and CLP + rSj-Cys (D), respectively.

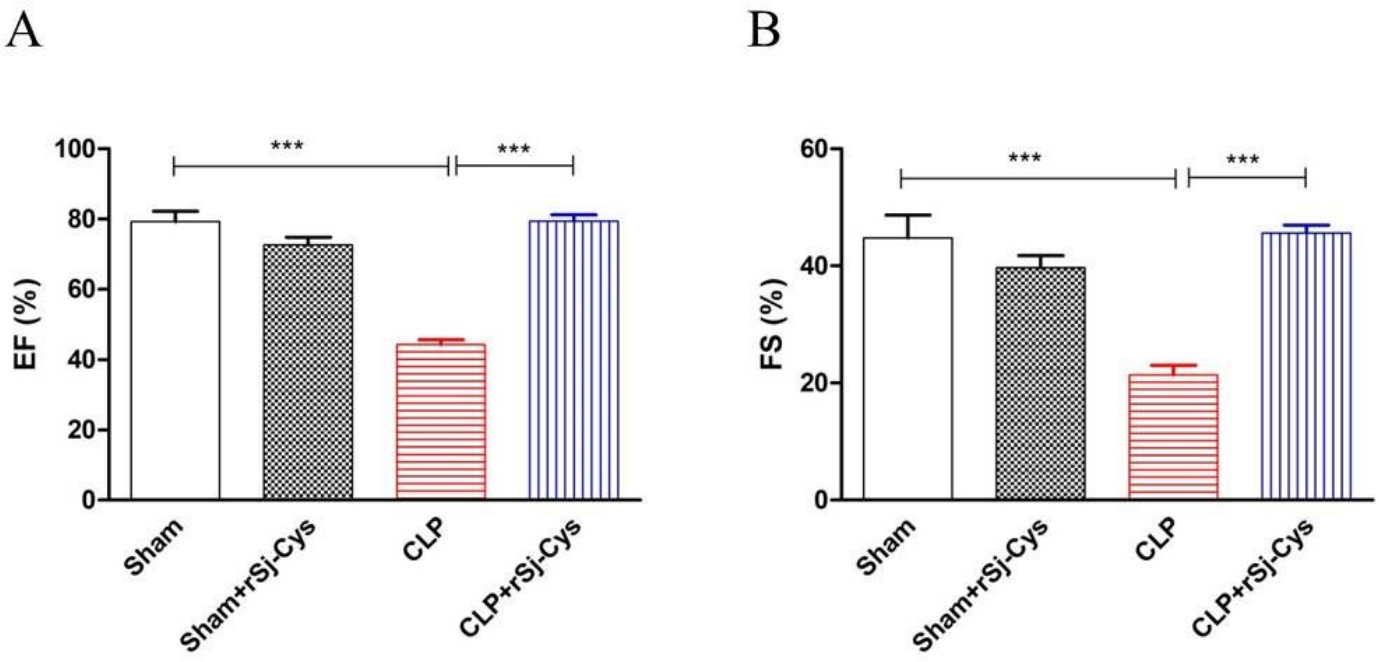

Figure 2

The improved left ventricular systolic function evaluated by EF (A) and FS (B) in CLP mice treated with rSj-Cys, compared with mice with sham-operation, sham + rSj-Cys and CLP mice without treatment (n = 6 mice per group). The data are presented as the mean ± SEM. ***P < 0.001.

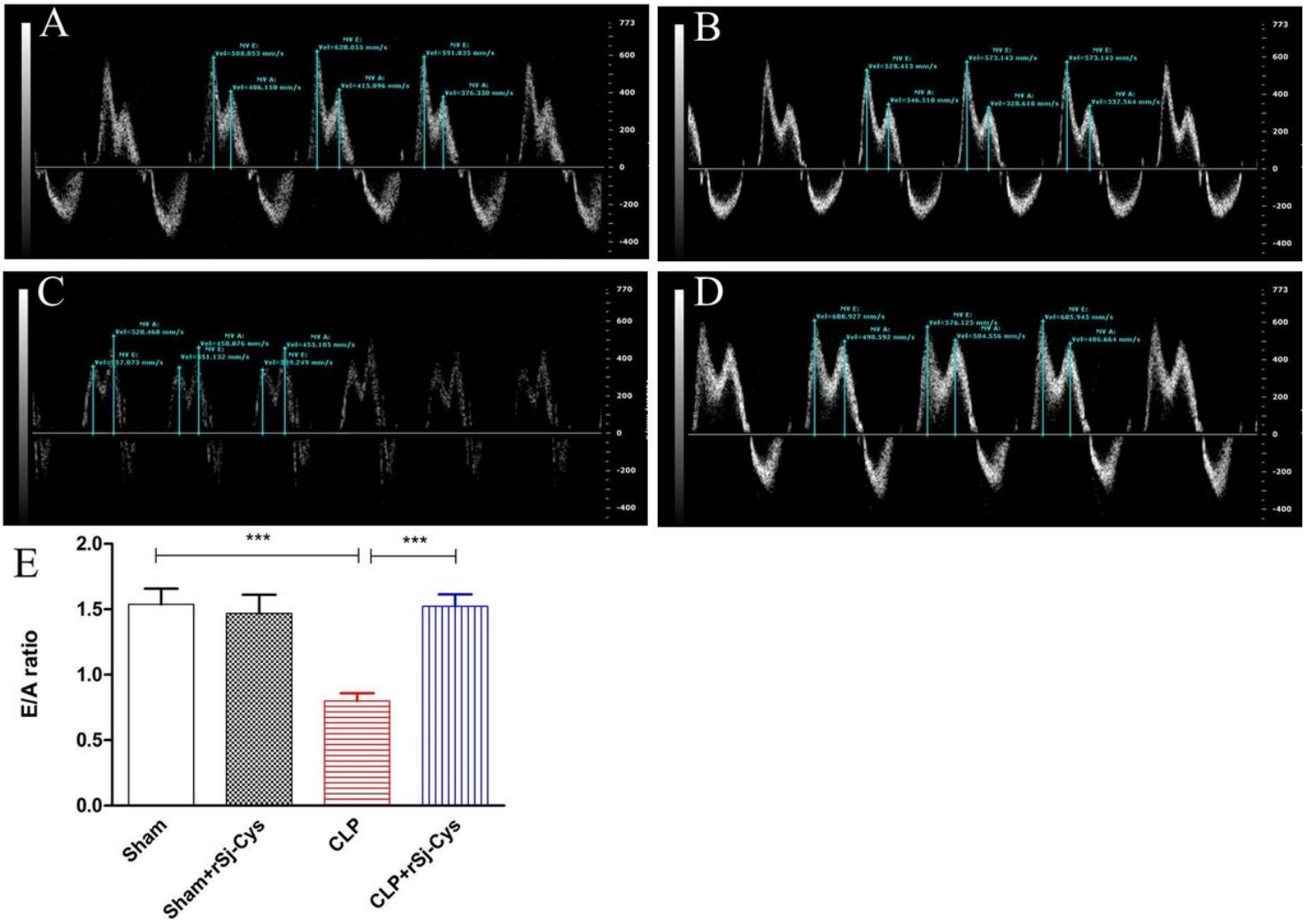

Figure 3

Treatment with rSj-Cys attenuated CLP-induced left ventricular diastolic dysfunction. Representative transmitral Doppler images for sham (A), sham + rSj-Cys (B), CLP (C) and CLP + rSj-Cys (D) groups. The E wave represents peak early-diastolic transmitral velocity, and the A wave indicates peak late-diastolic transmitral velocity. The changes of E/A ratio were used to assess the alteration in left ventricular diastolic function. (n = 6 mice per group). The data are presented as the mean ± SEM. ***P < 0.001.

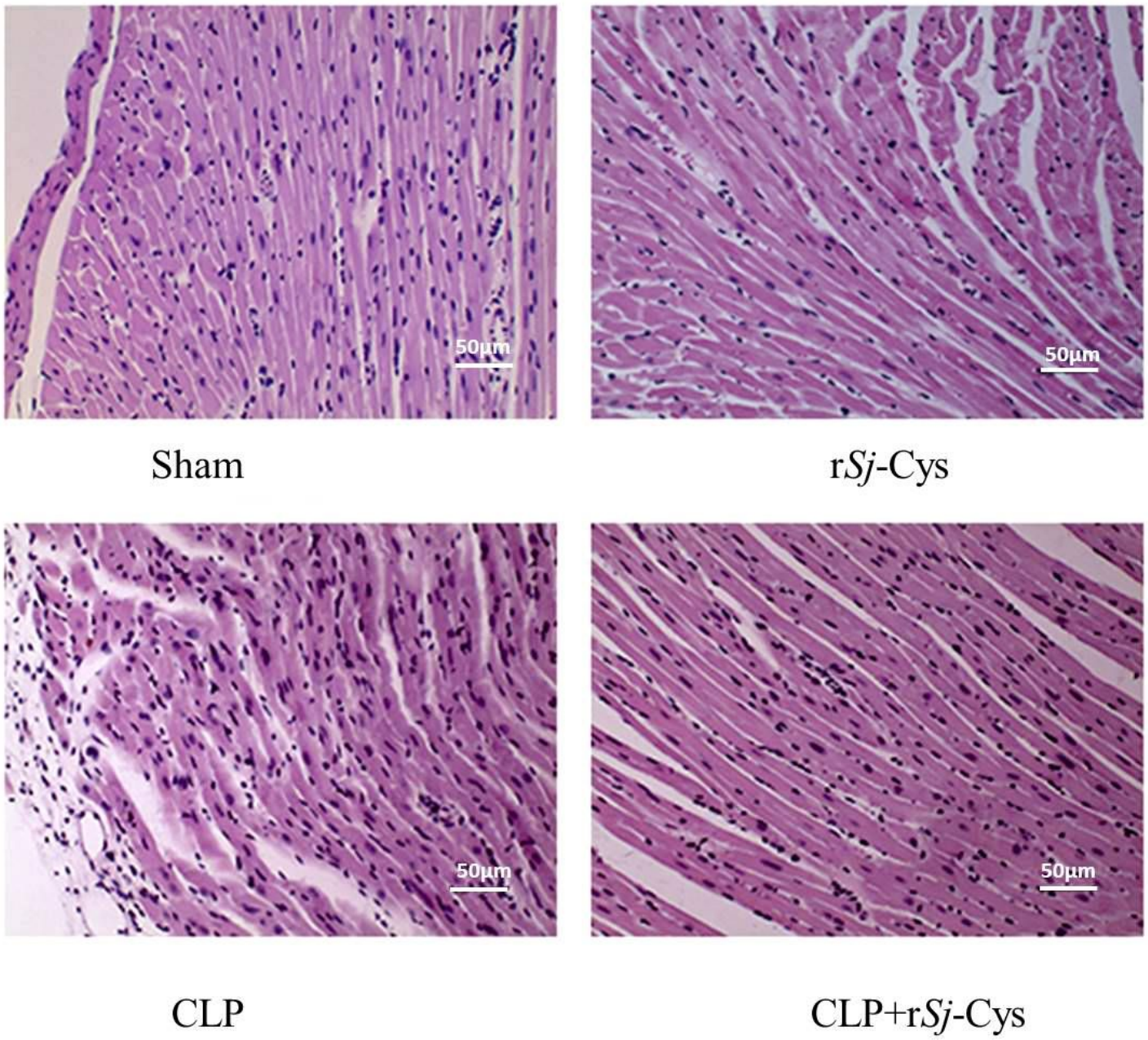

Figure 4

Histopathological and morphological variations in the cardiac tissue of mice with treatment of sham surgery (A), sham + rSj-Cys (B), CLP (C) and CLP + rSj-Cys (D) (n = 6 mice per group). (×200; Scale-bars: 50 μm).

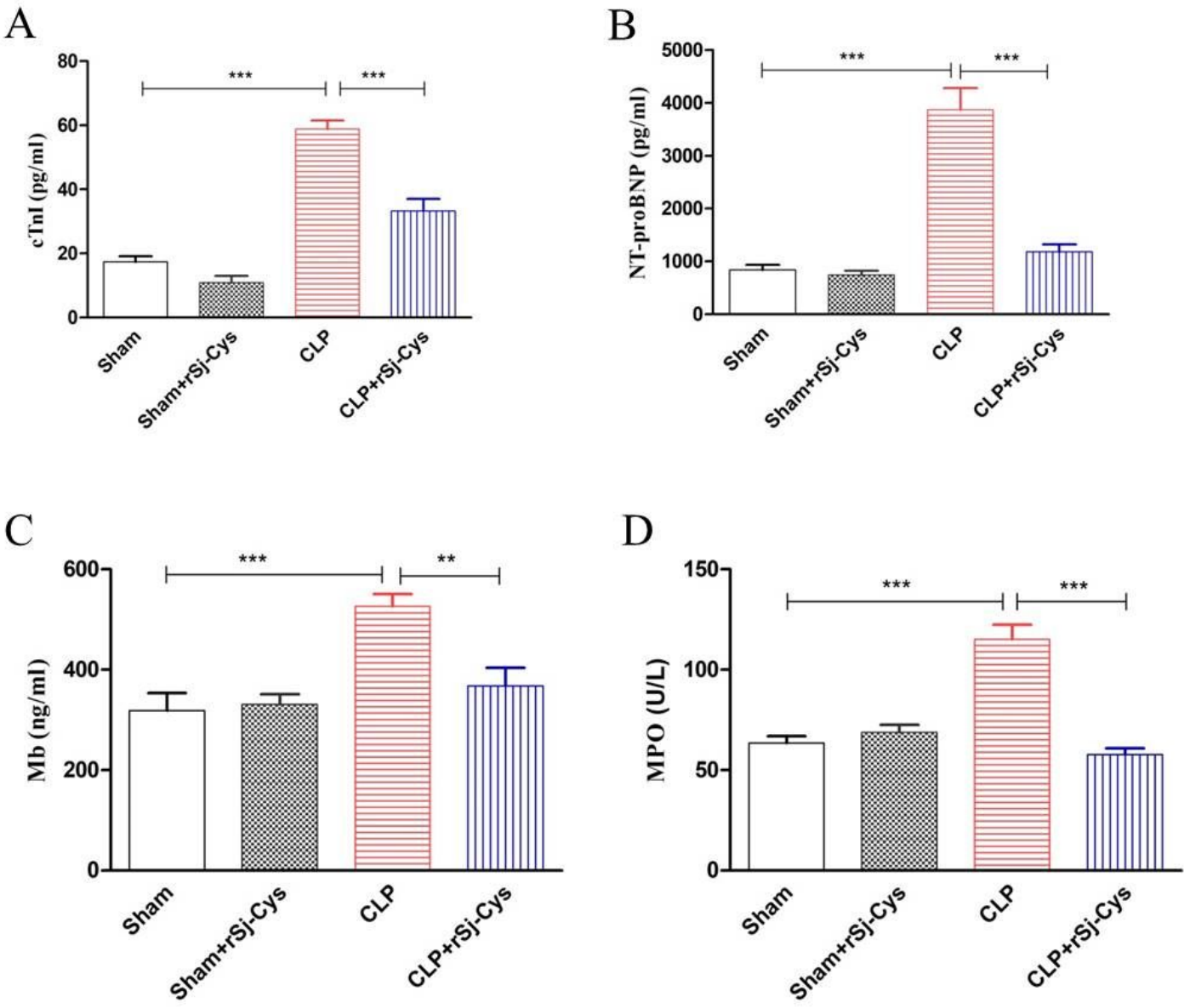

Figure 5

Treatment with rSj-Cys reduced CLP-sepsis induced myocardial injury in the BALB/c mice. The levels of cTnI (A), NT-proBNP (B), Mb (C) in serum and MPO activity in heart tissue (D) were sigficantly reduced in CLP mice treated with rSj-Cys. The data are presented as the mean ± SEM. **P < 0.01, ***P < 0.001.

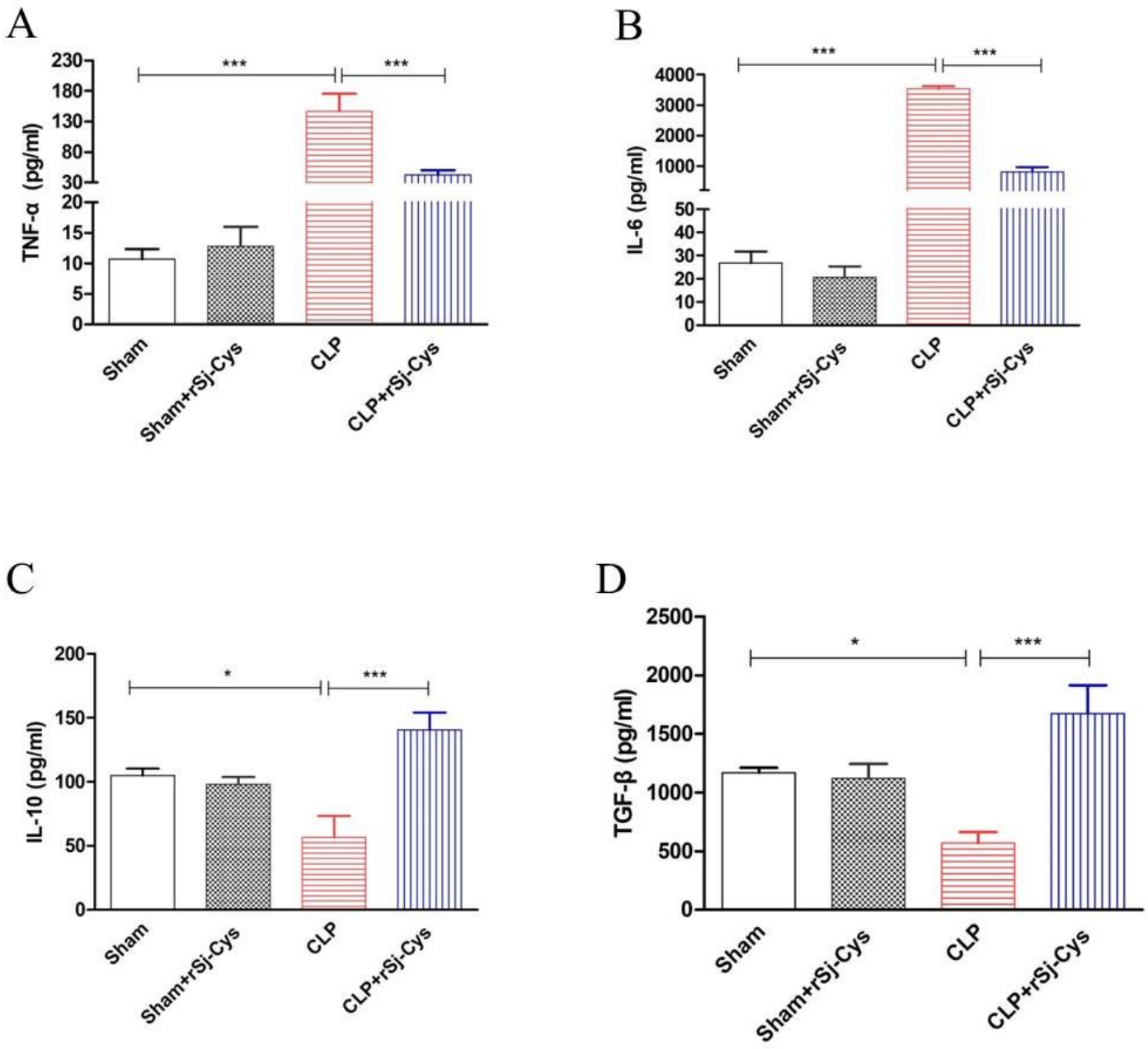

Figure 6

Treatment with rSj-Cys reduced the secretion of pro-inflammatory cytokines TNF-α (A) and IL-6 (B), and stimulated the secretion of regulatory cytokines IL-10 (C) and TGF-β (D) in mice with CLP-induced sepsis. The levels of these cytokines in sera of experimental mice were measured by ELISA 12 h after the surgery and treatment. The results are shown as the mean ± SEM for each group (n = 6 mice per group). *P < 0.05, ***P < 0.001.

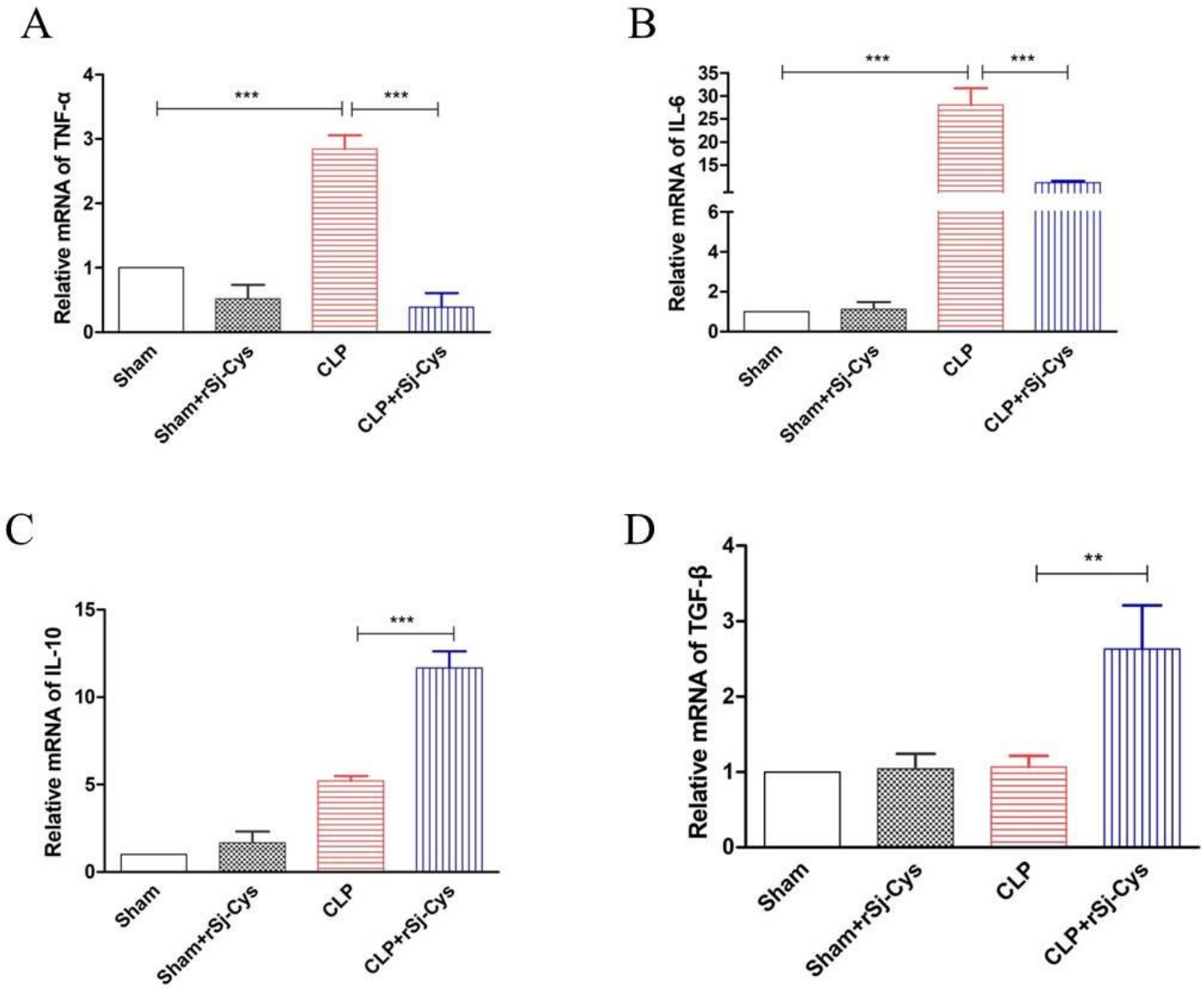

Figure 7

Treatment with rSj-Cys reduced the mRNA expression of TNF-α (A), IL-6 (B) and increased the mRNA expression of IL-10 (C) and TGF-β (D) in heart tissues of mice with CLP-induced sepsis measured by qPCR12 h after the treatment. The data are shown as the mean ± SEM for each group (n = 3 mice per group). **P < 0.01, ***P < 0.001.

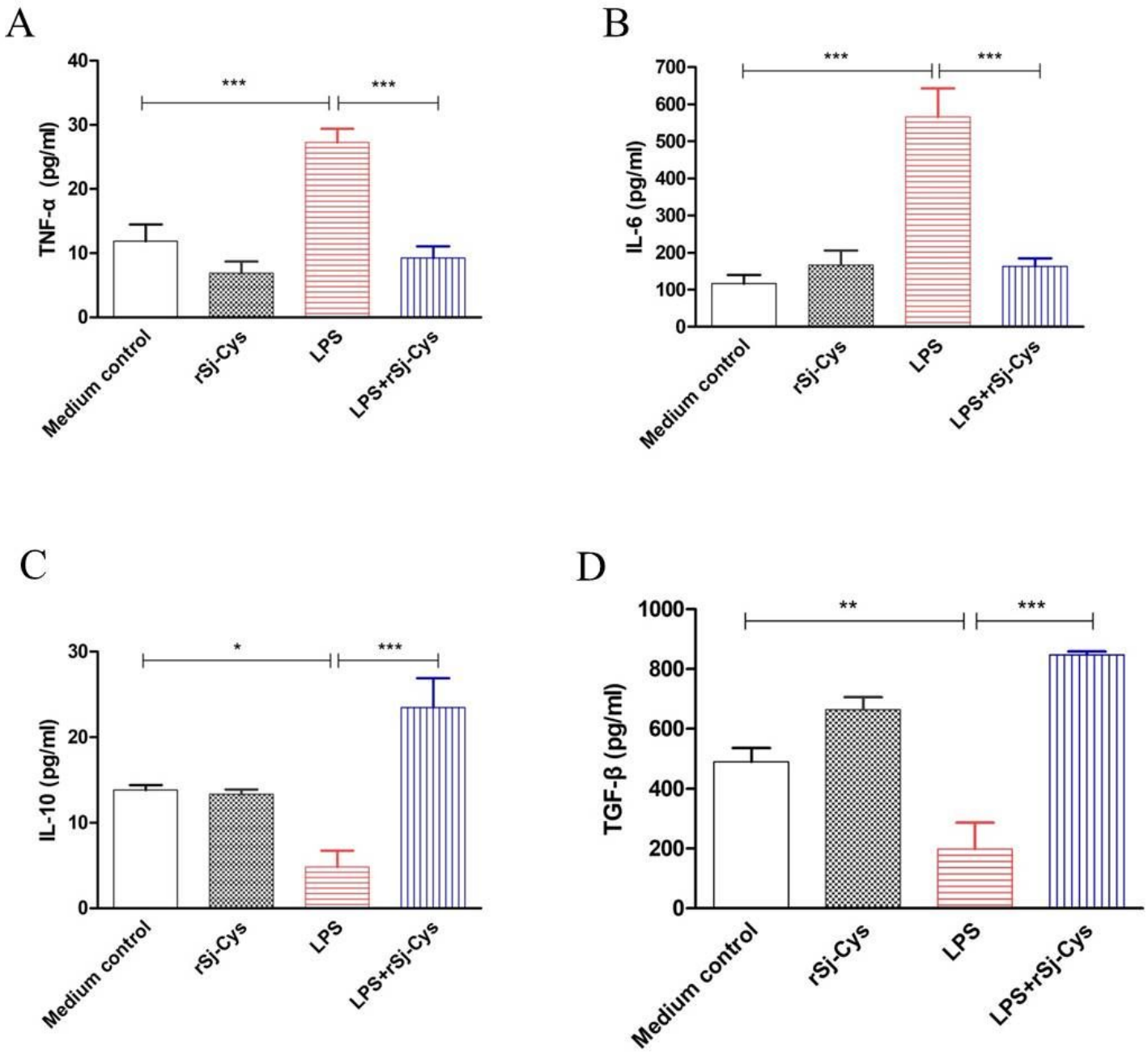

Figure 8

Incubation with rSj-Cys inhibited the pro-inflammatory cytokines TNF-α (A) and IL-6 (B) and stimulated regulatory cytokines IL-10 (C) and TGF-β (D) released by LPS-induced H9C2 cells. The levels of these cytokines in supernatant were measured by ELISA 24 h after incubation. The results are shown as the mean ± SEM for each group (n = 3 per group). *P < 0.05, **P < 0.01, ***P < 0.001.

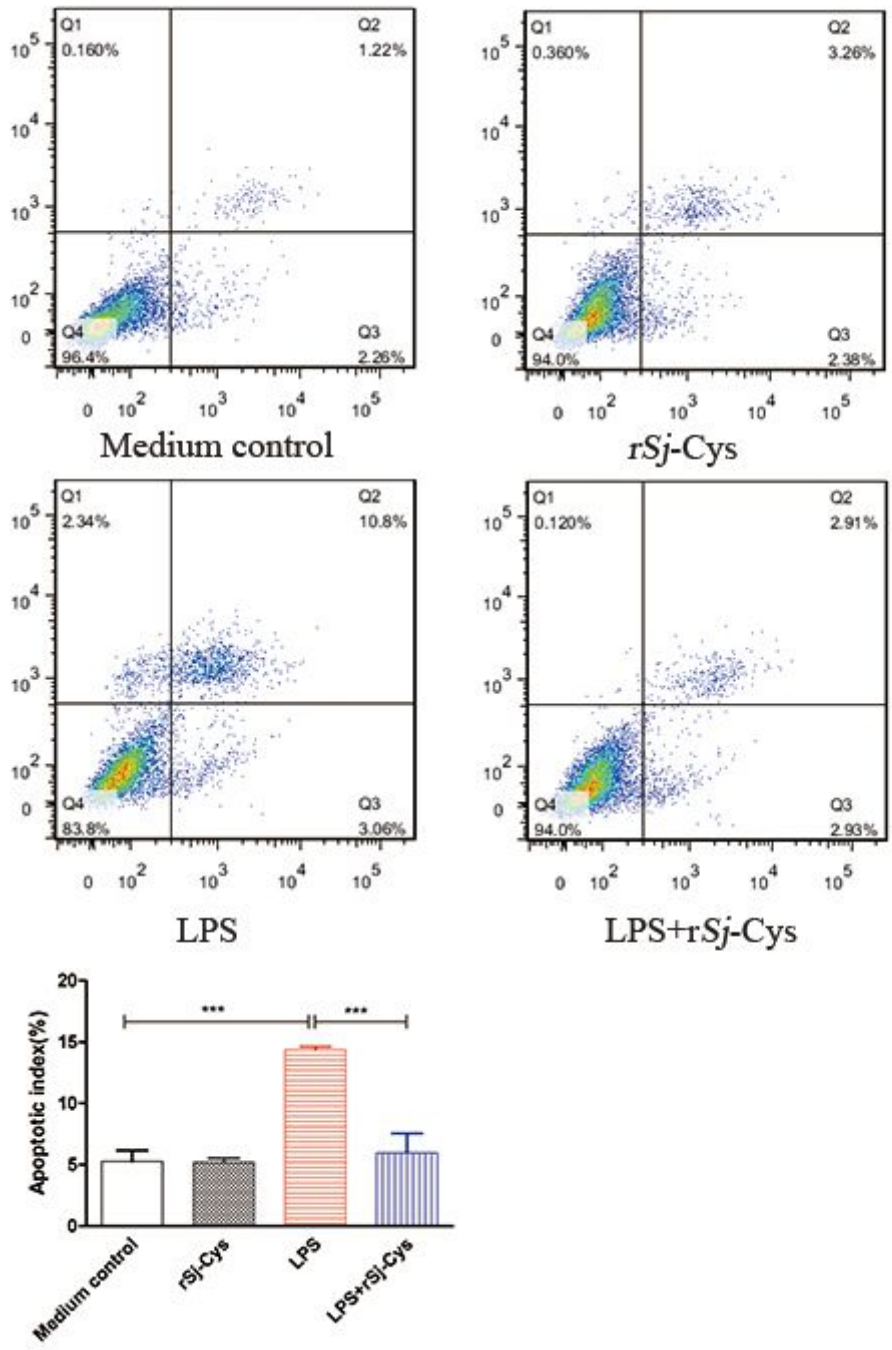

Figure 9

rSj-Cys reduced LPS-induced cardiomyocyte apoptosis measured by flow cytometry. Representative flow cytometry images showed the reduced cardiomyocyte apoptosis in rSj-Cys + LPS co-incubated H9C2 cells. The normal H9C2 cells in blank medium or medium with rSj-Cys were used as controls. Data are expressed as mean ± SEM from three independent experiments) (n = 3 per group). ***P < 0.001.

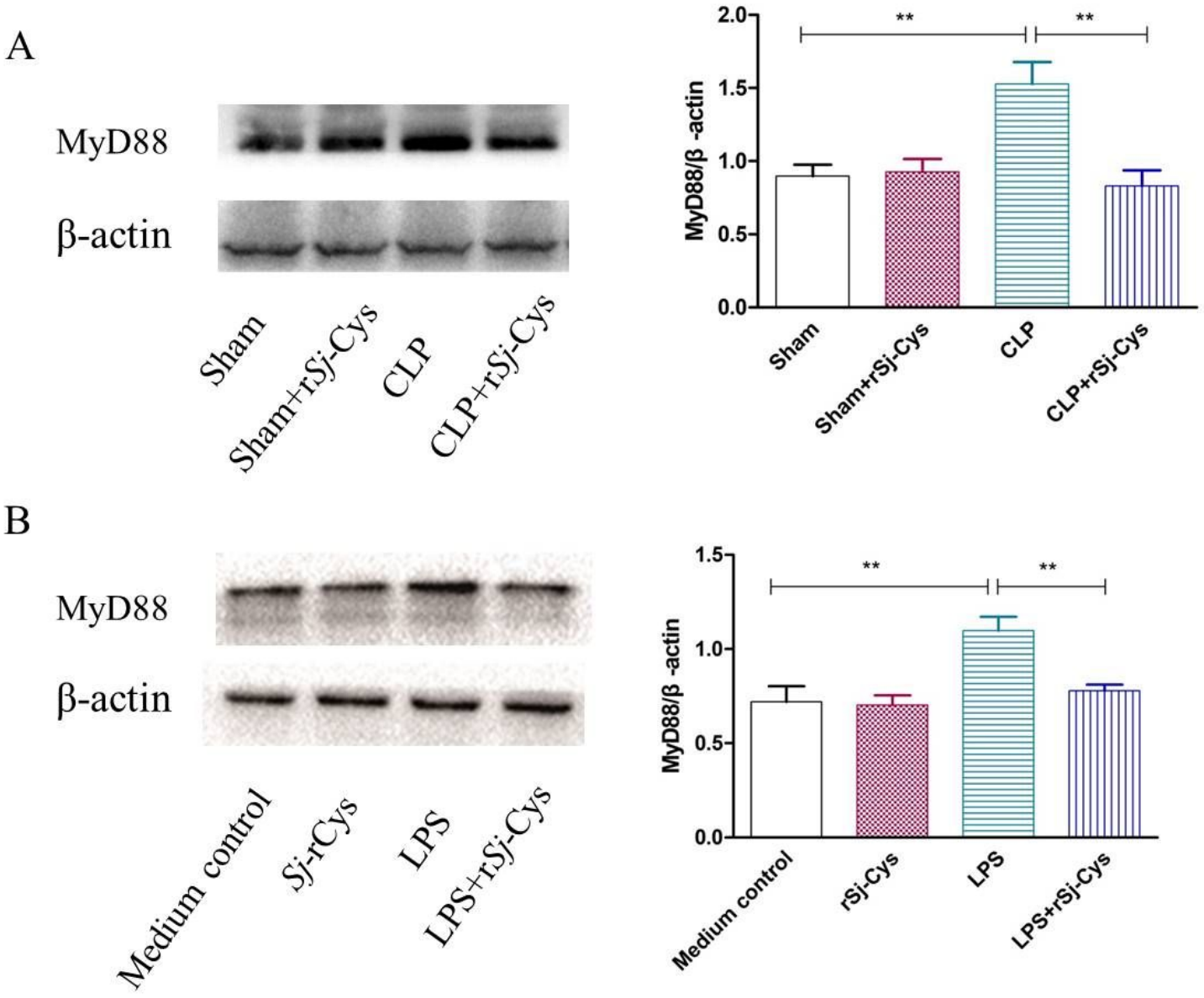

Figure 10

Effects of rSj-Cys on LPS-stimulated MyD88 expression in the cardiomyocytes. rSj-Cys treatment suppressed the expression of MyD88 in the myocardial tissues of mice with CLP-induced sepsis (A) (n = 6 mice per group) and in LPS-incubated H9C2 cells (B) (n = 3 per group) measured by Western blot. The β-actin was measured as control. The density ratio of MyD88/β-actin is shown on the right. The results are shown as the density mean ± SEM for each group. **P < 0.01.