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

Shifang Gao1,2†, Huihui Li2,3†, Hong Xie2, Shili Wu4, Yuan Yuan2,3, Liang Chu1, Siying Sun2, Huijuan Yang2, Lingqin Wu2, Yongsheng Bai2, Qiao Zhou2, Xin Wang2, Bin Zhan5, Hu Cui1* and Xiaodi Yang2,3*

**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. Although many studies have confirmed that helminth-derived proteins have strong immunomodulatory functions and could treat inflammatory diseases, there is no report on the therapeutic effect of *Schistosoma japonicum*-produced cystatin (*Sj*-Cys) on sepsis-induced cardiac dysfunction.

**Methods:** A model of sepsis-induced myocardial injury was established by cecal ligation and puncture (CLP) in mice. Upon CLP operation, each mouse was intraperitoneally treated with 10 µg of recombinant *Sj*-Cys (*r*Sj-Cys). Twelve hours after CLP, the systolic and diastolic functions of the 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, macrophages 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 in heart tissue of mice with sepsis and LPS-stimulated H9C2 cardiomyocytes. In addition, the therapeutic effects of *r*Sj-Cys on LPS-induced cardiomyocyte apoptosis were also detected. The levels of M1 biomarker iNOS and M2 biomarker Arg-1 were detected in heart tissue. 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 *r*Sj-Cys treatment, the sepsis-induced heart malfunction was largely improved. The inflammation and injury of heart tissue were significantly alleviated, characterized as 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 downregulated pro-inflammatory cytokines (TNF-α and IL-6) and upregulated regulatory inflammatory cytokines (IL-10 and TGF-β), possibly through inhibiting the LPS-MyD88 signal pathway.

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

**Keywords:** Cystatin, *Schistosoma japonicum*, Myocardial dysfunction, Immunoregulation
Background
Sepsis is a 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 cardiomyocyte 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 the 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 proportion of patients who cannot survive from severe sepsis. Other alternative approaches to better control sepsis and reduce sepsis-related myocardial dysfunction is greatly needed.

Parasitic helminths co-evolve with mammalian hosts and develop some strategies to survive within hosts. These strategies include modulating the host immune system to downregulate the immune response to helminths (parasite-specific immunomodulation) [8], characterized by a dominant Th2-mediated immune response and activated regulatory T cells (Tregs) or monocyte responses [9–11]. The helminth-induced regulatory responses not only facilitate the survival of worms in the host, but also benefit the 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 [13–15]. Further evidence showed that helminth-secreted proteins can induce the host to actively produce immune regulation [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 immunoinflammatory diseases such as allergies and autoimmune disorders [18–20]. In particular, cystatins derived from various parasitic helminths have received most of the attention because they have been identified as strong immunomodulatory proteins [14] and successfully used as potential therapeutic agents for inflammatory and autoimmune diseases [21–25]. 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 the blood-feeding trematode Schistosoma japonicum [26]. Treatment with rSj-Cys significantly stimulated Tregs and inhibited the antigen-presenting functions of dendritic cells (DCs) [27]. It also inhibited the release of pro-inflammatory factors (TNF-α, IL-6) in LPS-stimulated macrophages [23]. Recombinant Sj-Cys has been used as a therapeutic agent to alleviate the severity of dextran sulfate sodium (DSS)-induced colitis in mice [21] and murine collagen-induced arthritis [22]. Our previous study has identified that rSj-Cys displayed the therapeutic effect of 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 lungs [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 rSj-Cys on sepsis-triggered cardiac dysfunction and we found that treatment with rSj-Cys 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.

Methods
Production of recombinant 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 using the calcium transfection method. The recombinant Sj-Cys (rSj-Cys) with a His-tag at the N-terminus was induced with 1 mM isopropylthio-β-galactoside (IPTG; Sigma-Aldrich, Steinheim, Germany) at 37 °C for 5 h, and purified from the soluble fraction of the induced bacteria using a HisPur™ Ni-NTA Spin Column (Thermo Fisher Scientific Inc., Waltham, 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 using a ToxinSensor™ Chromogenic Limulus Amebocyte Lysate (LAL) Endotoxin Assay Kit (GenScript Biotechnology, Nanjing, China) following the manufacturer’s protocol. The concentration of rSj-Cys was measured using a Bicinchoninic Acid Protein Assay Kit (Beyotime Biotechnology, Shanghai, China) and the recombinant protein stored at −80 °C until use.
Sepsis-induced cardiomyopathy

The mice were subjected to CLP surgery according to the method described previously [4]. Briefly, mice were fasted for 12 h, with only drinking water available, 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 suture (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 (Syneture). Following CLP, sterile normal saline (300 μl) was injected sub-dermally for fluid resuscitation. Sham mice underwent the same process except for CLP.

Treatment of sepsis-induced cardiomyopathy with rSj-Cys

A total of 6 CLP-operated mice were treated intraperitoneally with 10 μg of rSj-Cys in a total volume of 200 μl 30 min after surgery. The same number of CLP-operated mice were given 200 μl of PBS only as a control. As normal controls, 12 mice that underwent sham surgery were divided into two groups; 6 received the same amount of rSj-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 centrifuged at 3000 × rpm for 15 min at 4 °C and stored at −80 °C until use. All mice were euthanized and hearts collected for histopathological staining and measurement.

Echocardiography

Echocardiographic evaluation was performed using a high-resolution echocardiograph (Vevo 2100; VisualSonics, Toronto, Canada) for the differently treated mice groups. Briefly, a mixture of 1% isoflurane and oxygen was inhaled via a nose cone, and each mouse was carefully kept under mild anesthesia and subjected to M-mode and Doppler echocardiography according to the method described previously [28]. The ejection fraction (EF%) and fractional shortening (FS%) of the left ventricle were calculated from M-mode tracing to reflect left systolic function. Peak early-diasstolic transmitral velocities (E wave) and peak late-diasstolic transmitral velocities (A wave) across the 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 were performed by the same skilled operator and data averaged from 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 h. Fixed left heart ventricles were sectioned and stained with hematoxylin and eosin (H&E) stain. H&E stained sections were observed under light microscopy (200 × magnification) (Nikon, Tokyo, Japan) for pathological changes.

Biochemical analysis

The heart-released myoglobin (Mb), cardiac troponin I (cTnl) 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 cTnl 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 using 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 (Bioengineering 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 and IL-6) and regulatory (IL-10 and 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, TGF-β, iNOS and Arg-1 mRNA expression by quantitative real time PCR (qRT-PCR)

Total RNA from the left ventricular myocardium was extracted with QIAzol reagent (Ambion, Austin, TX, USA). Then cDNAs were reverse-transcribed from 2 μg total RNA using a reverse transcription kit (RevertAid First Strand cDNA Synthesis Kit; Thermo Fisher Scientific Inc.). The cDNA was used as a template for qRT-PCR using the SYBR Green Super Mix Kit (Takara Bio Inc., Tokyo, Japan). All samples were duplicated and the
qRT-PCR 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 change in gene expression. The primers (GAPDH, TNF-α and IL-6) were designed and synthesized by Sangon Biotech (Shanghai, China). The forward and reverse primers of target genes are listed in Additional file 1: Table S1 [29–31].

**Cell culture and treatment**

H9C2 rat embryo cardiomyocytes were purchased from the 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, Niayet, France) and 1% penicillin/streptomycin (Gibco, Grand Island, NY) at 37 °C, 5% CO₂. Cultured H9C2 cells were treated with rSj-Cys (0.5 µg/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 rSj-Cys treatment, or cells incubated with rSj-Cys or medium alone were used as controls. After 24 h of incubation, the culture was centrifuged at 1000× rpm for 15 min 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 apoptosis was measured by annexin V-FITC and propidium iodide (PI) staining in accordance with the manufacturer’s instructions (Invitrogen, Thermo Fisher Scientific). Flow cytometric analysis was performed on a CYTEK DxP AthenaTM Analyzer (Cytek Biosciences, California, USA). The results were analyzed with FlowJo V7.6.5 software.

**Detection of MyD88 by western blotting**

MyD88 expression level in treated H9C2 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 were centrifuged at 12,000× rpm for 15 min at 4 °C. Supernatants were collected and protein concentration was quantified using a BCA assay kit (Pierce, Rockford, IL, USA). Equal amounts of cell extracts or heart homogenates were separated by 12% SDS-PAGE and electrophoretically transferred onto PVDF membranes. After blocking with 5% skimmed milk for 2 h at room temperature, 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. Immune reactive protein bands were visualized using a Tanon 5200 Chemiluminescence Imaging System (Tanon, Shanghai, China).

**Statistical analysis**

All data are expressed as the mean±standard error of the mean (SE), and statistical analyses were performed 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; $P$-value of <0.05 was considered statistically significant.

**Results**

**Treatment with rSj-Cys alleviated sepsis-induced myocardial malfunction**

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

To assess the left ventricular diastolic function, the 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: $F_{(3, 23)} = 10.99, P < 0.0002$) (Fig. 1b, d). Treatment with rSj-Cys significantly recovered the E/A ratio to a similar level of mice which had received sham surgery or sham + rSj-Cys (ANOVA: $F_{(3, 23)} = 10.99, P < 0.0002$) (Fig. 1b, d). These results indicate that treatment with rSj-Cys also improves sepsis-induced diastolic malfunction in mice. No significant difference was observed between the sham group and the sham rSj-Cys-treated group in terms of E/A ratio (ANOVA: $F_{(3, 23)} = 10.99, P < 0.0002$) (Fig. 1d).
Fig. 1 Treatment of rSj-Cys improved the sepsis-induced myocardial malfunction. Representative M-mode echocardiograms obtained from mice 12 h after treatment of sham-operation, sham + rSj-Cys, CLP and CLP + rSj-Cys, respectively (a). The improved left ventricular systolic function evaluated by EF and FS after treatment with rSj-Cys (c). The improved CLP-induced left ventricular diastolic dysfunction was shown as representative transmitral Doppler images. The E wave represents peak early-diastolic transmitral velocity, and the A wave indicates peak late-diastolic transmitral velocity (b). The changes of the E/A ratio were used to assess the alteration in left ventricular diastolic function (d). (n = 6 mice per group). Data are presented as the mean ± SE. ***P < 0.001
Treatment of rSj-Cys reduced sepsis-induced pathological heart abnormalities
The morphological structures and pathology of the myocardial tissues of mice 12 h after CLP surgery were determined by H&E staining. The results showed that the sham surgery group and sham with rSj-Cys group had no significant inflammatory cell infiltration with normal appearance of the myofibrillar structure (Fig. 2a). However, the heart tissue in mice with CLP showed significant inflammation, myocardial fiber arrangement disorder and highly recruited inflammatory cell infiltration. Of note, tissue sections from the CLP + rSj-Cys mice group showed significantly improved muscle fiber structure with reduced inflammatory cell infiltration compared with CLP group without rSj-Cys treatment (Fig. 2a). The pathological results indicate that rSj-Cys effectively alleviates CLP-induced cardiac lesions and inflammation in mice.

Administration of rSj-Cys reduced sepsis-induced heart injury
In myocardial injury, released Mb, cTnI, NT-proBNP and MPO into sera or heart tissue are usually used as biomarkers to evaluate the ischemic severity of heart injury [32–34]. Compared with the sham group, the CLP group showed a marked increase in MPO activity in the myocardial tissue homogenate and elevated levels of cTnI, Mb, NT-proBNP in sera (ANOVA: \( F_{(3, 23)} = 32.10, P < 0.0001; F_{(3, 23)} = 61.36, P < 0.0001; F_{(3, 23)} = 10.42, P < 0.0002; \) and \( F_{(3, 23)} = 42.79, P < 0.0001, \) respectively) (Fig. 2b). After being treated with rSj-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: \( F_{(3, 23)} = 32.10, P < 0.0001; F_{(3, 23)} = 61.36, P < 0.0001; F_{(3, 23)} = 10.42, P < 0.0002; \) and \( F_{(3, 23)} = 42.79, P < 0.0001, \) respectively) (Fig. 2b), while 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 rSj-Cys groups. The increased MPO activity in the heart tissue of CLP-induced sepsis mice was correlated with the increased inflammatory cell infiltration, especially neutrophils, in the heart tissue (Fig. 2b).

rSj-Cys inhibits pro-inflammatory cytokines and induces regulatory cytokines in mice with sepsis-caused heart injury
To understand the mechanisms underlying the improvement of sepsis-caused cardiac dysfunction with treatment of rSj-Cys, the levels of pro-inflammatory cytokines (TNF-α and IL-6) and regulatory cytokines (IL-10 and 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 and IL-6) dramatically increased in the sera of CLP-induced sepsis mice, compared to that in mice with sham surgery only or sham + rSj-Cys (ANOVA: \( F_{(3, 23)} = 18.39, P < 0.0001 \) and \( F_{(3, 23)} = 361.3, P < 0.0001, \) respectively) (Fig. 3a). Treatment with rSj-Cys significantly reduced the production of TNF-α and IL-6 in CLP-induced sepsis mice, compared with CLP mice without treatment (ANOVA: \( F_{(3, 23)} = 18.39, P < 0.0001 \) and \( F_{(3, 23)} = 361.3, P < 0.0001, \) respectively) (Fig. 3a). 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 rSj-Cys compared with the CLP group without treatment (Fig. 3a). The IL-10 and TGF-β levels were significantly lower in CLP-induced sepsis mice than that in sham surgery or sham + rSj-Cys mice (ANOVA: \( F_{(3, 23)} = 9.032, P < 0.0006 \) and \( F_{(3, 23)} = 9.789, P < 0.0004, \) respectively) (Fig. 3a). The mRNA expression levels of pro-inflammatory cytokines (TNF-α and IL-6) and regulatory cytokines (IL-10 and TGF-β) detected in heart tissue showed a similar pattern to that measured in sera (Fig. 3b). The results suggested that CLP-induced sepsis mice stimulated the secretion of pro-inflammatory cytokines (TNF-α and IL-6), but inhibited the regulatory immune pathway (lower levels of IL-10 and TGF-β). Treatment of rSj-Cys was able to significantly inhibit the activation of the pro-inflammatory pathway, possibly by activating the regulatory immune pathway. Interestingly, the mRNA level of the M1 macrophage marker iNOS was significantly reduced, and the M2 macrophage maker Arg-1 significantly increased in heart tissues of rSj-Cys-treated sepsis-mice (ANOVA: \( F_{(3, 11)} = 4.967, P < 0.0311 \) and \( F_{(3, 11)} = 77.27, P < 0.0001, \) respectively) (Fig. 3b), indicating that more macrophages shifted from M1 to M2 after being treated with rSj-Cys.

The inhibitory effect of rSj-Cys on LPS-induced inflammatory response in H9C2 cardiomyocytes
LPS is thought to be the major component to cause cardiac dysfunction in sepsis by inducing the innate immune inflammatory response [35]. In the present study, we identified that LPS significantly induced H9C2 cardiomyocytes to release pro-inflammatory cytokines IL-6 and TNF-α (ANOVA: \( F_{(3, 11)} = 20.78, P < 0.0004 \) and \( F_{(3, 11)} = 18.53, P < 0.0006, \) respectively), but inhibited the release of regulatory cytokines TGF-β and IL-10 (ANOVA: \( F_{(3, 11)} = 25.67, P < 0.0002 \) and \( F_{(3, 11)} = 14.41, P < 0.0001, \) respectively) (Fig. 3a, b). The inhibitory effect of rSj-Cys on LPS-induced inflammatory response was indicated by the significantly reduced TNF-α and IL-6 levels (ANOVA: \( F_{(3, 11)} = 9.032, P < 0.0006 \) and \( F_{(3, 11)} = 9.789, P < 0.0004, \) respectively) (Fig. 3a) and increased IL-10 and TGF-β levels (ANOVA: \( F_{(3, 11)} = 4.967, P < 0.0311 \) and \( F_{(3, 11)} = 77.27, P < 0.0001, \) respectively) (Fig. 3b). Moreover, the inhibitory effect of rSj-Cys on LPS-induced inflammatory response was further confirmed by the down-regulated pro-inflammatory cytokines TNF-α and IL-6 (ANOVA: \( F_{(3, 11)} = 20.78, P < 0.0004 \) and \( F_{(3, 11)} = 18.53, P < 0.0006, \) respectively) (Fig. 3a) and up-regulated regulatory cytokines IL-10 and TGF-β (ANOVA: \( F_{(3, 11)} = 25.67, P < 0.0002 \) and \( F_{(3, 11)} = 14.41, P < 0.0001, \) respectively) (Fig. 3b). The results indicated that rSj-Cys effectively inhibited the release of pro-inflammatory cytokines, possibly by activating the regulatory immune pathway and inhibiting the activation of pro-inflammatory cytokines.
the expression of MyD88 in cardiac tissue of sepsis mice (Fig. 4c). Treatment with \( rSj \)-Cys alone or \( rSj \)-Cys. The flow cytometry results revealed that incubation with LPS induced apoptosis in 14.4% of H9C2 cells, whereas co-incubation with \( rSj \)-Cys significantly reduced LPS-induced apoptosis to a level similar to cells without LPS (ANOVA: \( F_{(3, 11)} = 22.68, P < 0.0003 \)) (Fig. 4b). There was no significant difference in the apoptotic rate between the \( rSj \)-Cys-alone group and blank control group.

**rSj-Cys reduced LPS-induced cardiomyocyte apoptosis**

To further determine whether \( rSj \)-Cys reduced LPS-induced cardiomyocyte apoptosis, H9C2 cells were incubated with LPS alone or with \( rSj \)-Cys. The results revealed that incubation with LPS induced apoptosis in 14.4% of H9C2 cells, whereas co-incubation with \( rSj \)-Cys significantly reduced LPS-induced apoptosis to a level similar to cells without LPS (ANOVA: \( F_{(3, 11)} = 22.68, P < 0.0003 \)) (Fig. 4b). There was no significant difference in the apoptotic rate between the \( rSj \)-Cys-alone group and blank control group.

**rSj-Cys suppressed the expression 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 \( rSj \)-Cys on sepsis-induced inflammation and damage of cardiomyocytes, we detected the expression of MyD88 in heart tissue of mice with CLP-induced sepsis treated with \( rSj \)-Cys in vivo, and in LPS-stimulated H9C2 cells co-incubated with \( rSj \)-Cys in vitro. The elevated level of MyD88 was observed in cardiac tissue 12 h after CLP surgery compared with the sham surgery control (ANOVA: \( F_{(3, 11)} = 8.823, P < 0.0064 \)) (Fig. 4c). Treatment with \( rSj \)-Cys significantly reduced the expression of MyD88 in heart tissue of sepsis mice compared with mice without treatment (ANOVA: \( F_{(3, 11)} = 8.823, P < 0.0064 \)) (Fig. 4c). There was no effect of \( rSj \)-Cys on the expression of MyD88 in normal mice (sham control). Meanwhile, the expression of MyD88 was also increased in cardiomyocytes incubated with LPS for 24 h. Co-incubation with \( rSj \)-Cys significantly suppressed the expression of MyD88 in LPS-stimulated cardiomyocytes (ANOVA: \( F_{(3, 11)} = 8.550, P < 0.0071 \)) (Fig. 4d).

**Discussion**

Myocardial dysfunction is a fatal complication of patients with sepsis [18]. Studies have found that 50% of patients with sepsis have systolic and diastolic dysfunction in the heart left and right ventricle, possibly caused by endotoxin-induced myocardial injury [36] which has been confirmed both in animal and clinical observations [37]. In this study, we also confirm that CLP-induced sepsis caused serious myocardial damage characterized by a significant reduction of left ventricular systolic and diastolic functions in mice, which closely mimicked the pathological features of myocardial infarction observed in clinical patients [38]. We further confirm that LPS released by Gram-negative bacteria could cause apoptosis of H9C2 cardiomyocytes when co-incubated in vitro.

Cysteine proteases have been regarded as key molecules in regulating inflammation, cell apoptosis, cancer progression, protein degradation and antigen presentation [39–43]. Since cysteine proteases are largely involved in the inflammation and immune responses, their inhibitor, cystatin, could be a potential modulator for an 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 \( S. japonsicum \) 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-\( \alpha \) and IL-6 cytokines and induced M2 macrophage polarization [23]. Treatment with \( rSj \)-Cys significantly reduced TNBS-induced experimental colitis in mice through upregulation of Treg cells and related cytokines IL-10 and TGF-\( \beta \), and downregulation of pro-inflammatory cytokines TNF-\( \alpha \) and IL-6 in the colon tissues of mice [44].

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 h 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

\( P < 0.0014 \), respectively) compared with cells treated with PBS (Fig. 4a). After being treated with \( rSj \)-Cys, the LPS-induced IL-6 and TNF-\( \alpha \) were reduced to the level of cells unstimulated by LPS (ANOVA: \( F_{(3, 11)} = 20.78, P < 0.0004 \) and \( F_{(3, 11)} = 18.53, P < 0.0006 \), respectively), and TGF-\( \beta \) and IL-10 were significantly boosted compared to cells without \( rSj \)-Cys treatment (ANOVA: \( F_{(3, 11)} = 25.67, P < 0.0002 \) and \( F_{(3, 11)} = 14.41, P < 0.0014 \), respectively) (Fig. 4a). The \( rSj \)-Cys alone had no significant effect on the innate immune response of normal cardiomyocytes.
heart malfunction has been significantly improved, showing a recovered 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 the MPO level in heart tissue are the important biochemical markers of cardiac damage and injury in early septic shock [32, 33, 48, 49]. The CLP-induced sepsis model established in this study demonstrated significantly increased levels of cTnI, NT-proBNP and Mb in sera and a high level of MPO in heart tissue, indicating that the heart tissue and cells were seriously damaged as a result of the CLP-induced sepsis. The significantly increased inflammatory cell infiltration in heart tissue also demonstrated the serious inflammation occurring in the heart.

After being treated with rSj-Cys, the sepsis-induced heart malfunction has been significantly improved, showing a recovered left ventricular EF, FS and E/A ratio. The inflammation of heart tissue was also significantly reduced, as illustrated 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 the heart tissue and cells were seriously damaged as a result of the CLP-induced sepsis. The significantly increased inflammatory cell infiltration in heart tissue also demonstrated the serious inflammation occurring in the heart.

Another interesting finding in this study is that heart tissue from rSj-Cys-treated sepsis-mice expressed more Arg-1 and less iNOS compared to the mice without rSj-Cys treatment. Arg-1 is the biomarker for M2 alternatively activated macrophages that release anti-inflammatory cytokines to suppress immune responses and restore tissue homeostasis whereas iNOS is the biomarker of M1 classically activated macrophages responsible for the pro-inflammatory response. The shifting of
Fig. 3 Treatment with rSj-Cys reduced the pro-inflammatory cytokine (TNF-α and IL-6) and boosted regulatory cytokine (IL-10 and TGF-β) levels in sera (a) and the similar mRNA expression pattern observed in heart tissues (b) of mice with CLP-induced sepsis 12 h after treatment. The mRNA expression level of M1 macrophage marker (iNOS) was reduced and that of M2 macrophage marker (Arg-1) increased in heart tissues (b). The data are shown as the mean±SE for each group (n = 3 mice per group). *P < 0.05, **P < 0.01, ***P < 0.001
pro-inflammatory M1 macrophages to anti-inflammatory M2 macrophages in the heart tissue of sepsis mice after being treated with rSJ-Cys may provide another mechanism involved in the reduced inflammation and heart damage caused by sepsis. M2-type macrophages have been reported to attenuate experimental inflammation in dinitrobenzene sulfonic acid (DNBS)-induced colitis in mice [61, 62].

LPS associates with its receptor, the 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 [63]. Although the mechanism leading to sepsis-induced cardiac arrest remains controversial, there is increasing evidence supporting that TLR-mediated innate immunity and inflammatory responses play a key role in cardiac dysfunction caused by sepsis or septic shock [64–66]. Activation of the TLR4 signaling pathway may directly lead to myocardial cells’ dysfunction. The invaded bacteria or other external stimulus first trigger innate immunity and then induce TLR4 expression by upregulating the MyD88-mediated pathway and activating the transcription of nuclear factor-κB (NF-κB), resulting in the production of various inflammatory mediators, such as cytokines, chemokines and antimicrobial peptides [67]. 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 heart [7, 68] and in mice [69]. To investigate whether rSJ-Cys-involved anti-inflammatory and anti-apoptosis effects through inhibiting the MyD88-dependent signaling pathway, the level of MyD88 in CLP-induced septic heart tissue and in LPS-stimulated H9C2 cells was measured. Our study found that the expression of MyD88 increased in CLP-induced myocardial tissue or LPS-stimulated H9C2 cells, and treatment with rSJ-Cys significantly reduced the expression of MyD88 in these cardiomyocytes. It is under investigation whether treatment of rSJ-Cys reduces TLR-2...
or TLR-4 activation that results in downregulation of MyD88.

Our results suggest the possible mechanism of rSj-Cys involved in the alleviation of septic cardiomyopathy is that treatment of rSj-Cys stimulates Tregs and/or cardiomyocytes to produce regulatory cytokines such as IL-10 and TGF-β, and promotes the differentiation of M1 to M2 macrophages in heart tissue, thereby suppressing the production of pro-inflammatory cytokines via inhibiting the MyD88 activation signal pathway as shown for other helminth-derived proteins [70–73].

Conclusions

The present data show that rSj-Cys strongly alleviated excessive inflammation and protected against sepsis-induced cardiac dysfunction. Therefore, rSj-Cys could be considered as a potential therapeutic agent for the prevention and treatment of sepsis associated cardiac dysfunction.

Supplementary information

Supplementary information accompanies this paper at https://doi.org/10.1186/s13071-020-04104-3.

Abbreviations

CLP: cecal ligation and puncture; LPS: lipopolysaccharides; Mb: myoglobin; cTnl: 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 transmitial velocities; A wave: peak late-diastolic transmitial velocities; L.V: 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 cells; Tregs: regulatory T cells; DNBS: dinitrobenzenic sulfonic acid; DSS: dextran sulfmate sodium; TLR4: toll-like receptor 4; NF-κB: nuclear factor-κB; SE: standard error of the mean; SPF: specific pathogen free; SDS-PAGE: sodium dodecyl sulfate polyacrylamide gel electrophoresis; PVDF: polyvinylidene fluoride.

Acknowledgments

We thank Dr Li He at the Basic Medical School of Wuhan University, for providing the recombinant plasmid. We thank Dr Qin Gao at the Basic Medical College of Bengbu Medical College, for providing H9C2 cells. We thank Dr Li He at the Basic Medical School of Wuhan University, for providing the recombinant plasmid. We thank Dr Qin Gao at the Basic Medical College of Bengbu Medical College, for providing H9C2 cells. We thank Dr Li He at the Basic Medical School of Wuhan University, for providing the recombinant plasmid. We thank Dr Qin Gao at the Basic Medical College of Bengbu Medical College, for providing H9C2 cells.

Authors' contributions

XX, HC, and SG conceived and designed the study. SG, HL, HK, SS, HY, LW, YB, QZ and XW performed the experiments. YY, SW and LC analyzed the data. SG wrote the manuscript. BZ, YY and HC critically revised the manuscript. All authors read and approved the final manuscript.

Funding

This project was supported by the Scientific Research Innovation Platform Team of University (No. 2015–40), the Cardiovascular Injury and Protection Foundation and Clinical Application Innovation Team (No. BYKC201906), the Science Foundation of Anhui Province (No. gsbj2D15, 201904a07020017 and 2018H174), the Program of Natural Science Foundation of the Anhui Higher Education Institutions (No. KJ2019A0383), the Science Foundation of Bengbu Medical College (No. BYTM2019002), the Postgraduate Scientific Research Innovation Program of Bengbu Medical College (No. Byyx182), Byyx1920 and Byyx1903) and the National University Students’ Innovation and Entrepreneurship Training Program (No. 201910367025, 201810367012 and 201910367002).

Availability of data and materials

The datasets supporting the findings of this article are included within the article and its additional file.

Ethics approval and consent to participate

All procedures concerning 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 no. AMU26-08061).

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

Author details

1 Second Affiliated Hospital of Bengbu Medical College, Bengbu 233000, China. 2 Anhui Key Laboratory of Infection and Immunity of Bengbu Medical College, Bengbu 233000, China. 3 Basic Medical College of Bengbu Medical College, Bengbu 233000, China. 4 First Affiliated Hospital of Bengbu Medical College, Bengbu 233000, China. 5 National School of Tropical Medicine, Baylor College of Medicine, Houston, TX 77030, USA.

Received: 22 December 2019 Accepted: 27 April 2020

Published online: 18 May 2020

Additional file 1: Table S1. Primer sequences used for qRT-PCR analysis.

References

1. Fleischmann C, Scherag A, Adhikari NK, Hartog CS, Tsaganos T, Schlattmann P, et al. Assessment of global incidence and mortality of hospital-treated sepsis. Am J Respir Crit Care Med. 2016;193:259–72.
2. Becker JU, Theodosis C, Jacob ST, Wira CR, Groce NE. Surviving sepsis in low-income and middle-income countries: new directions for care and research. Lancet Infect Dis. 2009;9:577–82.
3. Buerke U, Carter JM, Schlitt A, Russ M, Schmidt H, Sibielus U, et al. Apoptosis contributes to septic cardiomyopathy and is improved by simvastatin therapy. Shock. 2008;29:497–503.
4. Rudiger A, Singer M. Mechanisms of sepsis-induced cardiac dysfunction. Crit Care Med. 2007;35:1599–608.
5. Wang Y, Zhang H, Chai F, Liu X, Berkshire M. The effects of escitalopram on myocardial apoptosis and the expression of Bax and Bcl-2 during myocardial ischemia/reperfusion in a model of rats with depression. BMC Psychiatry. 2014;14:349.
6. Merx MW, Weber C. Sepsis and the heart. Circulation. 2007;116:793–802.
7. Feng Y, Zou L, Chen C, Li D, Chao W. Role of cardiac- and myeloid-MyD88 signaling in endotoxin shock: a study with tissue-specific deletion models. Anesthesiology. 2014;121:1258–69.
8. Elliott DE, Weinstock JV. Helminth-host immunological interactions: prevention and control of immune-mediated diseases. Ann N Y Acad Sci. 2012;1247:83–96.
9. Babu S, Kumaraswami V, Nutman TB. Alternatively activated and immunoregulatory monocytes in human filarial infections. J Infect Dis. 2009;199:1827–37.
10. O’Regan NL, Steinfelder S, Venugopal G, Rao GB, Lucius R, Srikantam A, et al. Brugia malayi microfilariae induce a regulatory monocyte/macrophage phenotype that suppresses innate and adaptive immune responses. PLoS Negl Trop Dis. 2014;8:e2306.
11. Passos LS, Gazzinelli-Guimaraes PH, Oliveira Mendes TA, Guimaraes AC, Silva Lemos DD, Ricci ND, et al. Regulatory monocytes in human filariasis: insights from the modulation during human hookworm infection. BMC Infect Dis. 2017;17:253.
12. Li H, Wang S, Zhan B, He W, Chu L, Qiu D, et al. Therapeutic effect of Schistosoma japonicum cystatin on bacterial sepsis in mice. Parasit Vectors. 2017;10:222.
13. Anthony RM, Urban JF Jr, Alem F, Hamed HA, Rozo CT, Boucher JL, et al. Memory T(H2) cells induce alternatively activated macrophages to mediate protection against nematode parasites. Nat Med. 2006;12:955–60.

14. Bishn N, Khati V, Chauhan N, Kayan sandaram R. Cystatin from filarial parasites suppress the clinical symptoms and pathology of experimentally induced colitis in mice by inducing T-regulatory cells, B1-cells, and alternatively activated macrophages. Biomedicines. 2019;7:85.

15. Hubner MP, Layland LE, Hoerauf A. Helminths and their implication in sepsis - a new branch of their immunomodulatory behaviour? Pathog Dis. 2013;69:127–41.

16. Hewitson JF, Grainger JR, Maizels RM. Helminth immunoregulation: the role of parasite secreted proteins in modulating host immunity. Mol Biochem Parasitol. 2009;167:1–11.

17. McSorley HJ, Hewitson JF, Maizels RM. Immunomodulation by helminth parasites: defining mechanisms and mediators. Int J Parasitol. 2013;43:301–10.

18. Buck AH, Coakley G, Simbari F, McSorley HJ, Quintana JF, Le Bihan T, et al. A helminth immunomodulator reduces allergic and inflammatory responses by induction of IL-10-producing macrophages. J Immunol. 2014;192:5485–92.

19. Siles-Lucas M, Morchon R, Simon F, Manzano-Roman R. Exosome-containing microRNAs from helminth origin: new tools for allergic and autoimmune diseases therapy? Parasite Immunol. 2015;37:208–14.

20. Cascardo GG, Fura JA, de Pauw NC, Lemos Leite E, Ricci NG, Gazzei-Guimarães PH, et al. Hookworm proteins ameliorate dextran sodium sulfate-induced colitis in BALB/c mice. Inflamm Bowel Dis. 2011;17:2275–86.

21. Wang L, Yu Z, Wan S, Wu F, Chen W, Zhang B, et al. Exosomes derived from dendritic cells treated with Schistosoma japonicum soluble egg antigen attenuate DSS-induced colitis. Front Pharmacol. 2017;8:651.

22. Liu F, Cheng W, Pappoe F, Hu X, Wen H, Luo Q, et al. Characterization of cystatin attenuates murine collagen-induced arthritis. Parasitol Res. 2016;115:795–806.

23. Yang X, Liu J, Yu Y, Chen W, Song M, Zhan X, et al. Cloning, expression and characterization of a type II cystatin from Schistosoma japonicum, which could regulate macrophage activation. Parasitol Res. 2014;113:3985–92.

24. Jang SW, Cho MK, Park MW, Kang SA, Na BK, Ahn SC, et al. Parasitic helminth cystatin inhibits DSS-induced intestinal inflammation via IL-10(+)+F4/80(-) macrophage recruitment. Korean J Parasitol. 2019;47:245–54.

25. Schneffler C, Rauch S, Pillar S, Avagyan A, Wittd BM, Lodorkenkemper C, et al. A helminth immunomodulator reduces allergic and inflammatory responses by induction of IL-10-producing macrophages. J Immunol. 2008;180:4265–72.

26. He B, Cai G, Ni Y, Li Y, Zong H, He L. Characterization and expression of a novel cystatin gene from Schistosoma japonicum. Mol Cell Probes. 2011;25:186–93.

27. Chen L, He B, Hou W, He L. Cysteine protease inhibitor of Schistosoma japonicum—a parasite-derived negative immunoregulatory factor. Parasitol Res. 2017;116:901–8.

28. Semeniiuk LM, Kryski AJ, Severson DL. Echocardiographic assessment of cardiac function in diabetic db/db and transgenic db/db-hGLUT4 mice. J Mol Cell Cardiol. 2008;43:301–10.

29. Chen ZB, Tang H, Liang YB, Yang W, Wu JG, Hu XC, et al. Recombinant Trichinella spiralis 53-kDa protein activates M2 macrophages and attenuates the LPS-induced damage of endotoxemia. Innate Immun. 2016;22:419–32.

30. Alves-Filho JC, de Freitas A, Spiller F, Souto FO, Cunha FO. The role of neutrophils in severe sepsis. Shock. 2008;30:3–9.

31. Cheng Y, Yang C, Luo D, Li X, Le XC, Rong J. N-propargyl caffeamide skews macrophage towards a resolving M2-like phenotype against myocardial ischemic injury activating Nrf2/HO-1 pathway and inhibiting NF-κB signaling pathway. Exp Ther Med. 2019;18:779–85.

32. Nakao K, Nakao Y, Tachibana M, Taniuchi N. Role of Kupffer cells in sepsis-related death of mice. Am J Pathol. 1998;153:1327–36.

33. Kawasaki A, Takahashi S, Sudo T, Tanaka T, Nishimura H, Ohno Y, et al. Macrophage depletion in the dog. Circulation. 1983;67:1016–23.

34. Furr J, Ni Y, Le XC, Tsuchiya T, He L. Cystatin inhibits neutrophil recruitment, leukocyte chemotaxis, and cardiac troponins in sepsis-related deaths: a forensic perspective. Int J Legal Med. 2016;130:1035–43.

35. Xie J, Zhang L, Fan X, Dong X, Zhang Z, Fan W. MicroRNA-146a improves sepsis-induced cardiomyopathy by regulating the TRA-4/NF-kappaB signaling pathway. Exp Ther Med. 2019;18:779–85.

36. Alves-Filho JC, de Freitas A, Spiller F, Souto FO, Cunha FO. The role of neutrophils in severe sepsis. Shock. 2008;30:3–9.

37. Chen F, Wang J, Li K, Yao J, Li X, et al. Anti-TNF-α therapy attenuates murine collagen-induced arthritis. Parasitol Res. 2016;115:3795–806.

38. Sanchez-Villamil JP, D’Annunzio V, Finocchietto P, Holod S, Rebagliati I, Perez H, et al. Cardiac-specific overexpression of thioredoxin 1 attenuates mitochondrial and myocardial dysfunction in septic mice. Int J Biochem Cell Biol. 2016;81:323–34.

39. Lang A, Horler D, Baici A. The relative importance of cysteine peptidases in osteoarthritis. J Rheumatol. 2000;27:1970–9.

40. Turk V, Turk B, Turk D. Lysosomal cysteine proteases: facts and opportunities. EMBO J. 2001;20:4629–33.

41. Spray DC, Heuser JE, Hartburn G, Kneen R. Neutrophils in sepsis-related deaths: a forensic perspective. Int J Legal Med. 2016;130:1035–43.
58. Turdi S, Han X, Huff AF, Roe ND, Hu N, Gao F, et al. Cardiac-specific overexpression of catalase attenuates lipopolysaccharide-induced myocardial contractile dysfunction: role of autophagy. Free Radic Biol Med. 2012;53:1327–38.

59. Smallwood TB, Giacomin PR, Loukas A, Mulvenna JP, Clark RJ, Miles LJ. Helminth immunomodulation in autoimmune disease. Front Immunol. 2017;8:453.

60. Scumpia PO, Moldawer LL. Biology of interleukin-10 and its regulatory roles in sepsis syndromes. Crit Care Med. 2005;33:5468–71.

61. Hunter MM, Wang A, Parhar KS, Johnston MJ, Van Rooijen N, Beck PL, et al. In vitro-derived alternatively activated macrophages reduce colonic inflammation in mice. Gastroenterology. 2010;138:1395–405.

62. Faz-López B, Morales-Montor J, Terrazas LI. Role of macrophages in the repair process during the tissue migrating and resident helminth infections. Biomed Res Int. 2016;2016:6543603.

63. Tan S, Long Z, Hou X, Lin Y, Xu J, You X, et al. H2 protects against lipopolysaccharide-induced cardiac dysfunction via blocking TLR4-Mediated cytokines expression. Front Pharmacol. 2019;10:865.

64. Williams DL, Ha T, Li C, Kalbfleisch JH, Schweitzer J, Vogt W, et al. Modulation of tissue toll-like receptor 2 and 4 during the early phases of polymicrobial sepsis correlates with mortality. Crit Care Med. 2003;31:1808–18.

65. Feng Y, Zou L, Zhang M, Li Y, Chen C, Chao W. MyD88 and TIRf signaling play distinct roles in cardiac dysfunction and mortality during endotoxin shock and polymicrobial sepsis. Anesthesiology. 2011;115:S55–67.

66. Avila O, Fallach R, Shainberg A, Porat E, Hochhauser E. Toll-like receptor 4 stimulation initiates an inflammatory response that decreases cardiomyocyte contractility. Antioxid Redox Signal. 2011;15:985–909.

67. Kawai T, Akira S. Signaling to NF-kappaB by toll-like receptors. Trends Mol Med. 2007;13:460–9.

68. Fernandes CJ Jr, de Assuncao MS. Myocardial dysfunction in sepsis: a large, unsolved puzzle. Crit Care Res Pract. 2012;2012:896430.

69. Cain BS, Meldrum DR, Dinarello CA, Meng X, Joo KS, Banerjee A, et al. Tumor necrosis factor-alpha and interleukin-1beta synergistically depress human myocardial function. Crit Care Med. 1999;27:1309–18.

70. Puneet P, McGrath MA, Tay HK, Al-Riyami L, Reapecka J, et al. Retraction: The helminth product ES-62 protects against septic shock via toll-like receptor 4-dependent autophagosomal degradation of the adaptor MyD88. Nat Immunol. 2011;12:804.

71. Du L, Liu L, Yu Y, Shan H, Li L. Trichinella spiralis excretory-secretory products protect against polymicrobial sepsis by suppressing MyD88 via mannose receptor. Biomed Res Int. 2014;2014:898646.

72. Turner JD, Langley RS, Johnston KL, Egerton G, Wanjir, Taylor MJ. Wolbachia endosymbiotic bacteria of Brugia malayi mediate macrophage tolerance to TLR- and CD40-specific stimuli in a MyD88/TLR2-dependent manner. J Immunol. 2006;177:1240–9.

73. Martin I, Caban-Hernandez K, Figueroa-Santiago Q, Espino AM. Fasciola hepatica fatty acid binding protein inhibits TLR4 activation and suppresses the inflammatory cytokines induced by lipopolysaccharide in vitro and in vivo. J Immunol. 2015;194:3924–36.

Publisher’s Note
Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.