

ORIGINAL ARTICLE

Attenuation of Sepsis-Induced Cardiomyopathy by Regulation of MicroRNA-23b Is Mediated Through Targeting of MyD88-Mediated NF- κ B Activation

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Abstract— Myocardial cell injury or cardiomyopathy is associated with excessive inflammatory response and apoptosis of cardiac myocytes during sepsis. MicroRNA-23b (miR-23b) is a multifunctional miRNA that is considered to regulate immunosuppression in sepsis. The aim of this study was to examine the effect of miR-23b on cardiomyopathy induced by sepsis and to explore the potential mechanism involved. Sprague-Dawley rats were subjected to cecal ligation and puncture (CLP), and the level of miR-23b at different time points was measured by quantitative real-time polymerase chain reaction (qPCR). Then, we overexpressed miR-23b *in vivo* and *in vitro*. The rats were subjected to CLP 7 days after transfection. Cardiac function, inflammatory response, and heart tissues were examined 3 days thereafter. In an *in vitro* experiment, H9C2 cardiomyoblasts were stimulated with lipopolysaccharide (LPS) after transfection of miR-23b, following which apoptosis and the level of NF- κ B were analyzed. The expression of miR-23b was upregulated during polymicrobial sepsis, and transfection of miR-23b lentivirus improved the outcome of sepsis-induced cardiomyopathy by attenuating inflammatory responses and protecting

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ABBREVIATIONS: *BNP*, Brain natriuretic peptide; *CK-MB*, Creatine kinase-MB; *CLP*, Cecal ligation and puncture; *CO*, Cardiac output; *EF*, Ejection fraction; *ELISA*, Enzyme-linked immunosorbent assay; *EMSA*, Electrophoretic mobility shift assay; *ICAM-1*, Intercellular cell adhesion molecule 1; *LPS*, Lipopolysaccharide; *MIF*, Migration inhibitory factor; *miR-23b*, MicroRNA-23b; *NF- κ B*, Nuclear factor kappaB; *qPCR*, Quantitative real-time polymerase chain reaction; *SIC*, Sepsis-induced cardiomyopathy; *TLR*, Toll-like receptor; *VCAM-1*, Vascular cell adhesion molecule 1

against histopathological damage. In *in vitro* experiments, elevated miR-23b inhibited excessive apoptosis of cardiomyocytes, which may be because activation of the NF- κ B signaling pathway was inhibited by the decreased levels of TRAF6 and IKK β . Therefore, miR-23b improved sepsis-induced cardiomyopathy by attenuating the inflammatory response, suppressing apoptosis, and preventing NF- κ B activation *via* targeted inhibition of TRAF6 and IKK β . These results indicated that miR-23b may represent a novel therapeutic approach for clinical treatment of sepsis-induced cardiomyopathy.

KEY WORDS: microRNA-23b; sepsis-induced cardiomyopathy; inflammatory response; NF- κ B; TRAF6; IKK β .

INTRODUCTION

Sepsis is a life-threatening organ dysfunction syndrome caused by a dysregulated host response to infection [1], which subsequently results in systemic inflammatory response syndrome and progressive multiple organ dysfunctions, especially cardiac dysfunction [2]. Cardiovascular dysfunction or cardiomyopathy caused by sepsis has been revealed as the major cause of increased mortality in patients with septic shock [3, 4]. Studies have shown that the incidence and prevalence of cardiomyopathy induced by sepsis are as high as 50%, with the mortality reaching 70% [5]; survivors of sepsis also have a high risk of cardiovascular events [6], which are considered manifestations of severe sepsis or septic shock [7]. Hence, the specific pathogenesis and mechanisms of cardiomyopathy in sepsis warrant elucidation.

Sepsis-induced cardiomyopathy (SIC) is characterized by the reversible dysfunction of both the left and right sides of the heart throughout the cardiac cycle (systolic and diastolic) during sepsis [8], which involves a complicated response to pathogens including inflammatory response, mitochondrial dysfunction, altered metabolism, impaired energetics, and structural alterations [9, 10]. Although the myocardial suppression in sepsis has been investigated over past decades [11], the exact pathophysiological mechanisms leading to cardiac dysfunction, as well as effective therapeutic strategies for SIC, require further exploration. Innate immune and inflammatory responses activated by Toll-like receptors (TLRs), which mediate signaling and predominantly activate nuclear factor kappaB (NF- κ B), play an essential role in regulating the inflammatory responses in infectious diseases [12, 13]. Moreover, TLR-mediated NF- κ B signaling has been considered an important participant in SIC in recent years [14–16]. Therefore, targeting the regulation of the TLR-mediated NF- κ B signaling pathway could be an effective treatment for SIC.

MicroRNAs (miRNAs) are a class of small non-coding RNAs that have been identified as novel regulators

of gene expression at the posttranscriptional level. miRNAs exert strong regulatory effects on eukaryotic genes by contributing to cell proliferation, differentiation, development, metabolism, apoptosis, and other physiological activities [17, 18]. Recent studies have reported that miRNAs are also prognostic predictors of patients with sepsis [19, 20] and almost all cardiovascular processes during sepsis [21–23]. MicroRNA-23b (miR-23b) is considered a multifunctional miRNA that can prevent autoimmune diseases *via* regulation of inflammatory signaling pathways [24, 25] and induction of tolerogenic activity in dendritic cell responses by inhibition of NF- κ B signaling [26, 27]. Furthermore, Wu *et al.* found that miR-23b attenuates progression of sepsis by preventing the expression of inflammatory factors [28]. Considering that SIC is mostly associated with uncontrolled immune and inflammatory response, we speculate that miR-23b represents a therapeutic option for SIC.

In the present study, models of SIC were established using cecal ligation and puncture (CLP) or injection of lipopolysaccharide (LPS) and transfected with miR-23b to observe the effect of miR-23b on the inflammatory responses during SIC and subsequent outcome. We also investigated the effect of regulation of miR-23b and explored the underlying mechanism involved.

MATERIALS AND METHODS

Experimental Animals and CLP Polymicrobial Sepsis Model

Experiments were conducted on 8-week-old male Sprague-Dawley rats weighing 220–260 g (Vital River Laboratory Animal Technology Co., Ltd., Beijing, China). All rats were maintained in separated cages and provided pelleted rat chow with water *ad libitum*. Rats were housed in a temperature-controlled room with a 12-h light/dark cycle and allowed to acclimatize for 7 days before being

sacrificed. All experimental manipulations were undertaken in accordance with the Guide for the Care and Use of Medical Laboratory Animals (Ministry of Health, P.R. China, 1998), with the approval of the Scientific Investigation Board, Tianjin Medical University General Hospital, Tianjin, China. CLP was performed to induce sepsis in rats as previously described [29]. Briefly, rats were randomly divided into four groups: (1) miR-Con, (2) sham, (3) CLP, and (4) CLP + miR-23b. The survival was monitored for 7 days. At the study end point, rats were anesthetized and euthanized by opening the chest cavity and withdrawing blood by cardiac puncture.

Reagents

Ultrapure LPS (*Escherichia coli* 0111: B4) was obtained from Sigma Chemical Co. (St. Louis, MO, USA). Rat anti-MyD88 (E-11, sc-74532), anti-TRAF6 (D-10, sc-8409), anti-IKK α (B-8, sc-7606), anti-IKK β (G-8, sc-271782), anti-MIF (D-2, sc-271631), and anti-NF- κ B (F-6, sc-8008) antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). ELISA kits for CK-MB and BNP were purchased from R&D Corporation (R&D Systems Inc., Minneapolis, MN, USA). All other chemicals were of reagent grade.

Injection of LmiR-23b into Rat Hearts

The rats were anesthetized by 5.0% isoflurane and then maintained by inhalation of 1.5–2% isoflurane in 100% oxygen. Body temperature was maintained at 37 °C using a heating pad. An incision was made in the middle of the neck, and the right common carotid artery and right external carotid artery were carefully exposed. Then, a micro-catheter was introduced into the isolated common carotid artery and fed into the aortic root. One hundred microliters of LmiR-23b (1×10^8 PFU) or LmiR-Con was delivered through the micro-catheter. The micro-catheter was gently removed, and the common carotid artery was tightened before the skin was sutured. CLP was performed 7 days after delivery to initiate sepsis.

Echocardiography

Cardiac function was examined 3 days after CLP. Echocardiography was performed in rats under isoflurane anesthesia using an ultrasound system (Toshiba Aplio 80 Imaging System, Tochigi, Japan) with a 21-MHz probe to examine left ventricular function. The left ventricular ejection fraction (EF) and cardiac output (CO) were analyzed. All measurements were made by one specialist who was

blinded with respect to the identity of the tracings, and analysis was performed with the average values acquired over four consecutive cardiac cycles.

Enzyme-Linked Immunosorbent Assay

The whole myocardial samples were collected and centrifuged at 3000 r/min for 10 min. After that, all samples were stored at –80 °C until analysis. The concentrations of creatine kinase-MB (CK-MB) and brain natriuretic peptide (BNP) in serum were measured by ELISA kits (Sigma-Aldrich, St. Louis, MO, USA) following the manufacturer's protocols.

Histological Examination

Seven days after CLP, all animals were subjected to heart perfusion under anesthesia. Then, heart specimens were obtained, fixed in 4% paraformaldehyde, dehydrated, and embedded in paraffin. Five-micron-thick sections were cut using a microtome and stained with hematoxylin and eosin (H&E) for histological examination under a light microscope (Olympus, Tokyo, Japan). A scoring system was performed as previously reported [29]. Each category was graded on a 5-point scale: 0 = no lesions, 1 = one or few small lesions, 2 = many small or a few large lesions, 3 = multiple small and large lesions, 4 = massive lesions.

Construction of miR-23b Lentivirus Expression System

miR-23b and ~150 bp of flanking sequence were amplified from genomic DNA and cloned into a lentivirus expression system (Invitrogen, Carlsbad, CA, USA). After sequencing confirmation, the fragment was digested with *Eco*RI and *Bam*HI and cloned into lentiviral vector pCDH-CMV-MCS-EF1-copGFP (LVmiR-23b; System Biosciences, Palo Alto, CA, USA), resulting in LmiR-23b. The lentiviral miR-23b sponge (LV-sponge) was also constructed. Target cells were transfected using a polybrene reagent (Merck Millipore, Darmstadt, Germany) according to the manufacturer's instructions. Lentivirus expressing scrambled miRNA (LmiR-Con) served as a control.

Cell Culture and Transfection

H9C2 rat cardiomyoblasts obtained from the American Type Culture Collection (Rockville, MD, USA) were cultured in RPMI-1640 medium (Gibco, Eggenstein, Germany) with 10% fetal bovine serum (FBS) supplemented with 100 U/mL penicillin and 100 U/mL streptomycin in a 5% CO₂ atmosphere. The cells were plated in six-well plates at 5×10^5 cells/well and then transfected with

mirVana miR-23b mimics at a final concentration of 100 nM using the transfection reagent HiPerfect (Qiagen, Hilden, Germany). Cells were treated with 100 μ L LPS [10 mg of LPS in 50 mL phosphate-buffered saline (PBS)] after 7 days of transfection. For the culture experiments, the cells were divided into four groups: (1) control (medium), (2) LPS, (3) LPS + miR-23b, and (4) LPS + miR-con.

Quantitative Real-time Polymerase Chain Reaction

Total RNA was extracted from fresh rat hearts or cultured cells using an RNeasy Plus Mini Kit (Ambion, Foster City, CA, USA) according to the manufacturer's protocol. The purity and concentration of RNA were tested at a wavelength of 260 nm using a NanoDrop 2000c spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). cDNA was synthesized with stem-loop miRNA-specific reverse transcription primers using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA). qPCR was carried out on an Eppendorf Realplex 4 instrument (Eppendorf, Hamburg, Germany). miR-23b levels were quantified by qPCR using specific TaqMan assays. The specific primer sequences used for genes including TNF- α , IL-6, IL-1 β , VCAM-1, ICAM-1, NF- κ B, MyD88, TRAF6, IKK α , IKK β , and β -actin were obtained from Univ-bio (Shanghai, China) and are shown in Supplementary Table 1. The relative expression of each gene was normalized to that of β -actin.

Electrophoretic Mobility Shift Assay

Nuclear proteins were isolated from heart tissues, and NF- κ B binding activity was evaluated using a LightShift Chemiluminescent electrophoretic mobility shift assay (EMSA) kit (Thermo Fisher Scientific). The binding reaction mixture was incubated at room temperature for 15 min and analyzed by electrophoresis, after which the proteins were immobilized using a nylon membrane. The biotin end-labeled DNA was detected using the streptavidin-horseradish peroxidase conjugate and chemiluminescent substrate.

Western Blot

Western blot was performed as described previously [13]. In brief, collected heart tissue samples were lysed, and proteins were separated using a protein extraction kit (Well-bio, Shanghai, China) according to the manufacturer's instructions. Membranes with diverse bands were probed with the appropriate primary antibodies after being transferred to blocking buffer (5% milk in Tris-buffered saline containing 0.05% Tween 20 [TBST]) and incubated

overnight. Then, the membranes were washed and incubated with horseradish peroxidase-conjugated secondary antibody (Bio-Rad, Hercules, CA, USA). The densities of the bands on the membranes were scanned and analyzed by enhanced chemiluminescent western blotting reagents.

Flow Cytometry

After 5 days of stimulation with LPS, the H9C2 myocardiocytes were harvested and suspended, and flow cytometry was performed as previously described [30]. Cardiomyocytes were stained with Annexin-V-FITC and propidium iodide (Bioscience) for 30 min at 4 $^{\circ}$ C in the dark. The apoptotic rate of myocardiocytes was determined by flow cytometry using FACScan (BD Biosciences, San Jose, CA, USA) after 1 h.

Confocal Microscopy Analysis

Myocardiocytes were stimulated with LPS for 5 days, washed twice, and then fixed with 4% paraformaldehyde in PBS for 30 min before being permeabilized with 0.02% Triton X-100 for 30 min at 4 $^{\circ}$ C. The activity of caspase-3 was detected by confocal microscopy as in our previous study [30]. Images were acquired with a laser scanning confocal microscope (Leica, Wetzlar, Germany).

Statistical Analysis

All data were analyzed using Prism version 7.0 software (GraphPad Software, La Jolla, CA, USA). Comparisons between groups were analyzed using one-way analysis of variance (ANOVA). Survival is shown graphically using Kaplan-Meier curves and was evaluated statistically with the log-rank test. A *P* value less than 0.05 was considered statistically significant.

RESULTS

Polymicrobial Sepsis Increases the Level of miR-23b in the Circulation, Myocardium, and Cardiac Myocytes

To determine whether polymicrobial sepsis affects the level of miR-23b, miR-23b expression in circulation and heart tissues was investigated. The level of miR-23b in circulation and heart tissues was significantly elevated and sustained at three different time points (Fig. 1a, b). In cardiomyocytes stimulated with LPS, we found that the expression of miR-23b was also obviously increased at different phases of sepsis (24 h, 48 h, and 72 h) (Fig. 1c). These results indicated that the expression of miR-23b is

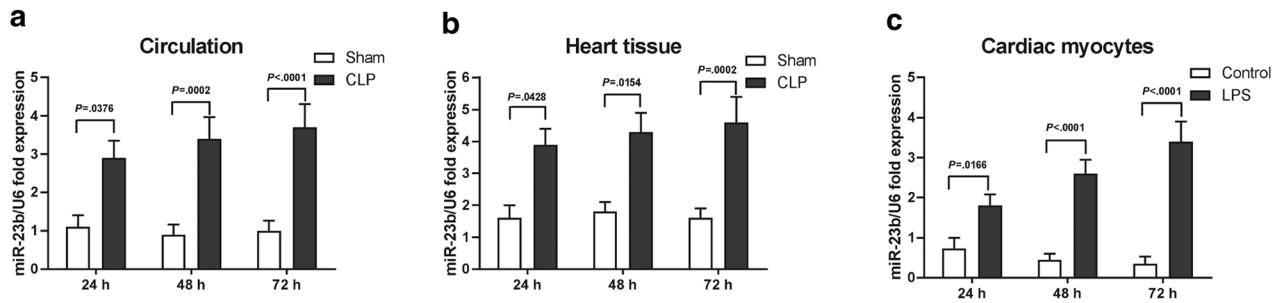


Fig. 1. Cecal ligation and puncture (CLP) and administration of lipopolysaccharide (LPS) increased the level of microRNA-23b (miR-23b) in circulation (a) and in H9C2 cardiomyoblasts (b). CLP increased expression of miR-23b in circulation and heart tissues during different periods of sepsis. Serum and heart tissues were collected for miR-23b assay by qPCR at 24 h, 48 h, and 72h after CLP. H9C2 cardiomyocytes (c) were treated with LPS (20 µg/mL), harvested at three timepoints, and evaluated for miR-23b expression by qPCR. Values are means ± SEM (n = 6 in each group) of four independent experiments.

upregulated by polymicrobial sepsis and possibly plays a role in sepsis.

Increased miR-23b Expression Attenuates Impaired Cardiac Dysfunction During SIC and Improves Survival Following CLP-Induced Sepsis

It has been suggested that functional rather than structural changes are involved in SIC [31]. To determine the

effect of miR-23b on cardiac function in SIC, we transfected LmiR-23b or LmiR-Con into the myocardium via the right carotid artery. In the present study, upregulation of miR-23b was efficiently established in heart tissue and cardiomyocytes *in vivo* and *in vitro*, respectively (Supplementary Fig. 1A and B). Cardiac function was measured by echocardiography or ELISA 3 days after CLP, and the survival rate was carefully documented for 7 days. We found that cardiac function was severely impaired during

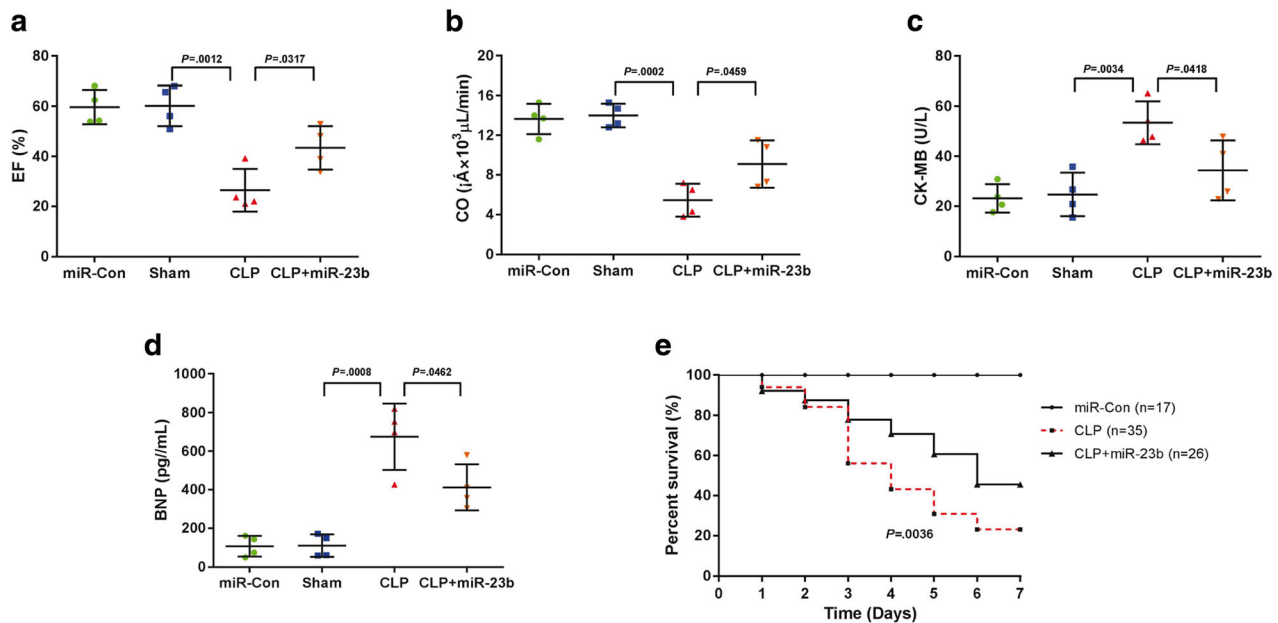


Fig. 2. Transfection with LmiR-23b attenuated cardiac dysfunction and improved survival in rats with SIC. LmiR-23b was transfected into the myocardium for 7 days. Cardiac function was examined 3 days after cecal ligation and puncture (CLP). Ejection fraction (EF) (a) and cardiac output (CO) (b) were tested by echocardiography, whereas the level of creatine kinase-MB (CK-MB) isoenzyme (c) and brain natriuretic peptide (BNP) (d) in serum was detected by ELISA. Data are means ± SEM (n = 6 in each group) of four independent experiments. Survival was monitored each day up to 7 days (e). Survival was analyzed by the log-rank (Mantel-Cox) test: $\chi^2 = 11.24$, $P = 0.036$.

sepsis, and LmiR-23b transfection improved cardiac dysfunction. Transfection of LmiR-23b into the myocardium improved the EF (Fig. 2a), CO (Fig. 2b), CK-MB (Fig. 2c), and BNP (Fig. 2d) during sepsis, and the survival rate (Fig. 2e) was also markedly increased compared with that in the CLP group. Furthermore, transfection of LmiR-Con did not affect CLP-induced cardiac dysfunction or mortality.

Transfection with LmiR-23b Decreases Secretion of Inflammatory Cytokines in the Myocardium

It is well known that cardiac dysfunction during *SIC* is likely due to excessive inflammation [32]. Therefore, the release of pro-inflammatory cytokines, such as TNF- α , IL-1 β , and IL-6, and macrophage migration inhibitory factor (MIF) in the myocardium was examined by qPCR and immunoblot, respectively, 3 days after the CLP procedure. We observed that transfection with LmiR-23b significantly suppressed the secretion of sepsis-induced inflammatory cytokines, including TNF- α (Fig. 3a), IL-1 β (Fig. 3b), and

IL-6 (Fig. 3c). MIF is recognized as a marker of endotoxin-induced myocardial dysfunction; the data indicated that the level of MIF in the myocardium was decreased in the CLP + miR-23b group compared with that in the CLP group (Fig. 3d). However, transfection of LmiR-Con did not affect CLP-induced inflammatory cytokines.

Transfection with miR-23b Reduces the Expression of Adhesion Molecules in the Myocardium and Protects Against Histopathological Damage Following *SIC*

We next examined whether transfection of miR-23b influences adhesion molecule expression and histological damage in the myocardium following *SIC*. As shown in Fig. 4a, b, the level of vascular cell adhesion molecule 1 (VCAM-1) and intercellular cell adhesion molecule 1 (ICAM-1) in CLP rats was markedly increased compared with that in the sham group, but transfection with miR-23b prevented sepsis-induced increases in their expression in the myocardium. Furthermore, CLP-induced histopathological

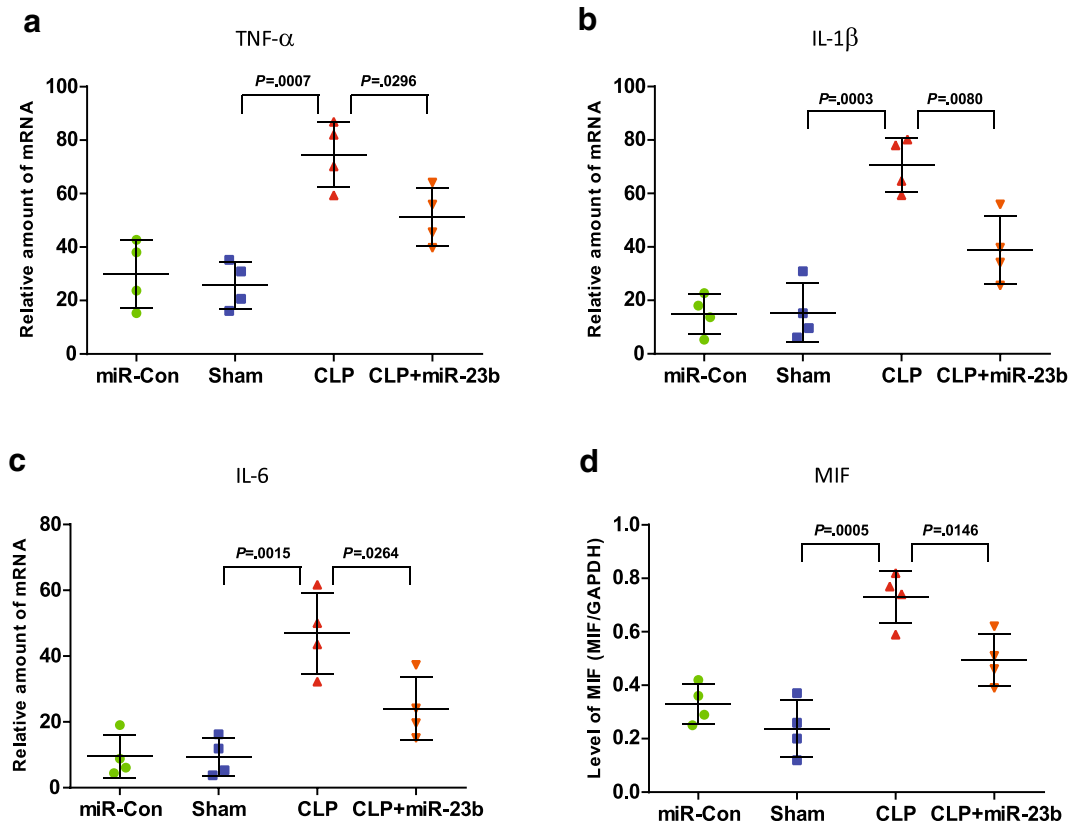


Fig. 3. Transfection with LmiR-23b inhibited the release of inflammatory cytokines in the myocardium. Inflammatory cytokines, including TNF- α (a), IL-1 β (b), and IL-6 (c), as well as migration inhibitory factor (MIF) (d), were analyzed 3 days after cecal ligation and puncture (CLP). Values are means \pm SEM ($n = 6$ in each group) of four independent experiments.

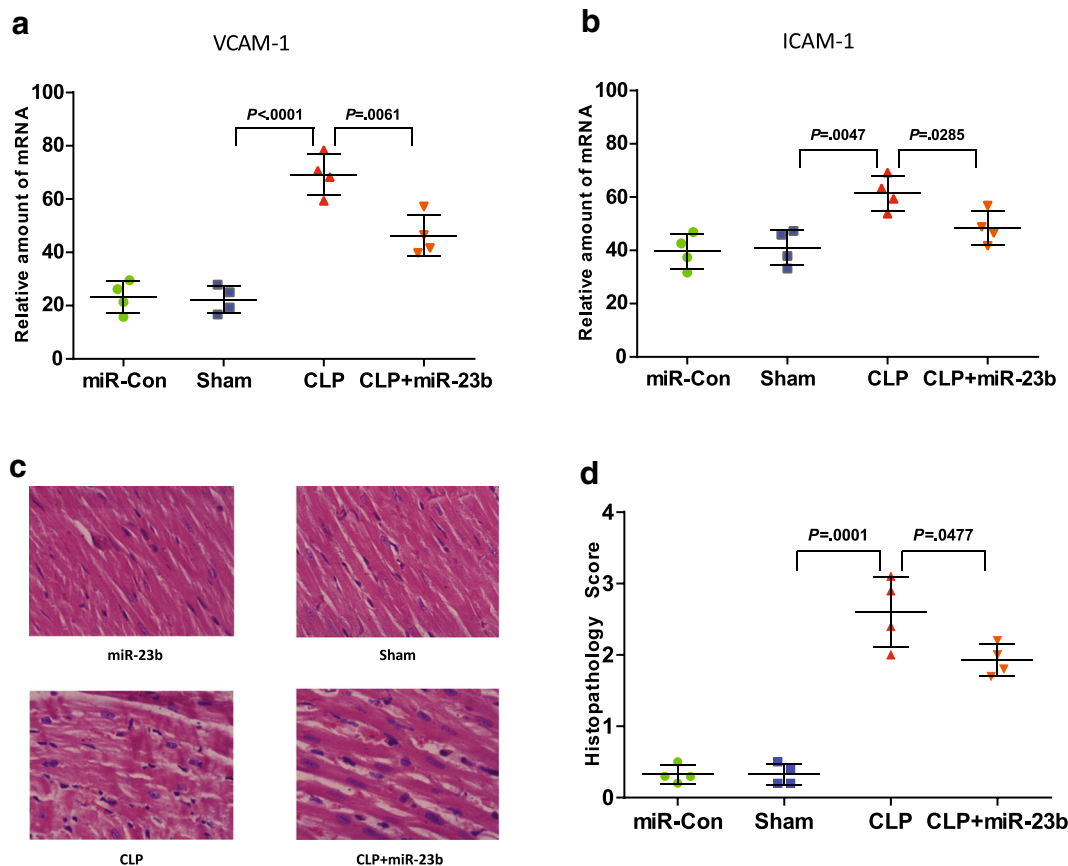


Fig. 4. Transfection with LmiR-23b reduced the level of VCAM-1 and ICAM-1 in the myocardium and protected against histopathological damage following *SIC*. The expression of VCAM-1 (a) and ICAM-1 (b) was detected after 3 days by qPCR. Values are means ± SEM ($n = 6$ in each group) of four independent experiments. Rats were euthanized, and heart tissues from each group were processed for histopathological evaluation following a heart tissue scoring system (c, d). Representative histological changes in tissues are shown by hematoxylin and eosin staining (magnification × 400).

damage was effectively alleviated under administration of LmiR-23b (Fig. 4c, d). However, transfection of LmiR-Con did not affect adhesion molecule expression or histopathological damage.

Transfection of LmiR-23b Decreases NF-κB Binding Activity and Inhibits Activation of the NF-κB Signaling Pathway

To explore the protective mechanism by which miR-23b alleviates *SIC*, we assessed the NF-κB signal binding activity and related expression of NF-κB, which is considered an important transcriptional factor that regulates the secretion of inflammatory cytokines during sepsis/septic shock [33]. The results showed that the levels of NF-κB, including NF-κB binding activity (Fig. 5a), mRNA (Fig. 5b), and protein (Fig. 5c), were significantly upregulated

during *SIC*, but transfection of miR-23b markedly decreased the levels of NF-κB, which indicated that miR-23b attenuated *SIC* by affecting NF-κB signaling.

Transfection of LmiR-23b Attenuates LPS-Induced H9C2 Myocardocyte Apoptosis *In Vitro*

Cardiac myocyte apoptosis is considered responsible for dysfunction in *SIC*; thus, we detected the apoptotic rate of H9C2 cells by flow cytometry and confocal microscopy. To clarify the role of miR-23b in *SIC*-induced myocardocyte apoptosis, cardiomyoblasts were treated with mirVana miR-23b mimics before LPS administration. We found that the apoptotic rate and activity of caspase-3 were significantly increased under stimulation with LPS. However, transfection of miR-23b markedly attenuated myocardial apoptosis and caspase-3 activity (Fig. 6a, b). These data indicated that

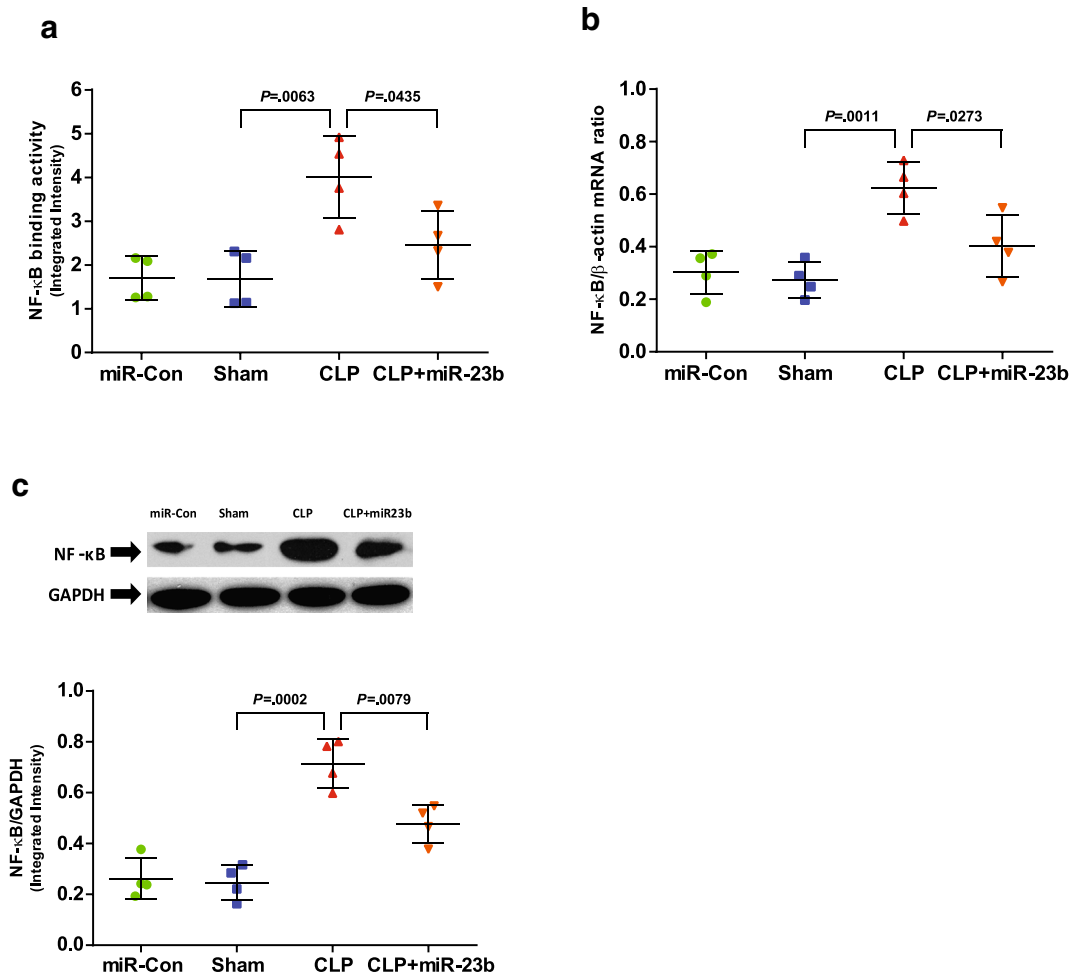


Fig. 5. Transfection with LmiR-23b reduced the level of NF- κ B in heart tissue during SIC. The levels of NF- κ B binding activity (a), mRNA (b), and protein (c) were detected after 3 days. Values are means \pm SEM ($n = 6$ in each group) of four independent experiments.

upregulation of miR-23b plays a protective role in LPS-induced myocardial apoptosis.

Modulation of miR-23b Affects Activation of the NF- κ B Signaling Pathway by Targeting TRAF6 and IKK β *In Vitro*

To further explore the protective mechanism of transfection with LmiR-23b in SIC, we assessed the NF- κ B signaling pathway *in vitro*. MyD88 is a critical receptor protein upstream of the signaling pathway, and TRAF6 and IKK (including IKK α and IKK β) are essential components in NF- κ B signaling pathway activation. As shown in Fig. 7, the expression of MyD88, TRAF6, and IKK β under LPS stimulation was significantly higher than in the control but markedly decreased in the LPS + miR-23b group (Fig.

7a, b, d). However, transfection of miR-23b did not significantly affect the level of IKK α (Fig. 7c). These results suggested that induction of miR-23b during early sepsis significantly inhibits NF- κ B signaling, with accompanying downregulation of MyD88, TRAF6, and IKK β .

DISCUSSION

In present study, we found that the level of miR-23b was upregulated during SIC, and transfection with miR-23b improved survival outcome and cardiac function, prevented histological damage by inhibiting the release of inflammatory cytokines, and attenuated apoptosis of cardiomyocytes. Transfection of LmiR-23b also inhibited activation of NF- κ B signaling by decreasing the level of

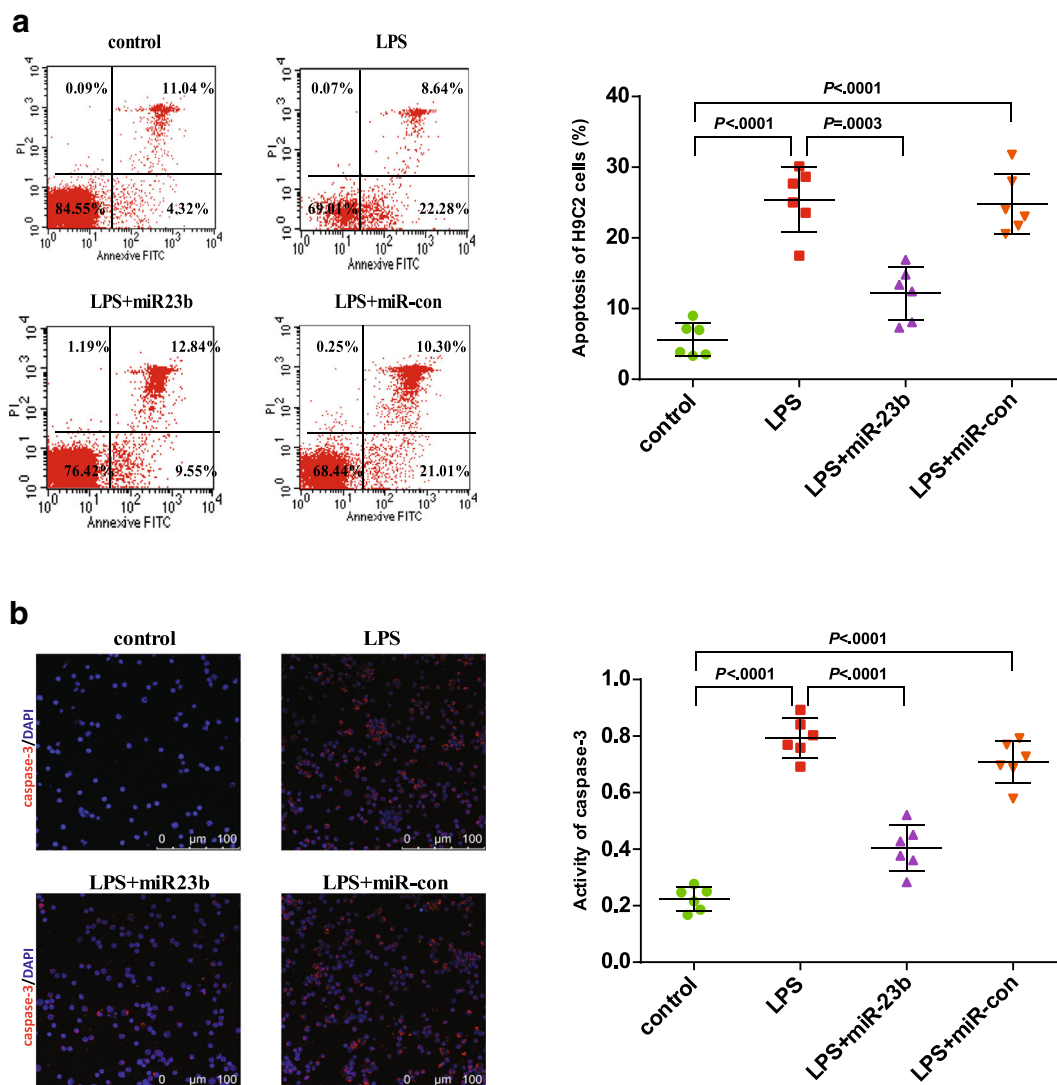


Fig. 6. Overexpression of miR-23b inhibited apoptosis of H9C2 cardiomyocytes under stimulation with lipopolysaccharide (LPS). The apoptotic rate of cells was measured by flow cytometry (a), and the activity of caspase-3 was detected by confocal microscopy(b). Caspase-3 was visualized by antibodies directly labeled with phycoerythrin (red). Values are means ± SEM of four independent experiments.

upstream molecules. Taken together, the protective effect of enhanced miR-23b expression is mediated through MyD88-mediated NF-κB signaling and targeting of TRAF6 and IKKβ.

The cardiovascular system plays an essential role in the outcome of patients with sepsis, and a previous study provided evidence of myocardial depression in cases of septic syndromes occurring over the last half century [34]. In the pathogenesis of SIC, excessive inflammatory responses and apoptosis of cardiomyocytes serve an essential function [9, 35]. Numerous studies reported that in CLP models, early

sepsis is characterized by elevated pro-inflammatory cytokine expression in the first 5 days after CLP, whereas late sepsis (after day 5) is characterized by reduced cytokine expression [36, 37]. In a recent study, miRNAs were highlighted as crucial factors in the development, homeostasis, and function of innate and adaptive immunity. In our study, we focused on miR-23b, as it represents a novel autoimmunity-regulating molecule with considerable therapeutic potential. In addition, miR-23b has been shown to inhibit inflammatory reactions in infectious diseases and attenuate vascular endothelial function during septic shock [28]. Zhang *et al.* reported that

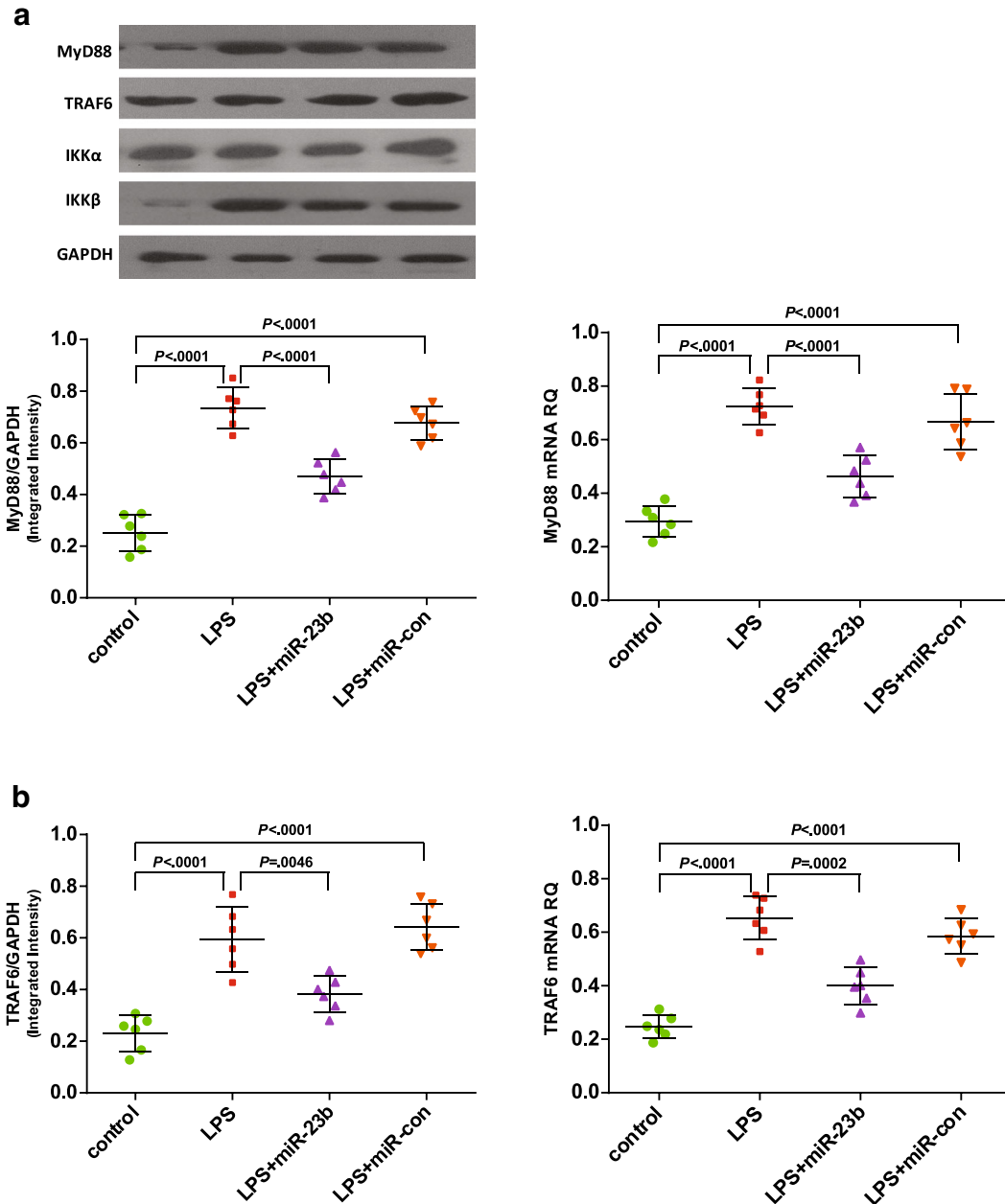


Fig. 7. Transfection of LmiR-23b affected the NF- κ B pathway under stimulation with lipopolysaccharide (LPS). The level of Myd88 (a), TRAF6 (b), IKK α (c), and IKK β (d) was measured by immunoblotting and qPCR assay. Values are means \pm SEM of four independent experiments.

inhibition of miR-23b significantly improved cardiac function and contributed to the activation of cardiac fibrosis in late sepsis [38]. However, clinical and experimental studies have revealed that myocardial dysfunction is an early and fatal complication of septic shock [39, 40]. We found that the expression of miR-23b was significantly increased in the

circulation, heart tissue, and H9C2 cardiomyocytes during *SIC*. To investigate the effect of miR-23b in early sepsis, we delivered LmiR-23b into the myocardium 7 days prior to CLP. Our study demonstrated that upregulation of miR-23b markedly improved cardiac function and survival, and the histological damage was obviously alleviated.

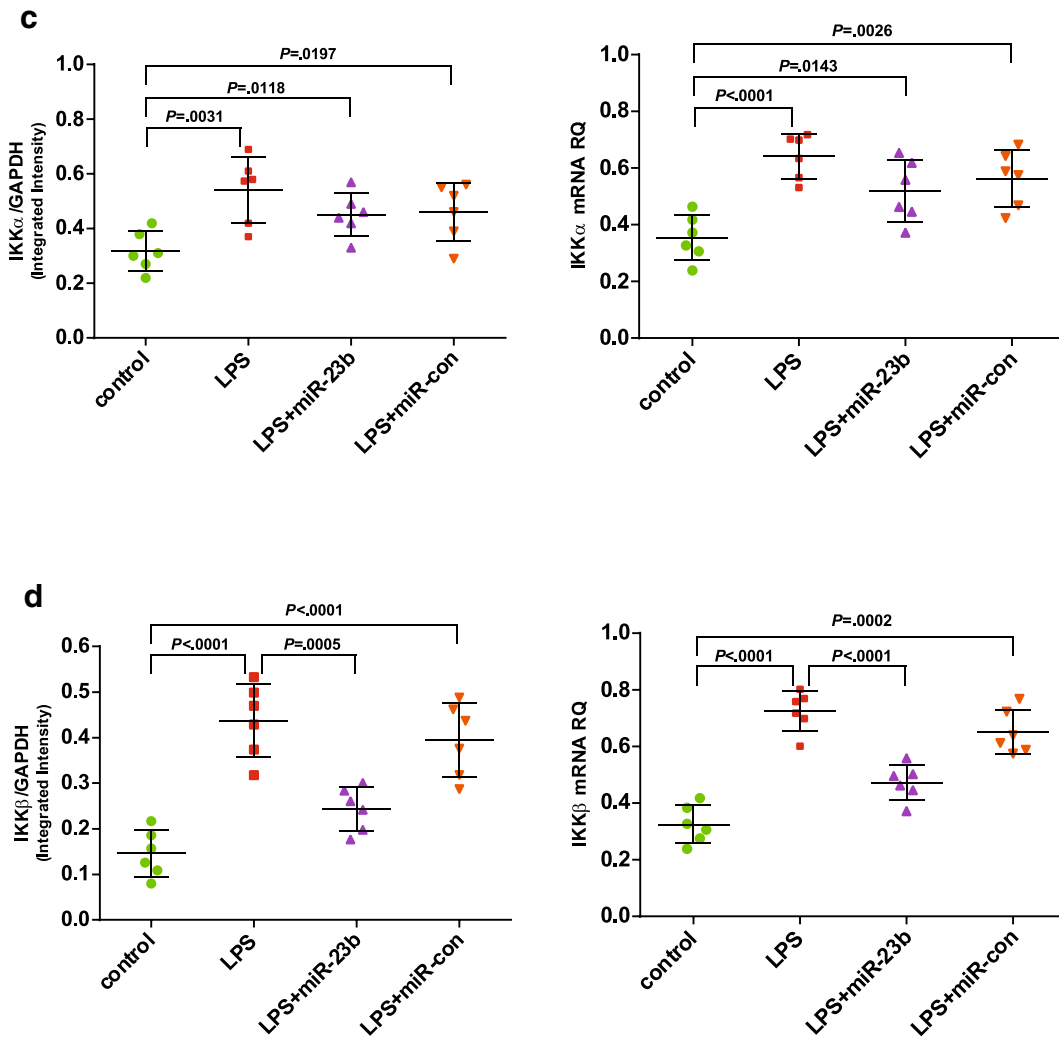


Fig. 7. (continued)

Inflammatory cytokines released by infiltrating macrophages and neutrophils, such as TNF- α and IL-1 β , are considered important suppressors of cardiac function [41]. We found that the protective effect of miR-23b largely depended on controlling the inflammatory responses by inhibiting secretion of inflammatory cytokines (including TNF- α , IL-1 β , and IL-6) in the myocardium. Increased expression of adhesion molecules, including VCAM-1 and ICAM-1, plays an essential role in mediating inflammatory cell infiltration into the tissues [41, 42]. In the present study, we observed that *SIC* resulted in significantly increased levels of VCAM-1 and ICAM-1 in the myocardium, but transfection of miR-23b downregulated this expression, suggesting that an increased level of miR-23b in

the myocardium suppresses sepsis-induced adhesion molecule expression, resulting in attenuation of the infiltration of macrophages and neutrophils into the myocardium.

Recent evidence has supported activation of the TLR-mediated NF- κ B pathway as a target of miRNAs [43], which could result in improved cardiac function and survival [44]. Ma *et al.* reported that cardiac function in *SIC* was improved by enhancement of miR-146a through targeting of the TLR-mediated NF- κ B pathway [45]. Furthermore, we and others previously reported that TLR-mediated NF- κ B activation contributes to the pathophysiology of polymicrobial sepsis [30] and cardiac dysfunction during *SIC* [46]. In the present study, we found that LmiR-23b transfection

prevented sepsis-induced myocardial NF- κ B binding activity and reduced the level of NF- κ B. More importantly, miR-23b transfection in cardiomyocytes directly targeted TRAF6 and IKK β , which are the key adapter molecules in the TLR/NF- κ B pathway. Furthermore, TRAF6 plays a crucial role in the induction of inflammatory responses *via* activation of IKK, leading to NF- κ B activation [32, 47]. The data revealed that miR-23b suppresses the expression of TRAF6 and IKK β , resulting in attenuation of the NF- κ B-mediated inflammatory response in *SIC*. Although LmiR-23b transfection did not significantly attenuate IKK- α levels in *SIC*, the expression of MyD88 (mRNA and protein), which functions upstream of the NF- κ B pathway, was markedly decreased.

Myocardial apoptosis contributes to cardiac dysfunction in sepsis [48], and inhibition of sepsis-induced cardiac apoptosis is associated with improved cardiac function in murine polymicrobial sepsis [15]. To confirm the effect of miR-23b on apoptosis in *SIC*, the apoptotic rate of cardiomyocytes was measured by flow cytometry, and activity of caspase-3 was evaluated by confocal microscopy in an *in vitro* experiment. LmiR-23b markedly decreased the apoptotic rate of cells and activity of caspase-3 in *SIC*, which indicated that enhancement of miR-23b could attenuate apoptosis of H9C2 cardiomyoblasts. Taken together, the present study demonstrated that LmiR-23b transfection significantly attenuates sepsis-induced myocardial apoptosis by suppressing activation of the TLR-mediated NF- κ B pathway.

There are several limitations in the present study. Although CLP is considered the gold standard model of sepsis, the healthy young rodents used herein are not representative of the elderly patients with comorbidities encountered in a clinical setting. In addition, common sepsis treatments, including antibiotics or vasoactive substances, were not used to avoid interference with the effect of miR-23b. Finally, miR-23b was transfected before CLP was performed, which is not representative of clinical practice, in which therapy is usually administered after the onset of sepsis. Further studies, particularly clinical trials, are necessary to clarify the efficacy of treatment involving miR-23b in *SIC*.

In conclusion, our data revealed that miR-23b is upregulated in the early stage of *SIC*, indicating its protective role in sepsis. Furthermore, the overexpression of miR-23b attenuated the inflammatory response and improved survival by decreasing secretion of inflammatory cytokines

and alleviating myocardial apoptosis, which occurred through MyD88-mediated NF- κ B signaling and targeting of TRAF6 and IKK β . These findings, together with our previous findings, indicate the importance of understanding modulation of miR-23b in the early stage of *SIC* as an appropriate approach to treatment.

AUTHORS' CONTRIBUTIONS

CC performed experiments, analyzed data, prepared figures, and wrote the manuscript. YZ, LJW, YFC, and STS performed experiments and analyzed data. CFY performed the histological examination of the heart tissues. HJ designed experiments, analyzed data, prepared figures, and wrote the manuscript. All authors read and approved the final manuscript.

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DATA AVAILABILITY

All data generated or analyzed during this study are included in this published article [and its supplementary information files].

COMPLIANCE WITH ETHICAL STANDARDS

Competing Interests. The authors declare that they have no conflicts of interest.

Ethics Approval and Consent to Participate. All experimental manipulations were undertaken in accordance with the Guide for the Care and Use of Medical Laboratory Animals (Ministry of Health, P.R. China, 1998), with the approval of the Scientific Investigation Board, Tianjin Medical University General Hospital, Tianjin, China.

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