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Deletion of TLR4 Ameliorates Inflammation Response and Apoptosis in Septic Cardiomyopathy (SCM)

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Sepsis; Septic cardiomyopathy;
Toll-like receptor 4; Apoptosis; Inflammation

1. Abstract

1.1. Background: Septic cardiomyopathy (SCM) is featured by severe myocardial dysfunction and remains one of the lethal complications in clinical sepsis. Toll-like receptor 4 (TLR4) signaling is known as a classical innate pathway in heart diseases, whereas the precise underlying mechanism of TLR4 in SCM remains elusive. This study was designed to examine the specific role of TLR4 in SCM with a focus on inflammation and apoptosis.

1.2. Methods: TLR4 deficiency (TLR4-/-) mice and wild type (WT) littermates were subjected to lipopolysaccharide (LPS, 4 mg/kg, 6 h) to establish SCM, echocardiography, carbonyl assay, and blood assay were performed to evaluate changes in myocardial function, protein oxidative damage, and cardiac injury markers (CK-MB, and troponin T). Protein and mRNA expression of TLR4 signaling molecules (TLR4, MyD88, and NF-κB), proinflammatory cytokines (TNF-α, IL-1β, and IL-6), and apoptotic markers (Bax, Bcl-2, and caspase 3) were assessed using RT-qPCR and Western blot. In vitro study, H9c2 cardiomyocytes were challenged with LPS (1 μg/ml, 24 h) in the presence or absence of TAK-242 (TLR4 inhibitor, 30 μM, 24 h) prior to assessment of biochemical indices.

1.3. Results: Our results indicated that cardiac function was significantly improved along with the reduced myocardial inflammation, oxidative damage, and apoptosis in TLR4-/- mice following LPS challenge. Taken together, our results demonstrated a role for the TLR4/MyD88/NF-κB signaling in the activation of proinflammatory responses and apoptosis in SCM.

1.4. Conclusion: TLR4 deficiency or inhibition ameliorated myocardial damage and dysfunction by inhibiting TLR4/MyD88/NF-κB signaling and reduced pro-inflammatory cytokines and apoptosis in SCM.

2. Introduction

The term “Sepsis” was originated from the Greek “σήψις” paraphrased as “decomposition of animal or vegetable organic matter in the presence of bacteria” and represents one of the oldest and most intricate syndromes in clinical medicine [1]. Hippocrates and Galen summarized that sepsis represents the dangerous, malodorous, biological decay in the body, which dramatically hampered the healing of injured tissues [2, 3]. With the advanced in medicine, sepsis has been recognized as a life-threatening organ dysfunction evoked by an abnormal host response to infection, and imposes a substantial burden on morbidity and mortality [4-6]. Epidemiological studies have estimated 48.9 million individuals afflicted with sepsis annually, leading to 11 million mortalities either from protopathy or deuteropathy, equivalent to 19.7% of worldwide mortality [7]. Furthermore, sepsis-associated morbidity

is expected to continuously rise given the aging world population [8]. Notwithstanding sepsis has been intensively studied from all aspects for decades, effective therapeutic approach remains challenging due to the extremely complicated molecular mechanisms of sepsis, and current clinical managements are limited to be supportive rather than curative [9]. Thus, it is imperative to explore the pathophysiological mechanisms of sepsis to guide novel therapeutic interventions.

As one of the lethal complications in clinical sepsis, septic cardiomyopathy (SCM) referring to myocardial dysfunction caused by sepsis is associated with increased mortality and poor prognosis [10, 11]. Although the large-scale studies and standard diagnostic criteria were not established in the past decades, the incidence of SCM in patients with sepsis was estimated from 13.8% to 40% [12, 13]. Compared with patients without cardiovascular impairment, the mortality would double or triple with up to 70%-90% in septic patients encountering heart dysfunction [14]. From a symptomatic perspective, SCM is acknowledged by decreased left ventricular ejection fraction (LVEF) [15]. Nevertheless, more in-depth studies unveiled various potential molecular mechanisms involved in SCM including inflammation response, cytokine overproduction, apoptosis damage, downregulation of adrenergic signaling, microvascular damage, oxidative and nitrosative stress, calcium overloading, endothelial dysfunction, mitochondrial dysfunction, autophagy [16-18]. As mentioned earlier, SCM is connected with proinflammatory responses triggered by pattern-recognition receptors (PRRs), such as Toll-like receptors (TLRs) [19]. Despite extensive efforts on SCM, there are still controversies regarding the pathological alterations.

As a type I transmembrane proteins, toll-like receptors (TLRs) are conserved PRR activated by diverse pathogen-associated molecular patterns (PAMPs), launching innate immune responses and inflammatory reactions [20]. The mammalian family of TLRs contains 13 members, among which TLR4 was first characterized in mammalians [21, 22]. Most importantly, level of TLR4 is the highest compared with other TLRs in the heart. In addition, TLR4 plays a critical role in myocardial inflammation and is involved in myocardial dysfunctions, such as myocarditis, myocardial infarction (MI), ischemia-reperfusion (I/R) injury, hypertension, and heart failure [23-25].

Beutler and colleagues previously revealed activation of TLR4 by lipopolysaccharide (LPS, endotoxin) [26], a major component of the outer membrane of Gram-negative bacteria, and one of the main contributors to infection and systemic inflammatory conditions in sepsis. TLR4 is activated by LPS in MyD88-dependent manner to trigger nuclear factor-κB (NF-κB)-dependent expression of inflammatory mediators such as IL-1, IL-6, and TNF-α [27-29]. TLR4 has been indicated to play a critical role in SCM to mediate the inflammatory response. Further evidence suggested that TLR4 knockout improves survival and cardiac function in sepsis [30-34]. Our previous studies found increased TLR4 level in septic mice, resulting in the release of proinflammatory factors, and apoptosis, ultimately triggering SCM [35]. However, the precise underlying mechanism remains unclear. Meanwhile, inhibition of TLR4 using TAK-242 resulted in prominent downregulation of TLR4 in rat myocardium with coronary microembolization, and ameliorated myocardial injury [36, 37]. We hypothesize that host innate immunity is tightly associated with inflammatory response and apoptosis accompanying SCM. This study was designed to examine the impact of TLR4 knockout on cardiac function, inflammation and apoptosis, as well as the role of the TLR4/MyD88/NF-κB signaling pathway. Our findings provide novel insights into the molecular mechanisms of SCM as well as cardiac dysfunction.

3. Results

3.1. TLR4 Deficiency Attenuates Sepsis-Induced Myocardial Dysfunction in Mice

To observe the effects of TLR4 deletion on cardiac function and myocardial injury in LPS-induced SCM, cardiomyocyte-specific TLR4 knockout (TLR4-/-) and WT mice were used and echocardiograms and cardiac injury indicators were evaluated (Figure 1). Echocardiography of cardiac function exhibited that LPS challenge overtly disturbed EF, FS, and LVEDS. Although TLR4 deficiency did not affect cardiac function ($p>0.05$ vs. WT group), it partially relieved LPS-induced cardiac injury ($p<0.05$ vs. LPS group, Figure-1a-d). In addition, LPS significantly elevated cardiac injury indicators such as CK-MB, and troponin T ($p<0.01$), the effect of which was reconciled by TLR4 deficiency ($p<0.05$ vs. LPS group, Figure-1e-f), with little effect from TLR4 deficiency itself. Meanwhile, oxidative damage and histological analysis of cardiac tissues were assessed. (Figure-1G-I) showed that LPS injection overtly increased levels of carbonyl protein, the effect of which was alleviated by TLR4 deletion with little effect from TLR4 deletion itself ($p<0.05$, Figure-1g). Given that LPS administration lasted for only 6 h, little difference was noted for H&E staining and pathological scores of cardiac tissues among any groups tested ($p>0.05$, Figure-1h-i). These data demonstrated that TLR4 deficiency attenuates sepsis-induced myocardial dysfunction.

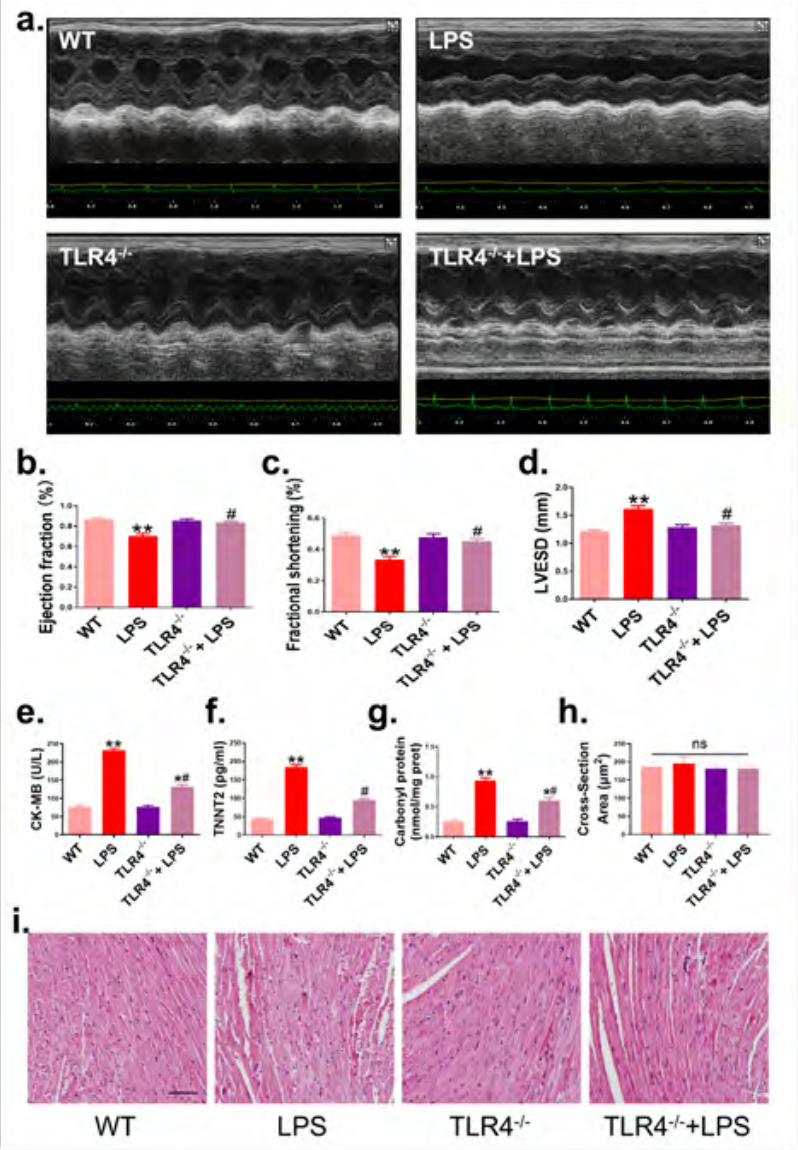

Figure 1: TLR4 deficiency attenuates sepsis-induced myocardial dysfunction in mice. Mice were challenged with intraperitoneall (i.p.) injections of either LPS (4 mg/kg) or vehicle PBS in shams. Blood and heart samples were harvested 6 h after LPS injection. a. Representative echocardiographic images (M mode) of each group. b-d. Echocardiographic data showing EF (ejection fraction), FS (fractional shortening), and LVESD (left ventricular end-systolic dimension) in each group. e-f. The concentration of CK-MB and TNNT2 in serum by ELISA analysis. g. The expression of oxidative carbonyl proteins in different cardiac tissues. h. The cross-section area of H&E in cardiac tissues. i. Representative images of hematoxylin and eosin-stained sections of the cardiac tissues in mice (original magnification, ×200). Scale bar=100 μm. (* denotes $p< 0.05$ versus WT, ** denotes $p< 0.01$ versus WT, $^{\#}$ denotes $p< 0.05$ versus LPS.)

3.2. TLR4 Deficiency Ameliorated Cardiac Inflammation in Mice with Sepsis-Induced Cardiac Injury

To illustrate the impact on the levels of inflammation of TLR4 deficiency in mice, ELISA and RT-qPCR assays were carried out to measure the levels of pro-inflammatory cytokines in serum and cardiac tissue, respectively. The results indicated that TLR4 deficiency did not affect the levels of pro-inflammatory cytokines including TNF-α, IL-1β, and IL-6 in serum and cardiac tissue (Figure-2a-f). After LPS administration, the levels of inflammatory factors like TNF-α, IL-1β, and IL-6 were significantly aggrandized in the serum and myocardium of mice in the LPS group compared with the control group ($p<0.01$). Meanwhile, the levels of pro-inflammatory cytokines including TNF-α, IL-1β, and IL-6 in serum and cardiac tissue were reduced in the TLR4-/-+LPS group compared with the WT group ($p<0.05$). Overall, TLR4 deficiency ameliorated cardiac inflammation in mice with sepsis-induced cardiac injury.

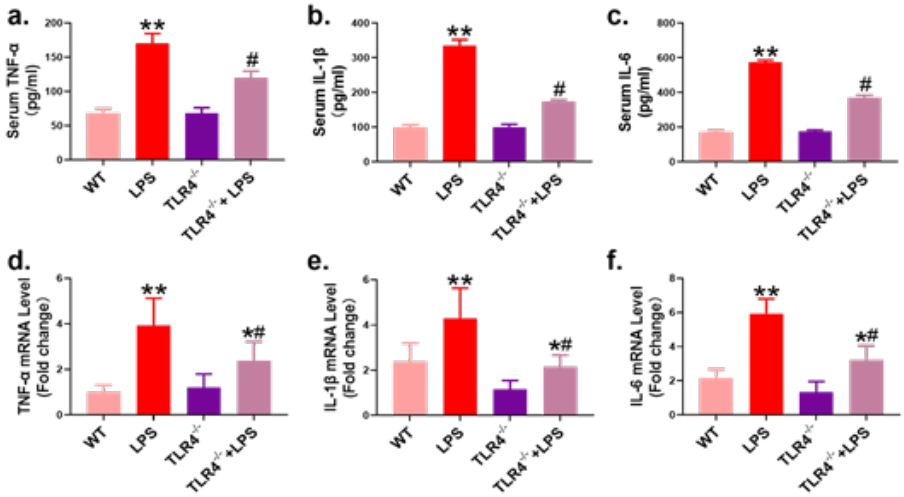

Figure 2: TLR4 deficiency ameliorated cardiac inflammation in mice with sepsis-induced cardiac injury. a-c. Pro-inflammatory cytokines including TNF-α, IL-1β, and IL-6 in serum were measured by ELISA assays. d-f. The mRNA expression of TNF-α, IL-1β, and IL-6 in myocardium tissue. (* denotes p< 0.05 versus WT, ** denotes p< 0.01 versus WT, # denotes p< 0.05 versus LPS.)

3.3. Effects of TLR4 Deficiency on TLR4/MyD88/NF-κB Signaling Pathway in Mice with Sepsis-Induced Cardiac Injury

To assess the effect of TLR4 deficiency on activation of TLR4 signaling in mice in SCM, RT-qPCR assay and western blot were performed to analyze whether TLR4 deficiency impacts the expression of TLR4, MyD88, NF-κB phosphorylation (p-NF-KB) in myocardial tissues of septic mice. RT-qPCR results indicated that relative mRNA expression levels of TLR4, MyD88, and NF-κB in cardiac tissues were significantly increased in response to LPS (p<0.01, Figure-3a-c). Moreover, protein levels of TLR4, MyD88, and p-NF-κB were increased in myocardial tissues of LPS-challenged mice compared with WT group. These findings indicated that TLR4 deficiency contributed to prominent reduction of LPS-induced upregulation of TLR4, MyD88, and p-NF-κB (Figure 3d).

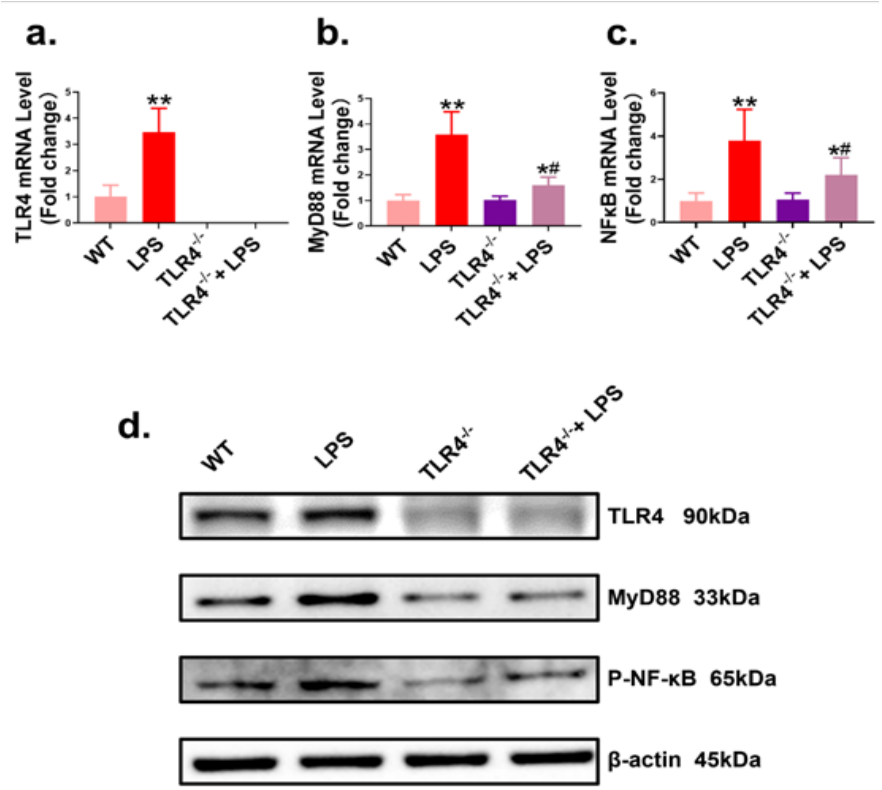

Figure 3: Effects of TLR4 deficiency on TLR4/MyD88/NF-κB signaling pathway in mice with sepsis-induced cardiac injury. a-c. The relative mRNA expression levels of TLR4, MyD88, and NF-κB in cardiac tissue were detected by RT-qPCR. d. Representative western blotting images of TLR4, MyD88, and p-NF-kB in each group. (* denotes p< 0.05 versus WT, ** denotes p< 0.01 versus WT, # denotes p< 0.05 versus LPS.)

3.4. TLR4 Deficiency Decreases Cardiac Apoptosis in Mice with Sepsis-Induced Cardiac Injury

We next performed TUNEL staining, caspase 3 activity, and Western blot to evaluate the impact of TLR4 deficiency on apoptosis in mice with LPS-induced sepsis. First, TUNEL staining assay was used to analyze number of apoptotic cardiomyocytes in LPS-treated mice. Results indicated that LPS led to increased number of TUNEL positive myocardial cardiomyocytes, suggesting antiapoptotic capacity of TLR4 deficiency in septic myocardium ($p<0.01$, Figure-4a-b). Furthermore, evaluation of caspase 3 activity and levels of apoptotic-related proteins confirmed that LPS evoked remarkable increase in caspase 3 activity and expression of pro-apoptotic protein Bax ($p<0.01$), accompanied by decreased anti-apoptotic protein Bcl-2 ($p<0.05$). On the contrary, TLR4 deficiency reversed effects mentioned earlier ($p<0.05$, Figure-4c-e). Moreover, TLR4 deficiency itself did not elicit any notable effect on TUNEL apoptosis, caspase 3 activity, and levels of Bax and Bcl-2 ($p>0.05$). These data showed that TLR4 deficiency decreased cardiomyocyte apoptosis in mice with sepsis-induced cardiac injury.

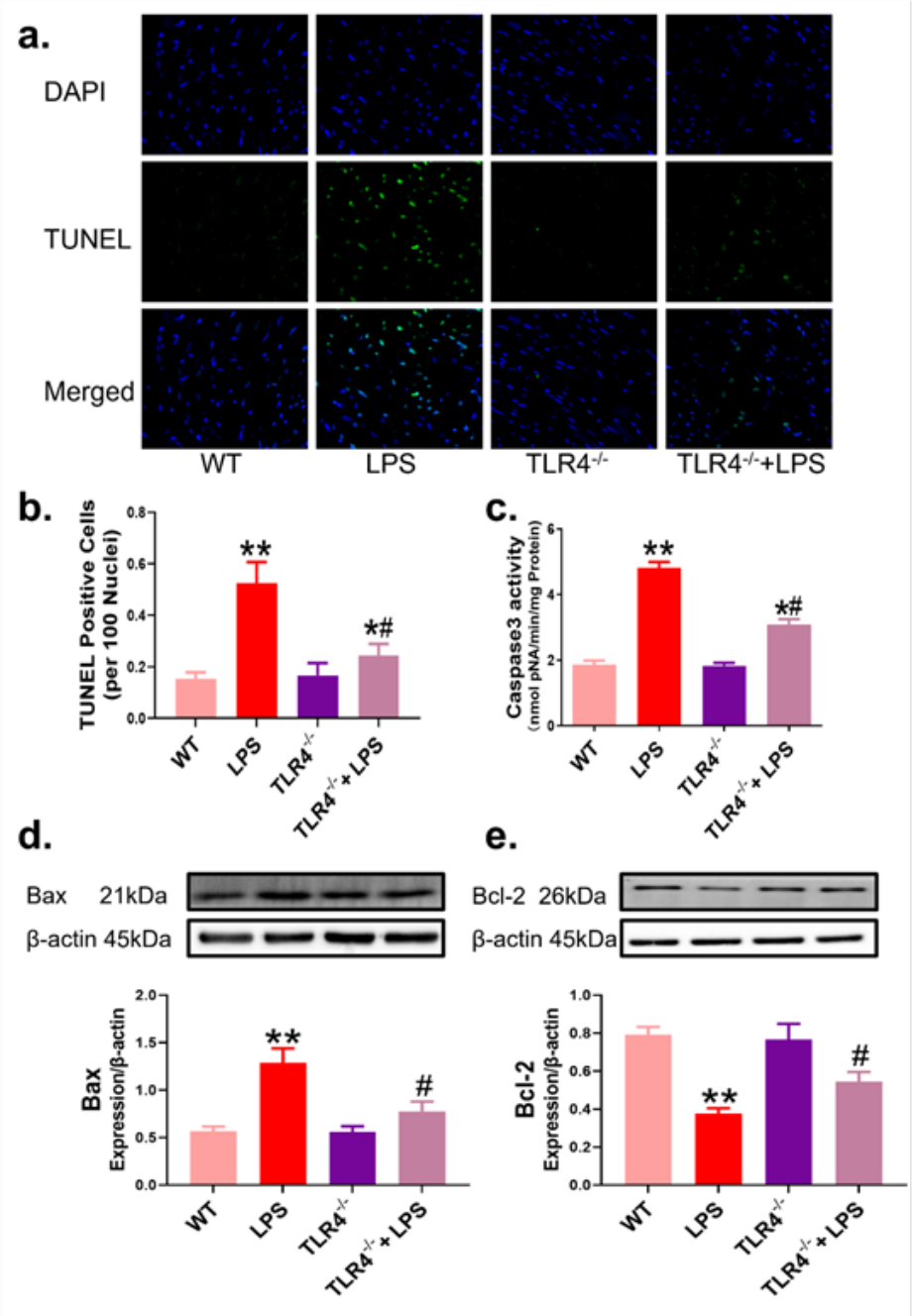

Figure 4: TLR4 deficiency decreases cardiomyocyte apoptosis in mice with sepsis-induced cardiac injury. a. Representative images of TUNEL staining of the cardiac tissues in mice (original magnification, ×400). b. The number of TUNEL positive cardiomyocytes. c. The activity of caspase 3. d. The relative expression of pro-apoptotic protein Bax. e. The relative expression of anti-apoptotic protein Bcl-2. (* denotes $p< 0.05$ versus WT, ** denotes $p< 0.01$ versus WT, # denotes $p< 0.05$ versus LPS.)

3.5. The Inhibition of TLR4 Alleviates Inflammation in an in Vitro Model of Sepsis-Induced Cardiac Injury

In vitro experiments were conducted to analyze the effect of TLR4 inhibition by evaluating H9c2 cardiomyocytes challenged with LPS (1μg/ml) for 24 h. Firstly, a dose-response experiment was carried out to explore the proper concentration of TAK-242 to repress the expression of TLR4 maximally in H9c2 cardiomyocytes and revealed with the Cell Counting Kit-8 assay. Hence, the H9c2 cardiomyocytes were exposed to 10, 20, 30, and 40 μM TAK-242 for 24 h, respectively. We found that the H9c2 cardiomyocytes challenged with TAK-242 for 24 h didn't have any obvious change in cell viability until the concentration exceeded 30 μM ($p>0.05$, Figure 5a). Therefore, otherwise mentioned, following H9c2 cardiomyocytes were treated with 30 μM TAK-242 for 24 h to repress the expression of TLR4. Subsequently, the effect of TLR4 inhibition on LPS-induced cell viability was evaluated by the CCK-8 assay. The results indicated that the cell viability of H9c2 cardiomyocytes decreased remarkably after the LPS challenge, and the situation was ameliorated due to the inhibition of TLR4 ($p<0.05$, Figure 5b). The levels of cardiac injury biological indicators and pro-inflammatory cytokines were recorded to further explore the effect of TLR4 inhibition during sepsis-induced cardiac injury in vitro (Figure 5c-g). These data showed that the LPS group accelerated the expression of cardiac injury biological indicators and pro-inflammatory cytokines ($p<0.01$). Simultaneously, the TAK-242+LPS group reversed the effects as previously mentioned ($p<0.05$). In addition, there were no significant differences between the control group and the TAK-242 group ($p>0.05$). Taken together, these results illustrated that the inhibition of TLR4 alleviated inflammation during sepsis-induced cardiac injury in vitro.

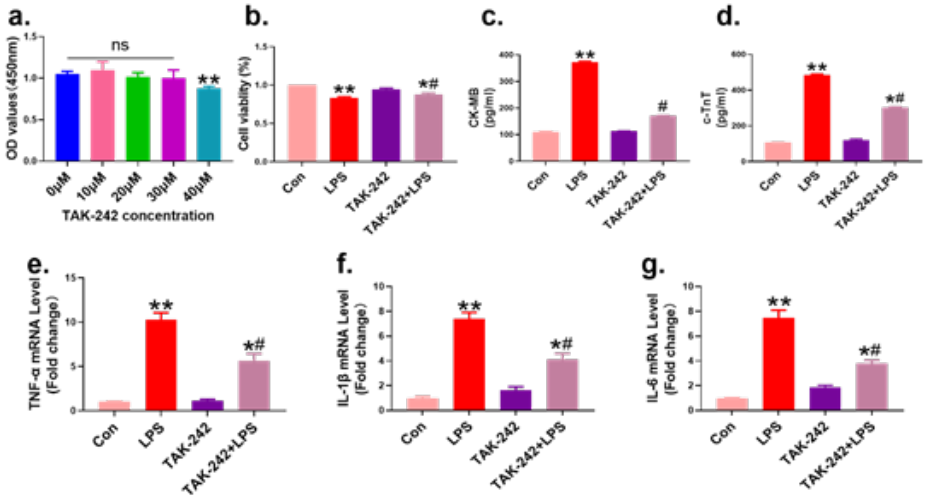

Figure 5: The inhibition of TLR4 alleviates inflammation and apoptosis in vitro model of sepsis-induced cardiac injury.a. The cell viability of H9c2 cardiomyocytes in different concentrations of TAK-242. b. The cell viability of each group. c-d. The concentration of CK-MB and cTnT in the supernatant of each group in H9c2 cardiomyocytes by ELISA analysis. e-g. The mRNA expression of TNF-α, IL-1β, and IL-6 of each group in H9c2 cardiomyocytes, respectively. (* denotes $p< 0.05$ versus Con, ** denotes $p< 0.01$ versus Con, # denotes $p< 0.05$ versus LPS.)

3.6. TLR4/MyD88/NF-κB signaling Pathway is Involved in Vitro with Sepsis-Induced Cardiac Injury

To further explore whether TLR4/MyD88/NF-κB signaling pathway participates in sepsis-induced cardiac injury of H9c2 cardiomyocytes, RT-qPCR and Western blot were carried out and shown in (Figure 6). The relative mRNA expressions of TLR4 and MyD88 were efficaciously inhibited by TAK-242. Compared with the Control group, the mRNA expressions of TLR4, MyD88, and p-NF-κB in the LPS group boosted remarkably ($p<0.01$, Figure 6a-c). Moreover, the protein expressions of TLR4, MyD88, and p-NF-κB in the LPS group increased with the protein expression relative levels of TLR4, MyD88, and p-NF-κB were inhibited by TAK-242 ($p<0.05$). However, the protein levels of TLR4, MyD88, and p-NF-κB in the TAK-242+LPS group were decreased notably compared with the LPS group ($p<0.05$). Meanwhile, compared to NF-κB, LPS treatment markedly active the expression levels of p-NF-κB ($p<0.01$, Figure 6d-h). The above data suggested that TLR4/MyD88/NF-κB signaling pathway participated in sepsis-induced cardiac injury in vitro.

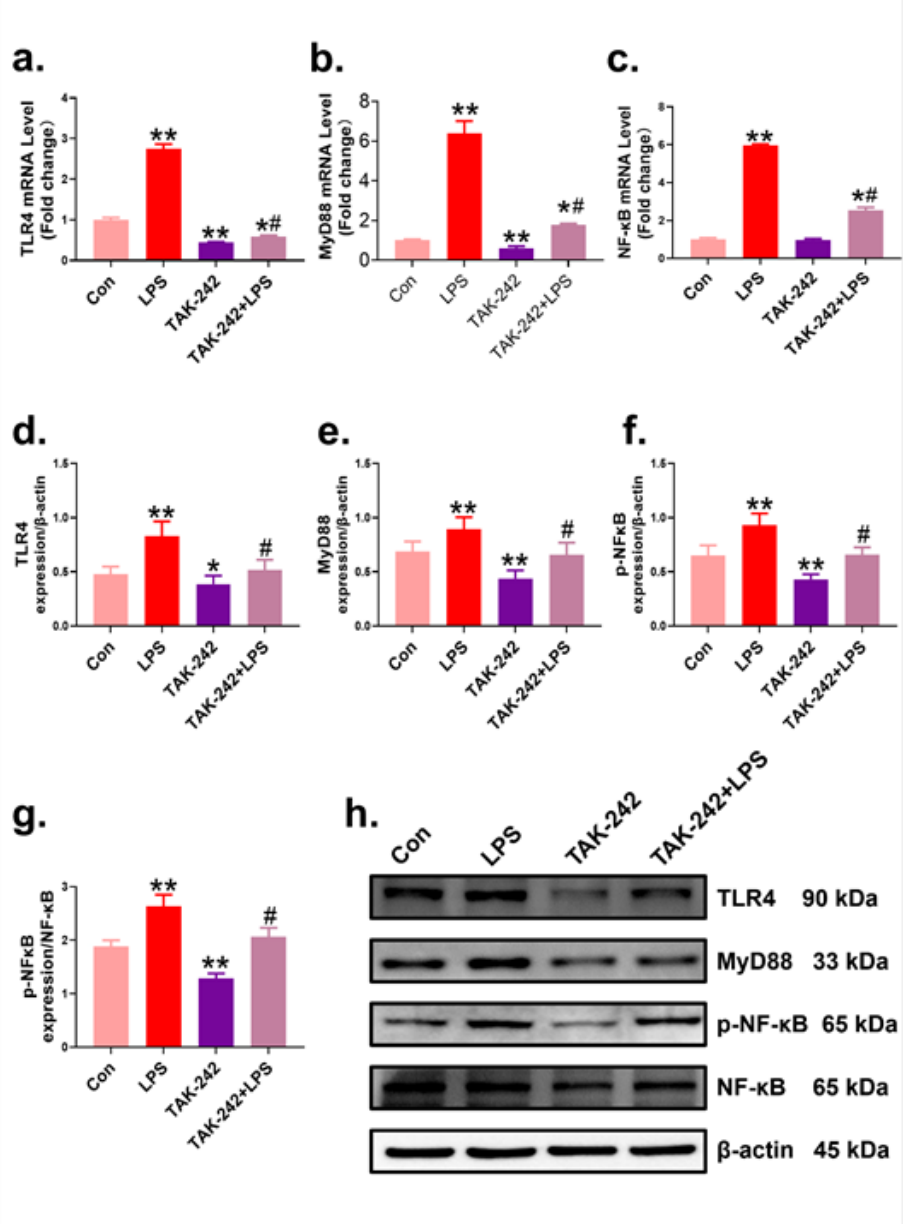

Figure 6: TLR4/MyD88/NF-κB signaling pathway is involved in sepsis-induced cardiac injury. a-c. The mRNA levels of TLR4, MyD88, and NF-κB in H9c2 cardiomyocytes were detected by fluorescence quantitative PCR respectively. d-f. The protein expression relative levels of TLR4, MyD88, and p-NF-κB in H9c2 cardiomyocytes were detected by Western blot. g. The protein expression relative level of p-NF-κB was compared with NF-κB. h. Representative images of Western blot of TLR4, MyD88, p-NF-kB, and NF-κB in each group. (* denotes $p<0.05$ versus Con, ** denotes $p<0.01$ versus Con, # denotes $p<0.05$ versus LPS.)

3.7. The Inhibition of TLR4 Reduces Myocardial Apoptosis in Vitro with Sepsis-Induced Cardiac Injury

Next, flow cytometry and Western blot were performed to determine the apoptosis level of H9c2 cardiomyocytes during sepsis. As depicted in (Figure 7), LPS administration raised the total number of apoptotic cardiomyocytes and the protein expression relative level of pro-apoptosis Bax in H9c2 cardiomyocytes ($p<0.01$). Meanwhile, the level of anti-apoptosis Bcl-2 in H9c2 cardiomyocytes reduced during LPS administration ($p<0.01$). Nevertheless, the inhibitory state of TLR4 by TAK-242 remarkably reversed the apoptosis of H9c2 cardiomyocytes after the LPS challenge ($p<0.05$).

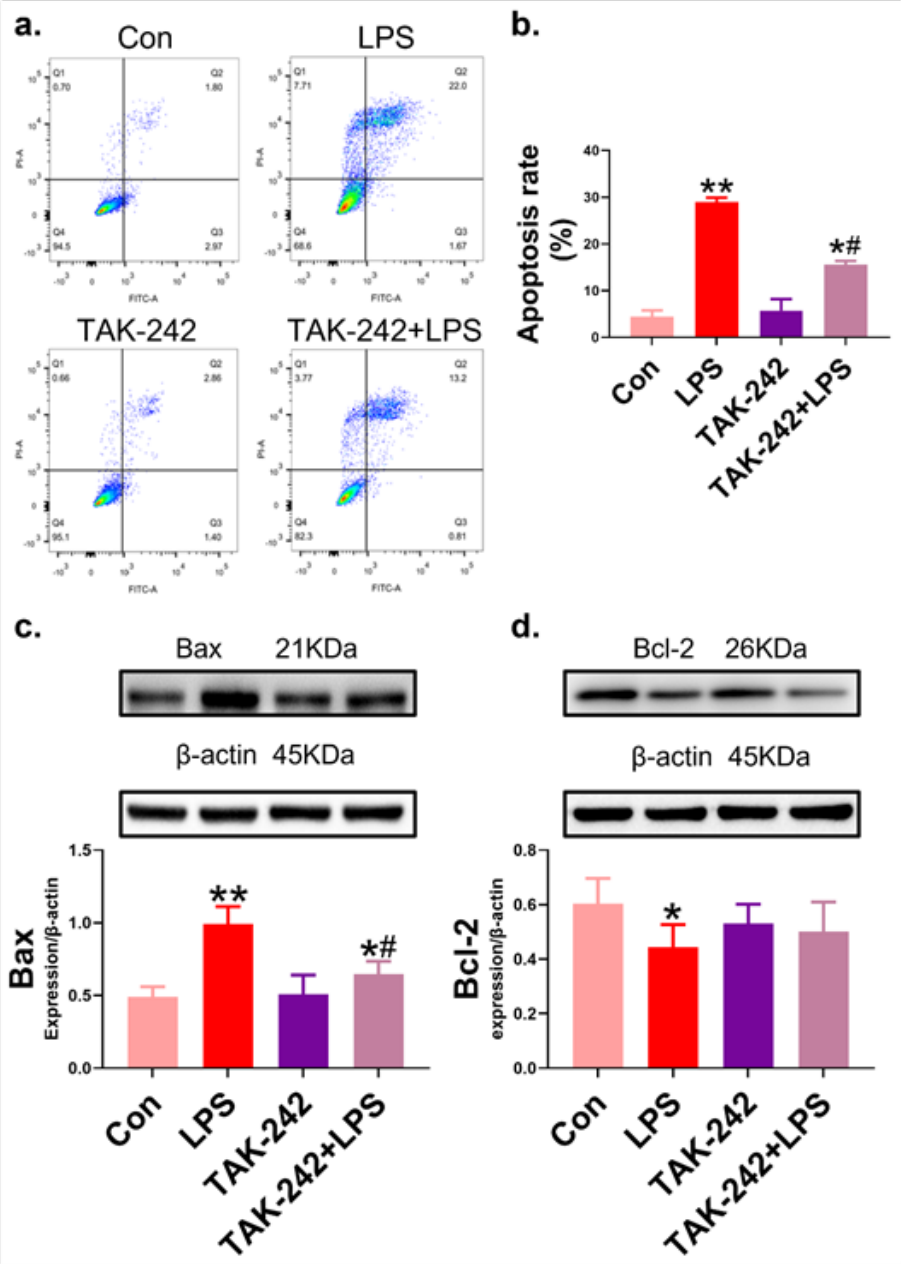

Figure 7: The inhibition of TLR4 reduces myocardial apoptosis in vitro with sepsis-induced cardiac injury. a. Representative images of flow cytometry. b. The apoptosis ratio in H9c2 cardiomyocytes of each group. c. The expression relative level of Bax (pro-apoptosis). d. The expression relative level of Bcl-2 (anti-apoptosis). (* denotes p< 0.05 versus Con, ** denotes p< 0.01 versus Con, # denotes p< 0.05 versus LPS)

4. Discussion

SCM is a lethal heart complication with high morbidity and mortality in patients with sepsis [45]. Diverse mechanisms were speculated for SCM, including uncontrollable inflammatory response, mitochondrial dysfunction, apoptosis, reactive oxygen species (ROS) accumulation, calcium dysregulation, ATP shortage, complement activation, metabolic reprogramming, ferroptosis, autophagy, mitophagy, endoplasmic reticulum (ER) stress, and myocardial edema [14, 46-50]. Little effective therapies or drugs are readily available for the clinical management of SCM largely due to the multifactorial characteristics of the disease along with the ambiguous pathogenesis [51]. Therefore, in depth understanding for the pathophysiology would benefit the diagnosis and treatment of SCM and related syndrome. In present study, our results revealed that TLR4 knockout ameliorated expression of proinflammatory cytokine and cardiomyocyte apoptosis in vivo in sepsis. Further measurements noted that the inflammatory response and cardiomyocyte apoptosis were closely related to activation of TLR4/MyD88/NF-κB signaling in septic mice and LPS-challenged H9c2 cardiomyocytes mimicking septic cardiomyopathy in the clinic reality. Furthermore, in vitro experiments indicated that inhibition of TLR4 by TAK-242 exerted a protective effect against LPS-challenged cardiomyocytes through refraining inflammation response and apoptosis via TLR4/MyD88/NF-κB signaling. Tak-

en together, these results revealed that TLR4 deficiency ameliorates septic cardiomyopathy by lessening activation of the TLR4/MyD88/NF-κB signaling pathway and corresponding manipulation of inflammatory responses and cardiomyocyte apoptosis.

As the dominating component of the outer membrane of Gram-negative bacteria, LPS is deemed a common molecular signature of bacteria and can be responded by the human immune system, and launches inflammatory responses and corresponding damage in cardiomyocytes mainly via TLR4 receptor, followed by receptor dimerization on the cytomembrane [28, 37, 52, 53]. TLR4 recruits MyD88 to tun on NF-κB and successive translocation of the nucleus, prior to amplification of inflammatory signals to upregulate the pro-inflammatory cytokines such as TNF-α, IL-1β, and IL-6 [54]. Furthermore, Tavener and associates first reported that TLR4 acted as the primary mediator in LPS-induced cardiomyocyte dysfunction back in 2004, although its specific pathogenesis remains unclear [55-60]. In recent years, numerous studies have confirmed a role for TLR4/MyD88/NF-κB signaling in various cardiovascular pathologies including heart failure, coronary microembolization, myocardial infarction, cardiac dysfunction in post-traumatic stress disorder, inactivation staphylococcus epidermidis-induced cornea inflammation, allergic asthma, streptococcus pneumonia, and spontaneous intestinal tumorigenesis [56-60]. For instance, our team previously reported that the TLR4/MyD88/NF-κB signaling participated in sepsis-induced acute liver injury [43]. However, little evidence has depicted a role for TLR4/MyD88/NF-κB signaling cascade in the regulation of inflammatory cascade and cardiomyocyte apoptosis under SCM. Therefore, this study revealed for the first time a role of the TLR4/MyD88/NF-κB signaling in SCM using TLR4 deficient murine model and H9c2 cardiomyocytes. Our results implied activation of TLR4/MyD88/NF-κB signaling in SCM, specifically in inflammatory responses of myocardium, resulting in deterioration of cardiac function and abnormal aggregation of protein carbonyl in mice. In addition, the TLR4 inhibitor resatorvid (TAK-242) was applied subsequently in H9c2 cardiomyocytes to verify the intravital results. The in vitro results also found that TLR4/MyD88/NF-κB signaling pathway was activated in H9c2 cardiomyocytes after LPS administration, resulting in not only cell death but also upregulated pro-inflammatory cytokines along with myocardial injury. Intriguingly, TLR4 deficiency or inhibition of TLR4 effectively reversed these pathological changes and detrimental effects on cardiac function.

In the present study, we assessed the apoptosis levels in cardiomyocytes of mice during sepsis by TUNEL assays, caspase 3 activity, and Western blot. Also, it was found that in the mice model of SCM, both TUNEL positive cells and caspase 3 activity were increased with the ascending expression level of pro-apoptosis protein Bax and the descending expression level of anti-apoptosis protein Bcl-2. Consequently, TLR4 deficiency exhibited the reversed function which embodied the abatement of TUNEL positive cells, caspase 3 activity, and expression of Bax, yet increased expression level of Bcl-2. Meanwhile, our in vitro results showed that inhibition of TLR4 reduced levels of apoptosis after LPS challenge in H9c2 cardiomyocytes, consistent with the results in vivo. All results together suggested that TLR4 deficiency protected the myocardium from septic damage by restricting apoptosis during SCM. In summary, our study demonstrates that the TLR4/MyD88/NF-κB signaling cascade participates in the activation of inflammatory responses and apoptosis in SCM. TLR4 deficiency or inhibition ameliorates myocardial damage and improves cardiac function by inhibiting the TLR4/MyD88/NF-κB signaling pathway and then reduces pro-inflammatory cytokines and apoptosis after SCM. These findings propose a relative solid mechanism underlying SCM and pave a new route to the prevention and management of SCM.

5. Materials and Method

5.1. Animals and Experimental Protocol

Male adult C57BL/10J mice and TLR4−/− mice (8 weeks old and weighing 20-25g) were purchased from GemPharmatech Co, Ltd. These mice were bred and maintained at the Animal Laboratory Center of the Nanfang Hospital. In our study, mice were randomly assigned to 4 groups wild type (WT) group (n=16), LPS group (n=16), TLR4−/− group (n=16), and TLR4−/−+LPS group. Male adult C57BL/10J mice and TLR4−/− mice were injected intraperitoneally with normal saline (NS) or lipopolysaccharide (LPS) (4 mg/kg) for 6h as described to induce septic cardiomyopathy [38]. After echocardiography assessment of left ventricular (LV) function, mice were euthanized via pentobarbital, and heart tissues were then harvested for subsequent experimentations.

5.2. Cell Culture and Treatment

H9c2 cardiomyocytes were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA) and cultured in a high-sugar DMEM (Gibco, Grand Island, NY, USA) containing penicillin/streptomycin (1%) with fetal bovine serum (10%) at 37℃ in a humidified incubator at 5% CO2. Four groups were included including control, LPS, TAK-242 group (or Resatorvid, a selective TLR4 inhibitor, No: HY-11109) was purchased from MedChemExpress (MCE, USA), and TAK-242+LPS group. When cell density reached nearly 70%-80%, septic cardiomyopathy was simulated in vitro by culturing H9c2 cardiomyocytes with 1μg/mL LPS for 24 h as grouping [39, 40]. For inhibitor treatment, H9c2 cardiomyocytes were incubated with 30 μM TAK-242 for 24 h prior to LPS stimulation [36, 41]. All experiments were repeated at least three times.

5.3. Cell Viability Assay

To evaluate cell viability, H9c2 cardiomyocytes were seeded in 96 well plate with 5×104/mL for CCK-8 assay (No: CK04, Dojindo, Japan) at 37℃ in a humidified incubator containing 5% CO2. Briefly, septic cardiomyopathy was simulated in cardiomyocytes, followed with 10 μl CCK-8 solution and 90 μl DMEM to each

well. Following incubation at 37°C for 1 h in a dark chamber, OD values were measured at 450 nm wavelength.

5.4. Echocardiography

Operators who were ignorant of the groups performed echocardiography on animals using an echocardiogram imaging system (15 MHz, VisualSonics Vevo 2100). Two-dimensionally directed M-mode images were obtained from long-axis views, collected the principle cardiac function parameters, including fractional shortening (FS), wall thickness estimated wall mass, volume, ejection fraction, heart rate, and cardiac output were collected, synchronously. Meanwhile, the left ventricular end-diastolic volume (LVEDV), the left ventricular end-diastolic dimension (LVEDD), left ventricular end-systolic dimension (LVESD), left ventricular ejection fraction (LVEF), and left ventricular fraction shortening (LVFS) were calculated by the computer algorithms.

5.5. Histopathological Examination of Myocardial Tissues

The heart tissues were dissected and fixed within 4% paraformaldehyde for 24 h to stabilize samples and subsequently embedded in paraffin as standard techniques. The tissue sections were stained with hematoxylin and eosin (H&E) and then observed by a microscope.

5.6. Terminal Deoxynucleotidyl Transferase DUTP Nick-End Labeling (TUNEL) Staining

Myocardial apoptosis was evaluated using TUNEL staining according to the instruction (Beyotime, China), and DAPI (blue) was used for nuclear staining of cardiomyocytes. All sections were sealed with fluorescence quenching sealing solution and ultimately observed under a fluorescence microscope [42]. The software Image J was utilized to assess apoptosis level in cardiomyocytes (quantitatively as percentage of TUNEL-positive cardiomyocytes).

5.7. Enzyme-Linked Immunosorbent Assay (ELISA)

The levels of TNF-α, IL-6, and IL-1β in serum were analyzed using ELISA kits (R&D Systems, Minneapolis, MN, USA) according to the instructions of the manufacturer. In addition, the levels of CK-MB (Cusabio Biotech CO., Ltd, Wuhan, China) and cTn (Cusabio Biotech CO., Ltd) in serum and supernatant were also measured by relevant ELISA kits according to the instructions.

5.8. Carbonyl Assay

To evaluate protein oxidative damage in the heart, carbonyl assay was performed according to the instruction of the micro protein carbonyl assay (Solarbio, China, BC1275). Briefly, heart tissues were mixed with extracting solution by a homogenizer. The supernatant was collected after centrifugation at 5000×g for 10 min at 4 °C. Next, secondary centrifugation was conducted at 12000×g for 10 min to collect the new supernatant. After determining the protein concentration by BCA detection kit (Thermo, USA), the samples were added to 96 well plate and incubated at 37°C for 1h away from light. After that, we collected the sediment centrifuged to perform the subsequent process in proper order according to the reagent instruction. Finally, the OD values at 370 nm wavelength were assessed and the content of carboxide based on corresponding calculation formulas was evaluated [43].

5.9. Cysteinyl Aspartate Specific Proteinase 3 (Caspase 3) Assay

Enzymatic activity of apoptosis-induced caspase 3 was measured with the corresponding kit (BestBio, Shanghai, China). Briefly, myocardial tissue was minced to homogenate in cold lysis buffer and then shook for 15 min on the ice. Next, the supernatant was collected after centrifugation at 12000×g for 10 min at 4 °C. Protein contents were estimated by using the BCA method. Caspase 3 activity was measured in a 96-well plate, and each well contained 50 μL of lysate, 40 μL of assay buffer, and 10 μL of caspase 3 colorimetric substrate reagent. The plate was incubated away from the light at 37 °C for 4 h. Finally, the OD values at 405 nm wavelength were measured [44].

5.10. Reverse Transcription-Quantitative PCR (RT-qPCR)

Total RNA was extracted from each group of myocardial tissues or H9c2 cardiomyocytes using the RNAiso Plus kit (Takara, Japan) according to the reagent instruction. After that, the PrimeScript™ RT reagent kit (Takara, Japan) was employed to synthesize cDNA by reverse transcription. Next, qPCR was conducted using an SYBR Premix Ex Taq kit on a QuantStudio™ 5 system (Thermo, USA). The amplification conditions included initial denaturation at 95 °C for 10 s, followed by 40 cycles at 95 °C for 5 s and 60 °C for 20 s. GAPDH was used as an internal reference for target genes. Ultimately, the relative expression levels of genes were calculated by using the $2^{-\Delta\Delta Ct}$ method. The primers used in animals (mouse) and cells (rat) were respectively synthesized by Sangon (Shanghai, China) and Thermo Fisher Scientific (Thermo, USA), and the sequences are listed in (Table 1).

5.11. Western Blot Analysis

The whole proteins were extracted from the heart and H9c2 cell, then quantitatively determined the protein concentration of the BCA detection kit (Thermo, USA) to complete the preparatory work for subsequent experiments. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE, 10%-12%) gels were employed to separate the proteins. These separated proteins were subsequently transferred onto undefiled polyvinylidene fluoride (PVDF) membranes. The membranes were blocked with TBS containing 5% bovine serum albumin (BSA) for 1h at room temperature and incubated overnight on a table concentrator with a primary antibody at 4℃. The membranes were washed three times (10 min) with tris-buffered saline (TBS) containing 0.1% tween-20, then incubated with horseradish peroxidase (HRP) - conjugated secondary antibodies (1:10000) for 1 h at room temperature. All the antibodies were purchased from Cell Signaling Technology (CST, Inc. MA, USA), the concentrations of antibodies were TLR4

(1:500), p-NF-κB p65 (1:1000), NF-κB p65 (1:1000), MyD88 (1:1000), Bax (1:1000), Bcl-2 (1:1000) and β-actin (1:1000). After that, all protein bands were washed three times and visualized through electrochemiluminescence with enhanced chemiluminescence reagent solution. GAPDH was used as the internal reference for target proteins.

5.12. Statistical Analysis

The data analyzed by GraphPad 9.0.0 software are presented as the mean ± SEM. Comparisons among multiple groups were analyzed by one-way analysis of variance (ANOVA) followed by Tukey's post hoc test. $p<0.05$ was considered statistically significant.

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