Abstract. Sepsis is a life-threatening organ dysfunction caused by a dysregulated host response to infection, and is a leading cause of mortality worldwide. Myocardial dysfunction is associated with poor prognosis in patients with sepsis and contributes to a high risk of mortality. However, the pathophysiological mechanisms underlying sepsis-induced myocardial dysfunction are not completely understood. The aim of the present study was to investigate the role of toll-like receptor 4 (Tlr4)/c-Jun N-terminal kinase (Jnk) signaling in pro-inflammatory cytokine expression and cardiac dysfunction during lipopolysaccharide (LPS)-induced sepsis in mice. C57BL/6 mice were pretreated with TAK-242 or saline for 1 h and then subjected to LPS (12 mg/kg, intraperitoneal) treatment. Cardiac function was assessed using an echocardiogram. The morphological changes of the myocardium were examined by hematoxylin and eosin staining and transmission electron microscopy. The serum protein levels of cardiac troponin I (cTni) and tumor necrosis factor-α (TnF-α) were determined by an enzyme-linked immunosorbent assay (ELISA). The Tlr4 and Jnk mRNA levels were analyzed via reverse transcription-quantitative PCR. TLR4, JNK and phosphorylated-JNK protein levels were measured by western blotting. In response to LPS, the activation of TLR4 and JNK in the myocardium was upregulated. There were significant increases in the serum levels of TNF-α and cTni, as well as histopathological changes in the myocardium and suppressed cardiac function, following LPS stimulation. Inhibition of TLR4 activation using TAK-242 led to a decrease in the activation of JNK and reduced the protein expression of TNF-α in plasma, and alleviated histological myocardial injury and improved cardiac function during sepsis in mice. The present data suggested that the TLR4/JNK signaling pathway played a critical role in regulating the production of pro-inflammatory cytokines and myocardial dysfunction induced by LPS.

Introduction

Sepsis, the most common cause of death among patients in the intensive care unit, is defined as life-threatening organ dysfunction caused by a dysregulated host response to infection (1). It is recognized as a global public health problem due to its high mortality and morbidity, as well as its substantial economic burden (2). Rudd et al (3) recently reported that an estimated 48.9 million cases of sepsis and 11.0 million sepsis-related deaths were recorded worldwide in 2017, representing 19.7% of all global deaths. Septic shock is a subset of sepsis that can lead to multi-organ dysfunction and rapid death (1). Myocardial dysfunction is recognized as a common complication of septic shock and contributes to adverse outcomes (4). Endotoxins, such as lipopolysaccharide (LPS), which is an important structural component of the gram-negative bacterial outer membrane, are important factors responsible for septic myocardial dysfunction (5). Previous studies have demonstrated that LPS can mimic the myocardial dysfunction of septic shock (6,7). A number of underlying pathophysiological mechanisms, including genetic, molecular, metabolic and structural mechanisms, have been proposed to be involved in sepsis-induced myocardial dysfunction (8). However, the specific mechanisms have not yet been elucidated.

Toll-like receptors (TLRs) are the first line of defense in the mammalian immune system and recognize pathogen-associated molecular patterns, such as lipoproteins, LPS, flagellin and nucleic acids of bacterial origin (9). Among these receptors, TLR4 has been identified as the only receptor for LPS and plays a key role in LPS-mediated inflammatory responses and myocardial dysfunction (6). The intracellular signaling
pathways activated after LPS-TLR4 binding have been classified as the myeloid differentiation primary response protein 88 (MyD88)-independent and TIR-containing adapter inducing interferon-β (TRIF)-dependent pathway (10). The MyD88-independent pathway induces the activation of nuclear factor-κB and mitogen-activated protein kinases (MAPKs), thus leading to the release of pro-inflammatory cytokines and regulation of cardiac function during sepsis (11). MAPKs, including p38, extracellular signal-regulated kinase (ERK)1/2, c-Jun N-terminal kinase (JNK) and ERK5 (12), transduce a variety of extracellular signals that regulate the cellular response implicated in proliferation, differentiation, apoptosis, stress and inflammatory response (13,14) and play an important role in regulating sepsis-induced cardiac dysfunction (15). JNK, also known as stress-activated protein kinase, is a member of the MAPK family. However, the role of the JNK signaling pathway in tumor necrosis factor-α (TNF-α) expression and myocardial dysfunction induced by sepsis has not yet been clearly defined. The majority of previous studies (16,17) have reported that the activation of the JNK signaling pathway promotes the production of pro-inflammatory cytokines and the development of LPS-induced cardiac dysfunction. Research from Peng et al (18) revealed that LPS activates JNK1, thus leading to the inhibition of TNF-α expression and improvement of the myocardial function in sepsis. In addition, only a few studies have investigated whether TLR4 mediates LPS-induced myocardial dysfunction by modulating the JNK signaling pathway. Thus, the aim of the present study was to examine the changes in cardiac function, myocardial histopathology and TNF-α expression during LPS stimulation. Furthermore, this study also attempted to explore the regulatory role of the TLR4/JNK signaling pathway in LPS-induced TNF-α expression and myocardial dysfunction. The results demonstrated that the production of TNF-α increased significantly, cardiac function decreased, and the activities of TLR4 and JNK in the myocardium were upregulated during LPS stimulation. Whereas, inhibition of TLR4 activation down-regulated JNK activation, reduced TNF-α expression and improved cardiac function in response to LPS. The present results suggested that TLR4 mediated myocardial dysfunction by regulating the JNK signaling pathway during sepsis.

Materials and methods

Animals. A total of 54 male wild-type C57BL/6 mice (8-12 weeks old and weighing 22-28 g), were purchased from the Laboratory Animal Center, Academy of Military Medical Sciences, [certificate no. SCXX (Jing) 2014-0013; Beijing, China]. The mice were housed in a specific pathogen-free environment at a constant temperature (22±1˚C) with 50% humidity and 12-h light/dark cycles. All mice had free access to food and water. All the experimental procedures were approved by the Animal Experiments Ethics Committee of Nankai University (Tianjin, China; approval no. 10011).

Experimental protocol. To induce sepsis, mice were injected intraperitoneally (i.p.) with 12 mg/kg LPS (from Escherichia coli, a phenol extract of serotype 011:B4; Sigma-Aldrich; Merck KGaA), a dose that was sufficient to induce cardiac dysfunction as determined in our preliminary experiment (19). Mice were randomly assigned to three groups: Sham group (n=18), LPS group (n=18) and TAK-242 group (n=18). Mice in the LPS and TAK-242 groups were i.p. treated with saline or a TLR4 inhibitor (TAK-242; 2 mg/kg), respectively, followed by LPS (12 mg/kg, i.p.) 1 h later. Mice in the sham group were injected with equal 0.9% saline. For time course experiments, mice were sacrificed at 3, 12 and 24 h after LPS or saline injection (n=6 for each time point). Left ventricular (LV) function was assessed at 3, 12 and 24 h after LPS injection during anesthesia. After measurement of LV function, mice were euthanized by excessive inhalation of isoflurane, and then the plasma and ventricular myocardium were collected and stored at -80˚C.

Echocardiographic assessment of LV function. A Vevo 2100 ultrahigh resolution small animal ultra-sound imaging system (VisualSonics, Inc.) with a MS-400 ultrasound scanning transducer was used to evaluate LV function (20-22). Briefly, mice were anesthetized with 1-1.25% isoflurane via a mask, and their chest was shaved before the animals were placed in the supine position on a 37˚C pad. Warm electrode gel was applied to the limb leads, allowing for the electrocardiogram and respiration rate to be recorded during ultrasound imaging. Briefly, an even layer of ultrasound coupling agent was spread on the mouse thoracic, and the console was used at a downward sloping angle of 30-45˚. Two-dimensional cine loops and guided M-mode frames were recorded from the parasternal long axis. The following parameters were measured as indicators of function: LV end diastolic diameter (LVEDD), LV end systole diameter (LVESD), LV end diastolic volume (LVEDV), LV end systolic volume (LVESV), LV ejection fraction percentage (LVEF%), and LV fractional shortening percentage (LVFS%). All data were analyzed off-line at the end of the study with the software in-built in the ultrasound system (20).

Histopathological examination of myocardial tissues. Immediately after the sacrifice of the mice, their hearts were removed, fixed in a 10% paraformaldehyde solution for 24 h at room temperature and embedded in paraffin. Serial sections (4 µm) of the ventricle were affixed to slides, deparaffinized, and stained with hematoxylin and eosin (H&E) for 5 min at room temperature. Pathological changes of the myocardial tissues were observed under a light microscope (magnification, x400).

Transmission electron microscopy. Transmission electron microscopy was performed as described in a previous study by Wang et al (23). After pretreatment, the samples were cut into thin sections and visualized at 120 kV with a H7650 transmission electron microscope (Hitachi, Ltd.). A total of 10-15 micrographs per sample were obtained using a Philips CM12 (Philips Medical Systems, Inc.) by random sampling.

Measurement of cardiac troponin I (cTnI) and TNF-α protein levels. cTnI and TNF-α protein levels in the plasma were determined using a mouse cTnI ELISA kit (Biotopped Life Sciences; cat. no. TOPEL02104) and a TNF-α ELISA kit (BioLegend; cat. no. 430907) according to the manufacturer's instructions.
Reverse transcription-quantitative PCR (RT-qPCR). Total RNA was extracted from the myocardial tissues using TRIzol® reagent (Ambion; Thermo Fisher Scientific, Inc.), following the manufacturer's instructions. RT of the purified RNA (4 µg) was performed using random primers and the RevertAid First Strand cDNA Synthesis kit (Thermo Fisher Scientific, Inc.), according to the manufacturer's instructions. TB Green Premix EX Taq II (Tli RNase H Pus) (Takara Bio, Inc.; cat. no. RR820A) in a CFX96 system (Bio‑Rad Laboratories, Inc.) was used to determine the mRNA expression of TLR4 and JNK. All reactions were performed in triplicate. The sequences of the sense and antisense primers used for amplification are listed in Table I. The relative expression of the target genes was determined by calculating the values of the Δ cycle quantification (ΔCq) by normalizing the average Cq value to that of the endogenous control (GAPDH), and then calculating the 2−ΔΔCq values (24).

Western blot analysis. The Whole Protein Extraction kit (Applygen Technologies, Inc.) was used to extract the whole protein of myocardial tissue, according to manufacturer's instructions. The concentration of whole protein was determined by calculating the values of the internal control. The total protein level was normalized to the GAPDH protein level, and the level of p-JNK to total JNK were presented.

Results

Changes in cardiac function. The beneficial effect of TAK-242 pretreatment on LPS-induced cardiac dysfunction was confirmed by quantitative analysis of echocardiograms. Representative echocardiograms in the different groups are presented in Fig. 1. The results of echocardiography analysis in various groups are summarized in Fig. 2. Compared with the values in the sham groups, the LPS groups showed a significant decrease in LVEDD at 24 h, and in LVEDV at 12 and 24 h (P<0.05), as well as a significant increase in LVESD and LVESV at 3 and 12 h (P<0.05), and a significant increase in LVEF% and LVFS% at all time points after LPS injection (P<0.05). However, compared with the values in the LPS groups, pretreatment with TAK-242 induced a significant increase in LVEDD and LVEDV at 12 and 24 h (P<0.05), alongside a significant reduction in LVESD and LVESV at 3 h (P<0.05), and a significant increase in LVEF% and LVFS% at 3 h following LPS injection (P<0.05).

Histological changes in the myocardium. To directly observe the effect of TAK-242 pretreatment on the LPS-induced alterations in myocardial structure, the histology of the LV myocardial tissues was examined (Fig. 3). H&E staining showed that distinct myocardial injury occurred in the myocardial tissues of the LPS groups, including myocardial interstitial edema, prominent hemorrhaging, rupture of myocardial fibers, myocardial cell swelling, degeneration, loss of transverse striations and infiltration of leukocytes. Under the transmission electron microscope, the myocardial ultrastructure in the LPS groups was damaged, which was characterized by myofibrillar disarray, cellular disorganization, disarrangement of sarcormere and/or disruption, mitochondrial swelling and cracking, disappearance of mitochondrial crista, as well as autophagic vacuoles. As time increased, the degree of myocardial damage in the LPS groups gradually worsened. However, the LPS-induced myocardial injuries were ameliorated by pretreatment with TAK-242.

Serum levels of cTnI and TNF-α. Significant increases in the enzyme cTnI, a myocardial injury marker, were observed in the LPS groups compared with the values of the sham groups, and the level of cTnI increased gradually with time (P<0.05).
Pretreatment with TAK-242 significantly reduced the level of cTnI compared with that of the LPS group (P<0.05). Compared with the levels of the sham groups, the serum TNF-α level increased significantly in the LPS groups (P<0.05) and reached the maximal level at 3 h following LPS injection. Pretreatment with TAK-242 induced a significant decrease in the serum TNF-α level (P<0.05), compared with that of the LPS groups (Fig. 4).

**Discussion**

The present study demonstrated that, in response to LPS, the activation of TLR4 and JNK in the myocardium was upregulated, and the serum levels of TNF-α and cTnI were increased. Pathological myocardial damage was observed using H&E staining and transmission electron microscopy, and cardiac function was shown to be reduced. Inhibition of TLR4 activation led to the reduction of JNK activation and protein expression of TNF-α in the plasma, and alleviated histopathological myocardial injury and improved cardiac function during sepsis in mice. The present data supported the notion that the TLR4/JNK signaling pathway plays an important role in myocardial dysfunction induced by sepsis.

Sepsis is a complicated syndrome that begins with a systemic immune response to an infection and can progress to septic shock leading to multiple organ failure and death (25). Although the identification of clinical biomarkers in patients

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**Figure 1.** Representative echocardiographic images (M-mode) in different groups. Left, representative echocardiographic images in sham group (top), LPS group (middle) and TAK-242 group (bottom) at 3 h after LPS or saline injection. Middle, representative echocardiographic images in sham group (top), LPS group (middle) and TAK-242 group (bottom) at 12 h after LPS or saline injection. Right, representative echocardiographic images in sham group (top), LPS group (middle) and TAK-242 group (bottom) at 24 h after LPS or saline injection. LPS, lipopolysaccharide.
to facilitate earlier diagnosis and rapid therapeutic intervention is likely to have the greatest impact on improving prognosis, understanding the underlying mechanisms of the disease and evaluating the therapeutic efficacy of novel interventions using animal models are also of critical importance. Animal models of sepsis are broadly divided into three categories: Toxemia models, bacterial infection models and host-barrier disruption models (26). The advantage of toxemia models is that there is a rapid onset of pathological changes, and they can have relatively low inter-animal variability because the exact dose and route of administration can be standardized (26). In addition, toxemia models are often used to study the basic biology of septic shock, and in particular, they are employed in mechanistic studies on the role of TLR signaling (26). Considering the aforementioned reasons, in the present study, the sepsis models were established by i.p. injection of 12 mg/kg LPS, a dose demonstrated to ensure cardiac dysfunction in our preliminary experiment (19).

TLR4 is a type I transmembrane protein and has a modular structure composed of an extracellular domain formed by 17-31 leucine-rich repeats and an intracellular domain that is known as the toll-IL-1 receptor domain, which is responsible for signaling transmission (27). Since the identification of TLR4 as the LPS receptor, it has been assumed that this molecule triggers all the responses to LPS (28). The TLR4 signaling pathway plays an important role in initiating the innate immune response and inflammation in sepsis. Typically, activation of TLR4 is preceded by binding of LPS to CD14 protein, and then CD14 transfers the LPS to the TLR4/myeloid differentiation factor 2 (MD-2) complex, which dimerizes and triggers MyD88- and TRIF-dependent production of pro-inflammatory cytokines (29). The TLR4 signaling pathway plays an important role in initiating the innate immune response and inflammation in sepsis. Typically, activation of TLR4 is preceded by binding of LPS to CD14 protein, and then CD14 transfers the LPS to the TLR4/myeloid differentiation factor 2 (MD-2) complex, which dimerizes and triggers MyD88- and TRIF-dependent production of pro-inflammatory cytokines (29). The TLR4 signaling pathway associated with MAPKs is an important signal transduction pathway that mediates sepsis-induced myocardial dysfunction. It has been reported that the activation of ERK, JNK and p38 is increased in response to LPS stimulation in cardiomyocytes (30), which is consistent with the results of the current study that found LPS administration increased the phosphorylation levels of JNK. A previous study demonstrated that TLR4/MyD88 triggers a signaling cascade that leads to
early-phase activation of MAPKs (29). In the present study, to investigate whether TLR4 plays a role in the activation of JNK in response to LPS, TAK-242 was employed to block TLR4 signaling. TAK-242 is a small-molecule compound that selectively inhibits the TLR4 signaling pathway by binding directly to the intracellular domain of TLR4 (27). Pretreatment with TAK-242 inhibited the activation of JNK induced by LPS, indicating that JNK is located downstream of TLR4 signaling.

Myocardial dysfunction is regarded as a well-recognized clinical manifestation of sepsis and septic shock, and is characterized by biventricular dilation, reduced ejection fraction (eF), and a recovery period of 7-10 days in patients with sepsis (31). Echocardiographic techniques have revealed that...
either systolic or diastolic dysfunction is commonly present in sepsis (32). Currently, two-dimensional echocardiography is the most common diagnostic tool for assessing myocardial dysfunction in sepsis (33), and the M-mode-derived fractional shortening, EF and ventricular dimensions are regarded as common parameters to evaluate cardiac function (20). Among these parameters, EF is most commonly employed to evaluate LV systolic function (34). Diastolic dysfunction occurs in ~50%
patients with sepsis and is associated with high mortality (35). LV dilation can be assessed by estimating the LVEDV, a simple measure of ventricular dilation that is associated with worse clinical outcomes (34). In the present study, suppressed cardiac function was confirmed by reduced EF% and FS% in response to LPS, and was improved by pretreatment with TaK-242 at 3 h following LPS injection, which suggested that TLR4/JNK signaling plays an important role in LPS-induced systolic dysfunction. However, the present study showed that TaK-242 failed to reverse the inhibitory effect of LPS on LVEF% and LVFS% at 12 and 24 h. The reasons for this finding may be because TaK-242 has been demonstrated to act early in the process of TLR4 signaling (36) or it may be related to the half-life of TaK-242. Further studies will explore whether TaK-242 can reverse the inhibitory effect of LPS on LVEF% and LVFS% at different time points through continuous and intermittent administration of TaK-242. Contrary to previous studies (37), the current data showed that the decreases in LVEDD and LVEDV were induced by LPS stimulation, which may be related to decompression of ventricular diastolic function and further studies are necessary to explore the reasons.

However, there were some limitations of the present study. The results showed that LPS had no significant effect on LVEDD, but in the TaK-242 group, there was either a decrease or an increase in LVEDD at 3 and 12 h, respectively. These results suggested that the effect of TaK-242 was independent of LPS exposure. TLR4 is an important pathway and is most commonly known for inducing inflammation in response to LPS, which is the most understood TLR4 agonist. However, it has also been established that TLR4, as a single protein, has a remarkably low affinity for LPS. Triggering the TLR4 pathway by LPS requires at least four co-receptors: MD-2, CD14 and CD11b bound to CD18 (38). In response to LPS, TLR4-expressing normal cells significantly increase the production of inflammatory factors due to the combined upregulation of TLR4, its intracellular adapters and co-receptors, which enhances cooperation among the pathway's components (38). TaK-242 is a small-molecule compound that selectively inhibits the Tlr4/JNK signaling pathway by binding directly to Cys747 in the intracellular TLR4 domain (27). Therefore, theoretically, the effect of TaK-242 cannot be independent of LPS exposure. In order to examine the effect of TaK-242 on various parameters, an additional group of sham animals treated with TaK-242 is needed, so further relevant experiments will be performed in the future.

Previous evidence suggests that structural abnormalities may play a critical role in the pathophysiology of sepsis-induced cardiac dysfunction (39). In previous years, cardiac biomarkers have been used to detect myocardial injury (40). Troponin-I, a sensitive and specific marker of myocardial injury, is the subunit of the troponin complex. Previous studies have demonstrated that cardiac troponin release in sepsis is related to LV dysfunction and poor outcomes (41,42). In the present study, consistent with previous findings (40,43), LPS-induced myocardial injury was confirmed by the loss of integrity of myocardial membranes on histological examination (Figs. 3 and 4) and elevation of the cTnI enzyme. Furthermore, myocardial injury and elevation of cTnI could be attenuated by TaK-242 administration. According to the current results, it could be suggested that the TLR4/JNK signaling pathway plays a critical role in myocardial injury induced by LPS.

Systemic inflammatory response syndrome (SIRS) is a key characteristic in the development of organ injury during sepsis and overactivated inflammatory response plays a critical role in sepsis (40). Although there is no definitive cause-and-effect association between systemic cytokine levels and survival outcomes in sepsis, numerous studies have reported that inhibition of the cardiac inflammatory processes is beneficial in sepsis-induced cardiac dysfunction (43,44). The production of pro-inflammatory cytokines stimulated by LPS has been demonstrated to be one of the primary underlying mechanisms of cardiac dysfunction (44). As a trigger of inflammation, TNF-α participates in the production of IL-1β, and together with IL-1β, it induces the formation of secondary inflammatory factors, such as IL-6, resulting in an inflammatory cascade (45). In addition, cytokines, in particular TNF-α, can also induce the release of additional inflammatory factors, such as inducible nitric oxide synthase (iNOS), cyclooxygenase-2 (COX-2) and reactive oxygen species (ROS), which eventually induce myocardial dysfunction (46,47). Administration of TNF-α directly suppresses myocardial function in animal and human cardiomyocytes (48,49), and treatment with anti-TNF-α protects cardiac function in sepsis animal models and patients with sepsis (50,51). In the present study, LPS increased the production of TNF-α in mouse serum, which is supported by a previous finding that inflammatory cytokines such as TNF-α were induced by LPS in mice (30). Inhibition of TLR4 by pretreatment with TaK-242 significantly reduced the protein levels of TNF-α induced by LPS. These results indicated that TLR4/JNK signaling was an important pathway leading to TNF-α expression in response to LPS stimulation. However, the present study failed to include assays that demonstrated the differential expression of inflammatory-related proteins, such as iNOS, COX-2 and IL-6, and accumulation of ROS. Relevant experiments to determine the expression of inflammatory-related proteins will be performed in the future.

In conclusion, the present study demonstrated that the TLR4/JNK signaling pathway appeared to be of critical importance in sepsis, as its inhibition attenuated cardiac dysfunction and the overactivated inflammatory response. Therefore, this study indicated that the TLR4/JNK signaling pathway plays an important role in regulating myocardial dysfunction in sepsis.

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Availability of data and materials
The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors’ contributions
PL and CC conceived, designed and coordinated the present study. LH performed the majority of experiments, analyzed
the data and drafted the manuscript. SS, YS, SL and JW helped perform the experiments. All authors reviewed the results, and read and approved the final manuscript.

Ethics approval and consent to participate

All the experimental procedures were approved by the Animal Experiments Ethics Committee of Nankai University (Tianjin, China; approval no. 1001).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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