Abstract. Increasing evidence suggest that NOD-like receptor protein 3 (NLRP3) inflammasome-mediated pyroptosis may be the underlying pathological mechanism of sepsis-induced cardiomyopathy. DDX3X, an ATP-dependent RNA helicase, plays a vital role in the formation of the NLRP3 inflammasome by directly interacting with cytoplasmic NLRP3. However, whether DDX3X has a direct impact on lipopolysaccharide (LPS)-induced cardiomyocyte injury by regulating NLRP3 inflammasome assembly remains unclear. The present study aimed to investigate the role of DDX3X in the activation of the NLRP3 inflammasome and determine the molecular mechanism of DDX3X action in LPS-induced pyroptosis in H9c2 cardiomyocytes. H9c2 cardiomyocytes were treated with LPS to simulate sepsis in vitro. The results demonstrated that LPS stimulation upregulated DDX3X expression in H9c2 cardiomyocytes. Furthermore, Ddx3x knockdown significantly attenuated pyroptosis and cell injury in LPS-treated H9c2 cells by suppressing NLRP3 inflammasome activation. Taken together, these results suggest that DDX3X is involved in LPS-induced cardiomyocyte pyroptosis, and DDX3X deficiency mitigates cardiomyocyte damage induced by LPS treatment.

Introduction

Sepsis is a clinical syndrome that occurs following infection or injury (1). If not timely and properly controlled, sepsis can develop into systemic inflammatory response syndrome and ultimately result in multiple organ dysfunction syndrome (MODS) (2). Cardiac dysfunction is a severe sepsis-related complication characterized by left ventricular dilatation, decreased ejection fraction and recovery in 7-10 days (3). The molecular mechanisms of cardiac tissue damage during sepsis remain elusive. In previous studies, we examined the role of inflammation, oxidative stress, apoptosis and autophagy in sepsis-related myocardial injury (4,5). Recently, pyroptosis, a specific type of programmed cell death, was reported as a common cause of sepsis-induced tissue damage (6-9). During the development of sepsis, pyroptosis can destroy the integrity of cell membranes, resulting in inflammatory cytokine secretion and augmented inflammatory responses (10,11). Thus, selective suppression of genes and proteins involved in pyroptosis may be a potential therapeutic strategy for sepsis or sepsis-induced cardiomyopathy.

The NOD-like receptor protein 3 (NLRP3) inflammasome is a multiprotein heteromeric complex that detected a variety of danger signals that originate not only from microorganisms but also from metabolic disorders. The assembly of the NLRP3 inflammasome contributes to the self-shearing of caspase-1, leading to the generation of activated caspase-1 fragments. Activated caspase-1 induces the maturation of the proinflammatory cytokines, pro-IL-1β and pro-IL-18. In addition, activated caspase-1 shears gasdermin D (GSDMD); the cleaved N-terminal domain of GSDMD translocates to the plasma membrane and forms pores, thereby facilitating the extracellular secretion of inflammatory cytokines into the circulation system and triggering the classical pathway of pyroptosis (12). Increasing evidence suggest that the NLRP3/caspase-1/GSDMD signaling pathway is involved in several pathophysiological mechanisms, such as innate immunity, myeloid proliferation, tumorigenesis and Alzheimer's disease (13-19). It has also been
reported that NLRP3 inflammasome activation requires two
signals, a priming signal and an activating signal. First, the
priming signal provided by pathogen- and danger-associated
molecular patterns activates the NF-κB signaling pathway
and subsequently upregulates the expression levels of NLRP3
and pro-IL-1β (20). The activating signal is then provided by
a variety of molecular or cellular events, including reactive
oxygen species (ROS) production, ionic flux and lysosomal
damage (21). Compared with the latter two models, the ROS
model was considered one of the most crucial signaling
paths for the activation of the NLRP3 inflammasome (22).
Mechanistically, increased intracellular ROS levels can lead
to the detachment of TXNIP from the TXNIP-Trx protein
complex, then free TXNIP can bind to NLRP3, resulting in
NLRP3 activation and promoting the assembly of the NLRP3
inflammasome (23).

DDX3X, an ATPase/RNA helicase of the DEAD-box
family, participates in several RNA metabolic processes (24).
This protein is also involved in cell cycle progression, apoptosis,
antiviral innate immunity and cancer development (25-28).
A recent study demonstrated that DDX3X is crucial for NLRP3
inflammasome assembly due to its direct binding interaction
with NLRP3, and Ddx3x knockdown in peritoneal macro-
phages suppresses NLRP3 inflammasome activation and
reduces pyroptosis (29). The binding site for DDX3X is in
the NACHT region of NLRP3, which exerts ATPase activity
required for NLRP3 oligomerization following activation.
This suggests that DDX3X plays an indispensable role in
facilitating the oligomerization of NLRP3 (30-32).

To the best of our knowledge, the role of DDX3X in lipo-
polysaccharide (LPS)-induced cardiomyocyte stress response
has not yet been investigated. Thus, the present study aimed
to determine whether DDX3X participates in LPS-induced
cardiomyocyte injury by regulating NLRP3 inflammasome
formation and subsequent pyroptosis.

Materials and methods

Cell culture and treatment. The H9c2 rat myocardial cell line
was purchased from the National Collection of Authenticated
Cell Culture (https://cellbank.org.cn). H9c2 cells were main-
tained in DMEM (cat. no. 12800017) supplemented with
1.5 g/l NaHCO₃, 10% fetal bovine serum (all purchased from
Gibco; Thermo Fisher Scientific, Inc.) and 1% antibiotics
contained in DMEM (cat. no. 12800017) supplemented with
1 mM phenylmethylsulfonyl fluoride (Beyotime Institute of Biotechnology) and 100 U/ml penicillin and 100 mg/ml streptomycin; Beijing Solarbio Science & Technology Co., Ltd.), at 37˚C with
5% CO₂.

LPS was purchased from MedChemExpress (cat. no. HY-D1056) and reconstituted in DMSO. When the cells
reached 70% confluence, LPS (1 µg/ml) was added to the
culture medium to mimic sepsis-induced cardiomyocyte
pyroptosis in vitro; the control group was treated with DMSO.

Cell transfection. Small interfering (si)RNA against rat
Ddx3x and scramble siRNA were purchased from Shanghai
GenePharma Co., Ltd. Scramble siRNA was used as the nega-
tive control for siRNA silencing. When H9c2 cells reached
50-60% confluence, siRNA was transfected into H9c2 cells
using Lipofectamine® 3000 (Invitrogen; Thermo Fisher
Scientific, Inc.), according to the manufacturer's instructions.

Briefly, 50 nM DDX3X siRNA or scramble siRNA in combi-
nation with 5 µl of Lipofectamine® 3000 was added to each
well. Following incubation with serum-free DMEM at 37˚C for
6 h, the medium was replaced with DMEM containing
serum. After additional incubation at 37˚C for 18 h, H9c2
cells were treated with LPS (1 µg/ml). The sense and antisense
siRNA sequences are listed in Table I.

Western blotting. Total protein extraction was performed
following treatment with LPS for 24 h. RIPA lysis buffer
supplemented with 1 mM phenylmethylsulfonyl fluoride
(Beyotime Institute of Biotechnology) was added dropwise
to each well to lyse the cells. The supernatant of the cell
lysates was collected following centrifugation at 11,588 x g for
15 min. For secretory protein extraction, cell medium
supernatant was collected and centrifuged at 600 x g for 5 min
at 4˚C and 11,588 x g for 15 min at 4˚C. The bicinchoninic
acid assay kit (Beyotime Institute of Biotechnology) was used
to quantify protein samples, according to the manufacturer's
instructions. Equal amounts of protein samples (30 µg) were
separated by 10, 12.5 and 15% SDS-PAGE (EpiZyme),
transferred onto PVDF membranes and blocked with 5% non-fat
milk at room temperature for 1 h. The membranes were
incubated with primary antibodies against rabbit anti-GAPDH
cat. no. 5174; 1:8,000; Cell Signalling Technology, Inc.), rabbit
anti-DDX3X (cat. no. 11115‑1‑AP; 1:1,000; ProteinTech Group,
Inc.), rabbit anti-Caspase1/P20/P10 (cat. no. 22915‑1‑AP;
1:1,500; ProteinTech Group, Inc.), rabbit anti-NLRP3 (cat.
no. 19771‑1‑AP; 1:1,000; ProteinTech Group, Inc.), rabbit
anti-IL-1β (cat. no. AF5103; 1:1,000; Affinity Biosciences)
and rabbit anti-Cleaved-IL-1β (cat. no. AF4006; 1:1,000;
Affinity Biosciences) overnight at 4˚C. GAPDH was used as
the internal control. The PVDF membranes were washed three
times with Tris-buffered saline with 1% Tween-20 (Beyotime
Institute of Biotechnology) and subsequently incubated with
Anti-rabbit IgG, HRP-linked Antibody (cat. no. 7074; 1:10,000;
Cell Signaling Technology, Inc.) at room temperature for 1 h.
Coomassie blue staining was performed by incubating the gels
with Coomassie blue staining solution (Beyotime Institute
of Biotechnology) at room temperature for 30 min and washing
them in Coomassie blue eluent (methylene:glacial acetic acid :
distilled water = 3:1:6) until clear bands appeared. The immu-
noblots were detected using chemiluminescence reagents
(MilliporeSigma) and the blots were scanned using a chemi-
luminescent analyzer (ProteinSimple). Relative immunoblot
intensities were analyzed using ImageJ v1.8.0.112 software
(National Institutes of Health).

Reverse transcription-quantitative (RT-q)PCR. Following
treatment with LPS for 24 h, TRIzol® reagent (Invitrogen;
Thermo Fisher Scientific, Inc.) was used to extract total
RNA from H9c2 cells. Equal amounts of total RNA (1-2 µg)
were reverse transcribed into cDNA using the HiScript III
RT SuperMix for qPCR (+gDNA wiper; Vazyme Biotech
Co., Ltd.). The following temperature protocol was used for
reverse transcription: 42˚C for 2 min, followed by 37˚C for
15 min and 85˚C for 5 sec. Primer sequences were purchased
from BGI (https://www.bgi.com/). qPCR was performed in a
20-µl reaction volume consisting of ChamQ Universal SYBR
tPCR Master Mix (Vazyme Biotech Co., Ltd.). RT-qPCR was
Measurement of caspase-1 activity. Caspase-1 activity was measured using the caspase-1 activity assay kit (Beyotime Institute of Biotechnology), according to the manufacturer's instructions. Absorbance was measured using an enzyme-labeled instrument (BioTek Instruments, Inc.) at a wavelength of 450 nm.

Determination of ROS levels. Following treatment with LPS, the culture medium was removed, and H9c2 cells were subsequently incubated with 1 ml of serum-free DMEM supplemented with 1 µl of fluorescent dichloro-dihydro-fluorescein diacetate (DCFH-DA; Beyotime Institute of Biotechnology) at 37°C for 30 min in the dark. Cells were washed twice with PBS and fluorescence was observed under an Olympus LCX100 imaging system (Olympus Corporation). The average fluorescence intensity was measured using ImageJ v1.8.0.112 software (National Institutes of Health).

Cell viability assay. Following treatment with LPS, Cell Counting Kit-8 (CCK-8) reagent (APExBIO Technology LLC) was added to a 96-well plate and H9c2 cells were incubated at 37°C for 4 h in the dark. Absorbance was measured using an enzyme-labeled instrument (BioTek Instruments, Inc.), at a wavelength of 450 nm. The average optical density (OD) was used to calculate cell viability using the following equation: Cell viability = (experimental group OD - blank control group OD)/(normal control group OD - blank control group OD) x100%.

Lactate dehydrogenase (LDH) cytotoxicity assay. Following treatment with LPS, the cell culture medium was collected and centrifuged at 11,588 x g for 15 min at 4°C, and the supernatant was collected. LDH activity was measured to evaluate the damage status of H9c2 cells using Lactate dehydrogenase assay kit (Nanjing Jiancheng Bioengineering Institute), according to the manufacturer's instructions. Absorbance was measured at a wavelength of 440 nm using an enzyme-labeled instrument (BioTek Instruments, Inc.). LDH activity was calculated according to the manufacturer's instructions.

Statistical analysis. Statistical analysis was performed using GraphPad Pro Prism 8.0 software (GraphPad Software, Inc.). All experiments were performed in triplicate and data are presented as the mean ± SEM. Unpaired Student's t-test was used to compare differences between two groups, while one-way ANOVA followed by Tukey's post hoc test were used to compare differences between multiple groups. P<0.05 was considered to indicate a statistically significant difference.

Results

DDX3X expression in LPS-treated H9c2 cardiomyocytes. RT-qPCR and western blot analyses were performed to detect DDX3X expression in H9c2 cells treated with different concentrations of LPS (0, 100, 500 and 1,000 ng/ml). The results demonstrated that the mRNA and protein expression levels of DDX3X increased following treatment with LPS for 24 h, with DDX3X mRNA levels significantly increased at LPS 500 (P<0.01) and 1,000 ng/ml (P<0.001; Fig. 1A-C). Taken together, upregulated DDX3X expression in LPS-treated cardiomyocytes suggests that DDX3X participates in the development of LPS-induced cardiomyocyte injury.

LPS induces pyroptosis by increasing intracellular ROS levels and activating the NLRP3 inflammasome. To determine whether LPS provides a priming signal to activate the NLRP3 inflammasome in H9c2 cardiomyocytes, the present study detected the expression levels of NLRP3 and pro-IL-1β. The results demonstrated that the protein and mRNA expression levels of NLRP3 and pro-IL-1β increased following treatment with LPS for 24 h, with NLRP3 mRNA levels significantly increased at LPS 100, 500 and 1,000 ng/ml (all P<0.001), and pro-IL-1β mRNA levels significantly increased at LPS 500 ng/ml (P<0.05) and 1,000 ng/ml (P<0.001; Fig. 2A-D). The present results suggested that LPS acted as a priming signal to promote the expression levels of NLRP3 and pro-IL-1β during NLRP3 inflammasome activation in H9c2 cardiomyocytes. It has been reported that ROS can supply activating signals for the activation of the NLRP3 inflammasome (22). Thus, the present study measured ROS levels in LPS-treated cardiomyocytes. As expected, LPS treatment induced the production of ROS, as ROS levels were positively correlated with increasing LPS concentrations (Fig. 2E). The
medium, in H9c2 cells treated with or without LPS or in the absence or presence of DDX3X. As presented in Fig. 3C and D, in the absence of LPS stimulation, no significant differences in the expression levels of NLRP3 and pro-IL-1β, the level of caspase-1 cleavage and the accumulation of cleaved IL-1β were observed between the Ddx3x-silenced and control groups. These results suggest that Ddx3x knockdown has no influence on the signaling pathway regulating NLRP3 inflammasome activation in the absence of LPS treatment. When H9c2 cells were stimulated with LPS 1,000 ng/ml, the levels of caspase-1 (P<0.01) cleavage and cleaved IL-1β (P<0.001) significantly decreased in DDX3X-deficient cells, while no significant difference in NLRP3 expression was observed between LPS-stimulated cells with normal or altered DDX3X expression (Fig. 3C). Caspase-1 activity also decreased (P<0.01) following Ddx3x knockdown in H9c2 cells treated with LPS 1,000 ng/ml (Fig. 3E). Taken together, these results suggest that Ddx3x knockdown blocks NLRP3 inflammasome activation but has no effect on NLRP3 expression in LPS-treated H9c2 cardiomyocytes.

Ddx3x knockdown attenuates pyroptotic cell death and cell injury in LPS-treated H9c2 cardiomyocytes. Cell cytotoxicity was assessed by detecting the levels of released LDH, and cell viability was detected via the CCK-8 assay. LDH release is a sensitive biomarker of cardiac injury (34); thus, the present study detected the levels of LDH in the supernatant of the culture medium. As presented in Fig. 4A, DDX3X deficiency reversed the increase in LDH level in cells treated with LPS. In addition, cell viability increased following Ddx3x knockdown in H9c2 cells treated with LPS (Fig. 4B). However, in H9c2 cells not treated with LPS, knockdown of Ddx3x had no significant effect on cell viability or LDH release. PI staining was performed to detect pyroptotic cell death in H9c2 cells. As expected, Ddx3x knockdown attenuated pyroptotic cell death in LPS-treated cardiomyocytes (Fig. 4C). Collectively, these results suggest that Ddx3x knockdown improves the viability of H9c2 cardiomyocytes treated with LPS. The interactions uncovered by the present results are presented in Fig. 5.

Discussion

To the best of our knowledge, the present study was the first study to demonstrate that DDX3X is an essential molecular component of the signaling pathway of LPS-induced cardiomyocyte pyroptosis and cell injury. The results demonstrated that DDX3X expression was significantly increased in LPS-treated cardiomyocytes in vitro. Notably, Ddx3x knockdown attenuated LPS-induced cardiomyocyte pyroptosis and cell injury.

Pyroptosis is a major pathophysiological mechanism in several cardiovascular diseases, such as atherosclerosis, ischemic heart disease, diabetic cardiomyopathy, and cardiac hypertrophy (35). During sepsis, pyroptosis protects the host by eliminating infected cells; however, overactivated pyroptosis can lead to systemic inflammation, resulting in septic shock, MODS and an increased risk of secondary infection (36). The present study detected the upregulation of NLRP3 and pro-IL-1β, and the activation of caspase-1 in H9c2 cells treated with LPS, demonstrating that LPS stimulation can directly
Figure 2. LPS induces pyroptosis by increasing intracellular reactive oxygen species levels and activating NLRP3 inflammasome. Reverse transcription-quantitative PCR analysis was performed to detect the mRNA expression levels of (A) NLRP3 and (B) IL-1β in H9C2 cardiomyocytes treated with different concentrations of LPS (0, 100, 500 and 1,000 ng/ml) for 24 h. Data are presented as the mean ± SEM (n=4). (C) Western blot analysis was performed to detect the protein expression levels of NLRP3 and pro-IL-1β in H9C2 cardiomyocytes treated with different concentrations of LPS (0, 100, 500 and 1,000 ng/ml) for 24 h. Representative blots (n=3). (D) Western blot analysis was performed to detect NLRP3 protein expression in H9C2 cardiomyocytes stimulated with LPS (1,000 ng/ml) for 0, 6, 12 and 24 h. Representative blots (n=3). (E) The DCFH-DA assay was performed to detect ROS production in H9C2 cardiomyocytes treated with LPS (1,000 ng/ml) for 6 h. Bar, 50 µm (n=3). (F) Western blot analysis was performed to detect intracellular protein levels of pro-caspase-1 and caspase-1 p20, and the level of the cleaved IL-1β in the supernatant of culture medium in H9C2 cardiomyocytes treated with different concentration of LPS (0, 100, 500 and 1,000 ng/ml) for 24 h. Representative blots (n=3). (G) Caspase-1 activity was detected in H9C2 cardiomyocytes treated with different concentrations of LPS (0, 100, 500 and 1,000 ng/ml) for 24 h. Data are presented as the mean ± SEM (n=3). *P<0.05, **P<0.01 and ***P<0.001. LPS, lipopolysaccharide; NLRP3, NOD-like receptor protein 3; F, forward; R, reverse; bp, base pair; TM, melting temperature.

Table II. Primer sequences used for quantitative PCR.

| Gene     | Sequence                        | Product size (bp) (5'–3') | TM (˚C) |
|----------|---------------------------------|---------------------------|---------|
| DDX3X-Rat | F: AAACCTTGGTCTTGCCACCTC       | 21                        | 57.57   |
|          | R: CCACGGCCTGCTACCTTTATAAG      | 21                        | 59.52   |
| NLRP3-Rat | F: GCTAAGAAGGACCAGCCAGA        | 20                        | 57.45   |
|          | R: TCCCAGCAAACCTATCCACT         | 19                        | 55.40   |
| IL-1β-Rat | F: CACCTCTCAAGCAGGCACAG         | 21                        | 59.76   |
|          | R: GGGTTCCATGGTGAAGTCAAC        | 21                        | 57.80   |
| GAPDH-Rat | F: GTATTTGGCGCTGGTCACC          | 20                        | 61.55   |
|          | R: CGCTCCTGGAAGATGGTGATGG       | 22                        | 61.85   |

NLRP3, NOD-like receptor protein 3; F, forward; R, reverse; bp, base pair; TM, melting temperature.
trigger pyroptosis by activating the NLRP3 inflammasome in H9c2 cardiomyocytes and lead to cardiomyocyte damage. Increasing evidence suggest that the NLRP3 inflammasome participates in sepsis-induced cardiomyopathy (34,37-39). The NLRP3 inflammasome mediates caspase-1 activation and the excretion of the proinflammatory cytokines, IL-1β and IL-18, resulting in pyroptosis and systemic inflammatory responses. NLRP3 inflammasome activation is essential for normal host defense against microbial infections. However, dysregulated activation of the NLRP3 inflammasome can give rise to severe auto-inflammatory states (37). Thus, activation of the NLRP3 inflammasome must be tightly regulated to provide adequate immune safeguards rather than damage to the host. Several mechanisms have been demonstrated to regulate inflammasome activation, including molecular post-translational modifications of NLRP3 and its interacting partners (21).
DDX3X is a functionally multifaceted helicase, which plays various roles in RNA metabolism, cell cycle control, stress granule formation, apoptosis, innate immunity, viral infection and cancer (40). A previous study suggested that the availability of DDX3X molecules regulated pyroptosis mediated by the NLRP3 inflammasome in macrophages, and that DDX3X was involved in NLRP3 inflammasome assembly through direct binding to the NACHT domain of NLRP3 (28). Inhibition of the ATPase activity of DDX3X by RK-33 does not affect NLRP3 inflammasome activation, suggesting...
that DDX3X exerts a scaffold function by facilitating the assembly of ASC specks, rather than a catalytic function (41). In the present study, DDX3X expression in cardiomyocytes increased following treatment with LPS, suggesting that DDX3X is involved in LPS-induced cardiomyocyte stress response. The results also demonstrated that Ddx3x knockdown inhibited LPS-induced NLRP3 inflammasome activation but did not affect NLRP3 expression. The results further confirmed that DDX3X promotes NLRP3 inflammasome assembly by interacting with NLRP3. The inhibition of NLRP3 inflammasome activation further hindered caspase-1 activation, which attenuated pyroptosis and cell death in LPS-treated cardiomyocytes. Taken together, these results suggest that DDX3X acts as a regulator of pyroptosis during cardiomyocyte stress response.

In conclusion, the results of the present study demonstrated that LPS stimulation induced DDX3X expression in cardiomyocytes, and Ddx3x knockdown attenuated LPS-induced cardiomyocyte pyroptosis and cell injury by suppressing NLRP3 inflammasome activation. However, no in vivo data were provided in the present study. Thus, further studies are required to confirm whether DDX3X may be a potential therapeutic target for sepsis-induced cardiomyopathy.

Acknowledgements

Not applicable.

Funding

The present study was supported by the National Nature Science Foundation of China (grant no. 81873473), the Academic Promotion Program of Shandong First Medical University (grant no. 2019QL014) and Shandong Taishan Scholarship (no grant number available).

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

DF, LG, JiL, HH, JuL and EH were involved in the study design. DF, YS and XM performed the experiments. DF analyzed the data and drafted the initial manuscript. All authors have read and approved the final manuscript. DF and EH confirm the authenticity of all the raw data.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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