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miR-206 alleviates LPS-induced inflammatory injury in cardiomyocytes via directly targeting USP33 to inhibit the JAK2/STAT3 signaling pathway

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Abstract

Previous reports have confirmed that miR-206 participates in inflammatory cardiomyopathy, but its definite mechanism remains elusive. This study aims to elucidate the potential mechanism of miR-206 in septic cardiomyopathy (SCM). The primary mouse cardiomyocytes were isolated and exposed to lipopolysaccharides (LPS) to construct a septic injury model in vitro. Then, the gene transcripts and protein levels were detected by RT-qPCR and/or Western blot assay. Cell proliferation, apoptosis, and inflammatory responses were evaluated by CCK-8/EdU, flow cytometry, and ELISA assays, respectively. Dual luciferase assay, Co-IP, and ubiquitination experiments were carried out to validate the molecular interactions among miR-206, USP33, and JAK2/STAT3 signaling. miR-206 was significantly downregulated, but USP33 was upregulated in LPS-induced cardiomyocytes. Gain-of-function of miR-206 elevated the proliferation but suppressed the inflammatory responses and apoptosis in LPS-induced cardiomyocytes. USP33, as a member of the USP protein family, was confirmed to be a direct target of miR-206 and could catalyze deubiquitination of JAK2 to activate JAK2/STAT3 signaling. Rescue experiments presented that neither upregulation of USP33 nor JAK2/STAT3 signaling activation considerably reversed the protective effects of miR-206 upregulation in LPS-induced cardiomyocytes. The above data showed that miR-206 protected cardiomyocytes from LPS-induced inflammatory injuries by targeting the USP33/JAK2/STAT3 signaling pathway, which might be a novel target for SCM treatment.

Keywords miR-206 · USP33 · JAK2/STAT3 signaling · Septic cardiomyopathy · Inflammation

Introduction

Sepsis is an infection-induced life-threatening organ disorder due to the dysregulation of host responses, which is one of the most frequent causes of mortality and morbidity, which accounts for about 50 million disease cases globally per year [1, 2]. The cause of sepsis is the imbalanced systemic inflammatory response due to infection, which can cause multiple organ dysfunction[3]. Increasing reports pointed out that myocardial dysfunction induced by the increasing cytokines, such as tumor necrosis factor-alpha (TNF-α) and Interleukin-1 beta (IL-1β), is a frequent side effect of patients with sepsis [4, 5]. Septic cardiomyopathy (SCM) is a non-ischemic myocardial dysfunction that occurs in septic patients and is one of the most severe complications of septic shock, dramatically leading to high mortality rates [6]. A previous study showed that SCM occurred at a highly variable rate (13.8–70%) in patients suffering from sepsis and sepsis shock[7]. Unfortunately, although several hypotheses have been revealed to elucidate the pathophysiological characteristics of SCM during the past decades[8], but the clinical efficacy of SCM is still not optimistic. It is vital to deeply elucidate SCM's

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pathogenesis to improve treatment measures and patient prognosis.

MircoRNA (miRNAs) are non-coding ribonucleic acids that can directly bind to the 3′ untranslated region (UTR) of downstream mRNA and induce mRNA degradation or suppress its translation [9]. A recent study revealed that 78 miRNAs were dysregulated in a rat septic shock model, and their different networks could potentially participate in and regulate the pathological process of SCM[10]. Moreover, Xie et al.'s experiment validated that miR-146a regulated the TLR-4/NF-κB signaling pathway to alleviate inflammatory response and improve cardiac disorder in SCM[11]. Another study also showed that miR-29c-3p was upregulated in the serum samples of sepsis patients, and suppression of miR-29c-3p could alleviate sepsis-induced cardiac symptoms and inflammation[12]. All those studies indicated that miRNAs play an essential role in SCM development. Recent findings exhibited that miR-206 was significantly upregulated in patients with septic shock relative to patients with sepsis, indicating that miR-206 was related to the severity and prognosis of patients with sepsis [13]. However, it is still unclear about the definite mechanisms of miR-206 in pathophysiology of sepsis.

Ubiquitin-specific protease 33 (USP33) is one of the deubiquitinating enzymes (DUBs) which participates in the ubiquitin–proteasome pathway. USP33 can remove the mono-ubiquitin or disassemble the polyubiquitin chains from the substrates[14]. Previous studies showed that USP33 could regulate E3 ligase von Hippel-Lindau protein (pVHL), which synergistically deubiquitinated Parkin RBR E3 Ubiquitin Protein Ligase PRKN(an E3 ligase) and inhibited PRKN-modulated ubiquitination of the downstream target proteins[15]. Additionally, USP33 could deubiquitinate and stabilize HIF-2α protein to enhance hypoxia response [16]. Increasing evidence has shown that USP33 participated in many biological processes, including mitophagy [15], cancer development [14, 16], autophagy, and innate immune response[17]. For example, dengue virus (DENV) infection could upregulate miR-148a to suppress USP33 expression, thus destabilizing ATF3 to aggravate neuroinflammation[18]. Similarly, SARS-CoV-2 spikes also led to a high amount of miR-148a to suppress USP33/IRF9 signaling to stimulate neuroinflammation[19]. However, until now, there have been no reports about how USP33 participates in bacterial infection during the development of sepsis.

Previous bioinformatic prediction discovered that USP33 3′-UTR had a putative site of miR-206, and USP33 had a deubiquitination region on JAK2, suggesting the potential regulatory association among miR-206, USP33, and JAK2. Thus, we hypothesized that miR-206 could alleviate the inflammatory injury of SCM by direct targeting USP33 and inactivating JAK2/STAT3 signaling. To verify the hypothesis, the primary mouse cardiomyocytes were obtained and administrated with lipopolysaccharides (LPS) to construct a septic injury model in vitro. The experimental data would be elucidate a novel molecular mechanism and provide a new target for clinical treatment of SCM.

Materials and methods

Isolation and culture of mouse cardiomyocytes

Male C57BL/6 mice (2-month-old, weight 25.0 ± 1.25 g) were purchased from Guangdong Medical Laboratory Animal Center (Guangzhou, Guangdong, China). All animal experiments were performed according to the Guidelines for the Care and Use of Laboratory Animals and the protocols approved by Laboratory Animal of the First Affiliated Hospital of Nanchang University Ethics Committee. Mice were housed in a sterile facility with a controlled temperature of 21.0 ± 1.0 °C, 12 h light-12 h dark cycles and received free access to water and a standard chow diet. Mice were euthanized by isoflurane and the descending aorta was surgically isolated after opening the chest cavity. After brief washes and perfusion by pre-cold 1 × Dulbecco's Phosphate Buffered Saline (DPBS) and Hank's Balanced Salt Solution (HBSS), heart blood was cleared from the heart ventricles. The primary cardiomyocytes were isolated using a Pierce™ Primary Cardiomyocyte Isolation Kit (88,281, Thermo Fisher Scientific, CA, USA). Briefly, the dissected individual-washed heart was minced and transferred to a dish in 500 µl ice-cold HBSS containing 0.2 ml cardiomyocyte isolation enzyme I (with papain) + 10 µl cardiomyocyte isolation enzyme II (with thermolysin). In an incubator, the tissues were digested at 37 °C with 5% CO_2 for 0.5 h. Subsequently, the lysate was collected gently and washed twice using 500 µl ice-cold HBSS. Then, 0.5 ml DMEM was added to the tube, and the pellet was resuspended and pipetted up and down 25–30 times using a sterile 1.0 ml pipette tip to generate a single-cell suspension. After pipetting, 1.0 ml complete DMEM was added to the tube to bring the volume to a total of 1.5 ml. After cell counting to determine the cell concentration, cells were seeded into 6-well plates at 2.5×10^5 cells/cm^2 in 2 ml complete DMEM. After 24 h, the medium was replaced with the prepared complete DMEM containing a cardiomyocyte growth supplement (1:100). After the first medium change. The medium was changed every other day until further assays.

To establish a septic injury model in vitro, the cardiomyocytes were exposed to LPS (Sigma, MO, USA) at 0.1, 1, 2, 5, and 10 µg/ml in a complete DMEM medium for 24 h. To activate JAK2 signaling, cardiomyocytes were pre-treated

with 50 nM Coumermycin A1 (a JAK2 signal activator) for 1 h before LPS treatment.

Cell transfections

The commercial mimics NC, miR-206 mimics, inhibitor NC, and miR-206 inhibitor were purchased from RiboBio (Guangzhou, Guangdong, China). The full length of USP33 was amplified and cloned into pcDNA3.1 (RiboBio) to construct recombinant pcDNA3.1-USP33 plasmids. For cell transfection, cardiomyocytes were seeded into 6-well plates and cultured at 37 °C, 5% CO_2 in an incubator. When cells reached 70% confluence, lipofectamine 3000 (Thermo Fisher Scientific, MA, USA) was used to transfect the above nucleotide or vectors for 48 h. Then the cells were collected for subsequent assays.

Cell counting kit-8 (CCK-8) assay

Cell viability of cardiomyocytes was measured using a commercial CCK-8 (Beyotime Biotechnology, Shanghai, China). Briefly, 5×10^3 cardiomyocytes were seeded into each well of the 96-well plate for reculture for 24 h. After indicated treatment, 10μl of CCK-8 solution was added to each well. After 1 h incubation, the absorbance was read at 450 nm in a microplate reader (Bio-TEK, USA).

5-ethynyl-2′-deoxyuridine (EdU) Staining

The cell proliferation was detected by a Click-iT™ Plus EdU Cell Proliferation Kit (Thermo Fisher Scientific). Briefly, cardiomyocytes were seeded in a PhenoPlate 96-well plate with a clear flat bottom and incubated with 10 μM EdU for 2 h. After fixation by 4% paraformaldehyde-DPBS solution for 10 min, the incorporated EdU was detected according to the detailed protocol. Cell nuclei were stained by DAPI (Thermo Fisher Scientific) in 1 X DPBS for 10 min. After washing with 1 × DPBS solution for 3 times, the stained cells were imaged with Operetta CLS High Content Analysis System 4.9 (PerkinElmer Inc, CA, USA).

Real time- quantitative PCR (RT-qPCR)

After different treatments, total RNA was extracted from cardiomyocytes using a RNeasy Mini Kit (Qiagen, CA, USA). 1.0 μg of total RNA was transcripted into cDNA using a High-Capacity cDNA Reverse Transcription Kit with RNase Inhibitor (Thermo Fisher Scientific). Then, One-Step qRT-PCR Kit (Thermo Fisher Scientific) was used for RT-qPCR assay on a Quant Studio™ 6 Flex Real-Time PCR Systems (Thermo Fisher Scientific). The relative expressions of target genes were normalized to the internal control GAPDH or U6 using the $2^{-\Delta\Delta Ct}$ method. The related primer sequences are provided in Table 1.

Table 1 The primer sequences used for RT-qPCR

Gene	Sequence (5′-3′)
miR-206 (Forward)	GGGTGGAATGTAAGGAAGT
miR-206 (Reverse)	CGTGTCGTGGAGTC
U6 (Forward)	GCTTCGGCAGCACATATACTAAAAT
U6 (Reverse)	CGCTTCACGAATTTGCGTGTCAT
USP33 (Forward)	GACGTGCGATAGGGTGTCTA
USP33 (Reverse)	AGTTGGGTGACTGGAGGAGT
GAPDH (Forward)	GGGAAACTGTGGCGTGAT
GAPDH (Reverse)	GAGTGGGTGTCGCTGTTGA

Western blot

The harvested cells after the different treatments were lysed in RIPA buffer (Beyotime Biotechnology) containing 10 μM PMSF on ice for 20 min. After centrifugation at 4 °C for 14,000 g × 10 min, the supernatant was transferred into a new sterile tube. Subsequently, protein concentrations were determined using a BCA Protein Assay Kit (Thermo Fisher Scientific). A total of 20 μg protein was separated by SDS-PAGE and transferred onto PVDF membranes (Millipore, Burlington, USA). After blocking with 5% non-fat milk, the membranes were incubated with the individual primary antibodies Rabbit anti-USP33 (ab237510, Abcam, MA, USA), rabbit anti-GAPDH (ab9485, Abcam), mouse anti-Bax (ab3191, Abcam), rabbit anti-Bcl-2 (ab182858, Abcam), rabbit anti-JAK2 (ab108596, Abcam), rabbit anti-STAT3 (ab68153, Abcam) and rabbit-STAT3 (phospho Y705) (ab267373, Abcam) at 4 °C overnight. After three washes by 1 × PBST, the membranes were incubated with HRP- conjugated rabbit anti-Goat IgG (31,403, Thermo Fisher Scientific) or Donkey anti-Mouse IgG (H + L) (A16011, Thermo Fisher Scientific) for 1 h at room temperature. Subsequently, the membranes were visualized by Pierce™ ECL Western Blotting Substrate (Thermo Fisher Scientific, MA, USA). The membranes were imaged by Image J software.

Enzyme-linked immunosorbent assay (ELISA)

After the different treatments, the cardiomyocytes were collected and centrifugated at 1000 g for 20 min to remove the particles, and the supernatants were obtained to examine the contents of inflammatory factors according to the manufacturer's instructions. The absorbance at 450 nm was recorded by a microplate reader (Bio-TEK, USA). The ELISA kit

including TNF alpha Mouse ELISA Kit (BMS607-3), IL-1 beta Mouse ELISA Kit (BMS6002), and IL-6 mouse ELISA kit (KMC0061) from Thermo Fisher Scientific.

Cell apoptosis

Cell apoptosis was measured by Bioscience™ Annexin V Apoptosis Detection Kits (Thermo Fisher Scientific). After the different stimulation, cells were collected by centrifugation at 1000 g × 3 min. After discarding the supernatant, the cells were resuspended and washed with pre-cold DPBS 2 times. Then, cell pellets were resuspended in 200 µl binding buffer (1x) with cell concentration at 2×10^5 cells/ml. Subsequently, 5 µl Annexin V-FITC was added to the cell suspension. After the mixture, cells were incubated at room temperature for10min in the dark. After three washes using 200 µl binding buffer, cells were resuspended in 190µl binding buffer (1x) with 10 µl propidium iodide (20 µg/ml). Apoptotic cells were analyzed using a CytoFLEX Flow Cytometer (Beckman Coulter, VWR, NJ, USA).

Luciferase reporter assay

The potential binding sequence of miR-206 in USP33 was predicted by the online software miRDB (http://www.mirdb.org/cgi-bin/target_detail.cgi?targetID=2562474). Subsequently, the wide-type (WT) or mutant-type (MUT) of USP33 containing the potential binding sequence of miR-206 was cloned into the pmirGLO luciferase reporter vector (Promega Corporation, WI, USA), named as site 1-WT/MUT, site2-WT/MUT, respectively. Subsequently, cardiomyocytes were cotransfected with the above WT- or MUT-luciferase reporter vectors with miR-206 mimics, miR-206 inhibitors, mimics NC or inhibitor NC using lipofectamine™ 3000 (Thermo Fisher Scientific). Forty-eight hours after transfection, the cells were harvested, and the luciferase activities were detected by a Luciferase Reporter Gene Assay System (Promega Corporation).

Co-immunoprecipitation (Co-IP)

Pierce™ Co-Immunoprecipitation Kit (Thermo Fisher Scientific) was used to examine the binding relationship between USP33 and JAK2. Cardiomyocytes were transfected with Flag-HA-USP33 (Addgene), or Myc-tagged JAK2 (OriGene Technologies, MD, USA), or cotransfected with Flag-HA-USP33 + Myc-tagged JAK2 plasmids using lipofectamine™ 3000 (Thermo Fisher Scientific), respectively. After 24 h transfection, cells were harvested and lysed by RIPA buffer (Beyotime). Then, the cell lysates were incubated with anti-Flag antibody (MA1-91,878, Thermo Fisher Scientific) for overnight at 4 °C. Then, 10 µl pre-treated protein A agarose beads were added and co-incubated for 4 h. Afterward, the mixed suspension was centrifuged at 3000 rpm for 3 min. Then the precipitated proteins were obtained and determined by western blot analysis. The endogenous and precipitated proteins were detected using anti-Flag (MA1-142-A555, Thermo Fisher Scientific) and anti-Myc antibodies (13–2500, Thermo Fisher Scientific).

In vivo ubiquitination assays

Cardiomyocytes were transfected with the HA-UB (V005498#, NovoPro, Shanghai, China) for 24 h. After indicated treatment, cells were treated with 10 µM MG132 (Sigma) for 2 h. Then the cells were harvested, lysed, and immunoprecipitated with an anti-JAK2 antibody (JAK2-201AP, Thermo Fisher Scientific). The HA antibody (26,183, Thermo Fisher Scientific) was used to examine the ubiquitination level of JAK2. The immunoprecipitated protein was determined by western blot assay.

Statistical analysis

All data were expressed as means ± standard deviation (Mean ± S.D.). All experiments were performed at least three times. The statistical analyses were performed using SPSS 20.0 (SPSS, Chicago, IL, USA). Student t test was used to analyze the difference between two group. One-way ANOVA of variance with post hoc Tukey test analyzed the differences. $P < 0.05$ was considered as statistical significance.

Results

MiR-206 was downregulated, and USP33 was upregulated in LPS-stimulated cardiomyocytes

To explore the viability of cardiomyocytes administrated with different concentrations of LPS (0, 0.1, 1, 2, 5, and 10 µg/ml), CCK-8 assay was carried out. As shown in Fig. 1A, LPS dramatically decreased the cell survival of cardiomyocytes in a dose-dependent manner. Upon the LPS concentration up to 5 µg/ml, the survival rate of cardiomyocytes was about 50%. Therefore, we used this concentration (5 µg/ml) in the subsequent experiments. Further RT-qPCR experiments showed that LPS stimulation induced significant downregulation of miR-206 (Fig. 1B) and remarkable upregulation of USP33 (Fig. 1C). Western blot results also revealed that LPS stimulation dramatically increased USP33 expression (Fig. 1D).

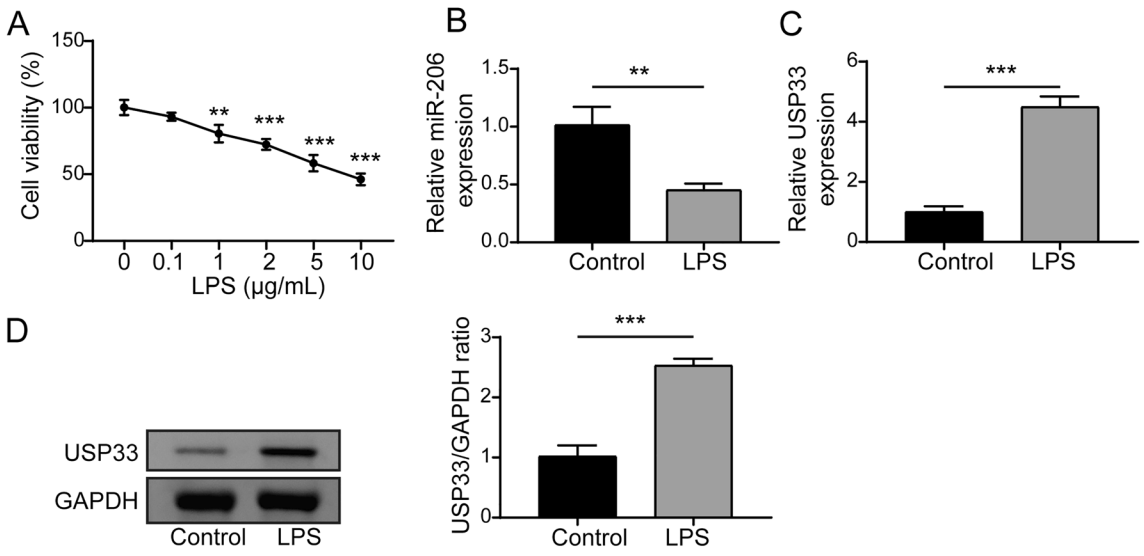

Fig. 1 MiR-206 was downregulated, and USP33 was upregulated in LPS-stimulated cardiomyocytes. Primary mouse cardiomyocytes were treated by LPS (0, 0.1, 1, 2, 5, 10 μg/ml) for 24 h. **A** Cell viability was analyzed by CCK-8 assay. (B-C) The expressions of miR-206 **B** and USP33 **C** were evaluated by RT-qPCR. **D** The protein levels of USP33 were measured by Western blot. Data are representative of three independent experiments. $*P<0.05$, $**P<0.01$, $***P<0.001$

Overexpression of miR-206 alleviated LPS-induced inflammatory injuries in cardiomyocytes

Subsequently, we explored the potential role of miR-206 in LPS-induced cardiomyocyte injuries. RT-qPCR assay showed that miR-206 was greatly decreased after LPS treatment, but miR-206 mimics transfection remarkably restored miR-206 expression (Fig. 2A). CCK8 and EdU assays showed that LPS significantly reduced cell proliferation in cardiomyocytes, while these effects were markedly abolished upon miR-206 overexpression (Fig. 2B and C). Compared with the control group, LPS treatment dramatically increased the levels of cytokines TNF-α, IL-1β, and IL-6, but miR-206 mimics significantly weakened the contents of these cytokines (Fig. 2D). Similarly, LPS stimulation enhanced the apoptosis rate of cardiomyocytes, whereas miR-206 mimics significantly attenuated LPS-induced cell apoptosis compared with mimics NC transfection (Fig. 2E). Western blot results also showed that LPS upregulated apoptosis-related protein Bax, but downregulated anti-apoptosis-related protein Bcl2. However, miR-206 overexpression markedly reversed these protein changes induced by LPS treatment (Fig. 2F). These findings suggested overexpression of miR-206 protected cardiomyocytes from LPS-induced inflammatory injuries.

USP33 was a direct target of miR-206

As shown in Fig. 3A–C, we found that miR-206 mimics highly upregulated miR-206 and downregulated USP33 expression compared to the mimics NC group. However, the miR-206 inhibitor strikingly decreased miR-206 expression but enhanced USP33 expression than the inhibitor NC group. Through the prediction of online software miRDB (http://www.mirdb.org/cgi-bin/target_detail.cgi?targetID=2562474), the potential binding site for miR-206 in USP33 3′-UTR was presented (Fig. 3D). Subsequently, the luciferase reporter assay showed that the cotransfection of miR-206 mimics with WT-USP33 (site1 or site2) exhibited remarkably decreased luciferase activity. In contrast, cells cotransfected with miR-206 inhibitor and WT-USP33 (site1 or site2) exhibited dramatically elevated luciferase activity. Moreover, cardiomyocytes cotransfected with miR-206 mimics or inhibitor with MUT-USP33 (site 1 or site2) showed no noticeable change in luciferase activity (Fig. 3E). Collectively, our results suggested that USP33 was a direct target of miR-206 in LPS-stimulated cardiomyocytes.

MiR-206 overexpression alleviated LPS-induced inflammatory injuries via inhibiting USP33

On the previous basis, we explored whether USP33 could involve in the regulation of miR-206 in LPS-induced inflammatory injuries. We found that miR-206 mimics significantly reduced USP33 levels relative to mimics NC in LPS-stimulated cardiomyocytes, while the inhibitory effects mediated by miR-206 mimics was reversed by USP33 overexpression (Fig. 4A). CCK8 assay showed that compared to the control, miR-206 mimics increased cell viability in LPS-stimulated cardiomyocytes, while this effect was dramatically abolished

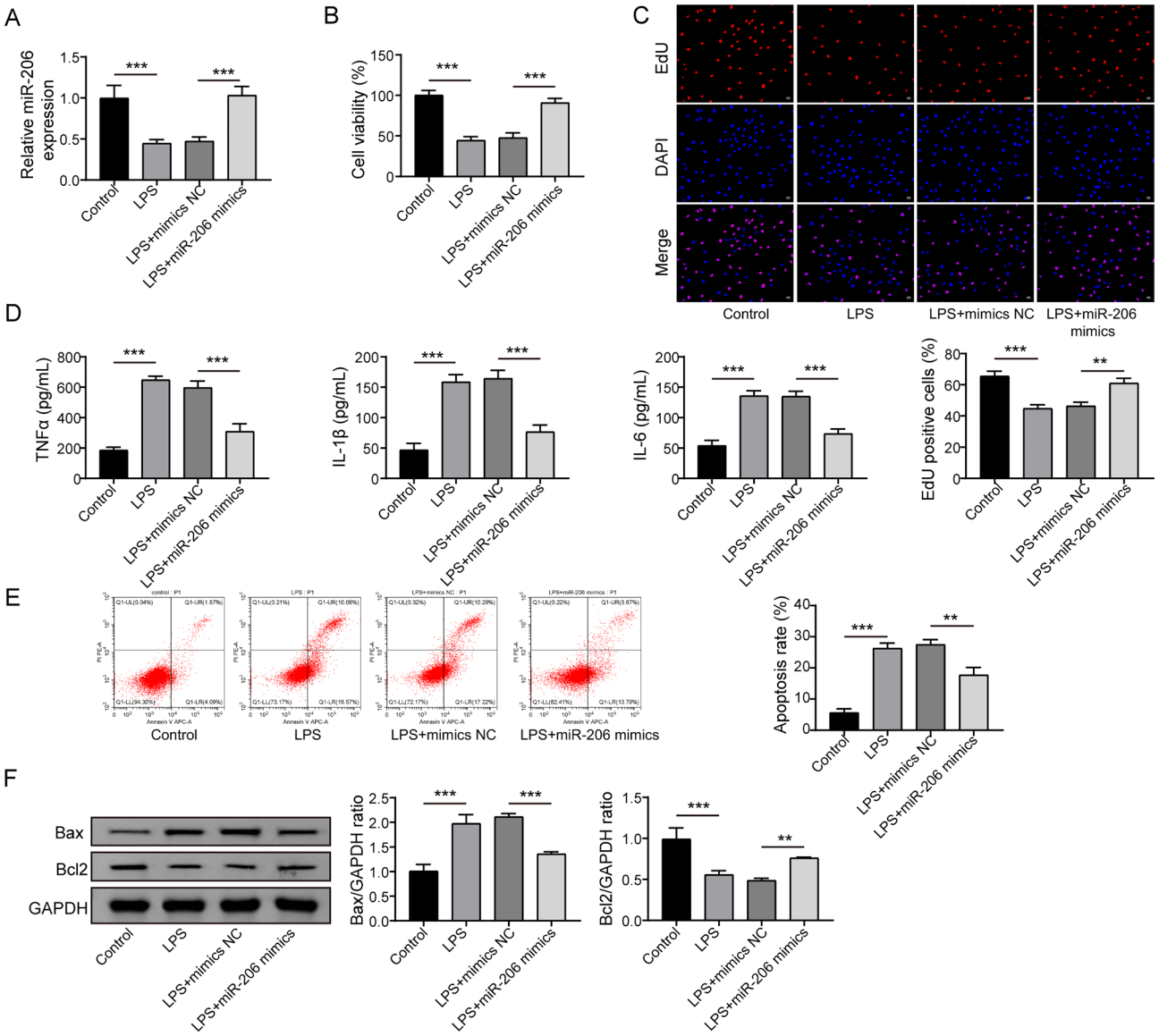

Fig. 2 Overexpression of miR-206 alleviated LPS-induced inflammatory injuries in cardiomyocytes. Primary mouse cardiomyocytes were transfected with mimics NC or miR-206 mimics for 48 h, followed by the treatment of LPS (5 μg/ml) for 24 h. **A** RT-qPCR determined the level of miR-206. (B-C) CCK-8 **B** or EdU incorporation **D** measured the cell viability. **D** ELISA was used to measure the levels of cytokines (TNF-α, IL-1β, IL-6). **E** Cell apoptosis was analyzed by flow cytometry assay. **F** The protein levels of apoptosis-related proteins Bax and Bcl-2 were measured by Western blot assay. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$. Data are representative of three independent experiments

by USP33 overexpression (Fig. 4B). Further ELISA and flow cytometry assays showed that overexpression of miR-206 significantly decreased cytokine levels and cell apoptosis. Notably, the regulatory roles of miR-206 overexpression were remarkably restrained by USP33 overexpression (Fig. 4C, D). Western blot results revealed that miR-206 overexpression decreased Bax level but increased Bcl-2 level compared with the LPS group, while these impacts were markedly reversed in the presence of pcDNA3.1-USP33 (Fig. 4E). Therefore, these findings showed USP33 overexpression reversed the protective role of miR-206 upregulation in LPS-induced cardiomyocytes.

USP33 activated JAK2/STAT3 pathway via deubiquitinating JAK2

To explore the underlying mechanism of USP33 in LPS-induced inflammatory injury, we used an online database to predict the potential protein target of USP33. From the UbiBrowser _V2 data, we found that JAK2 was a potential target for USP33, suggesting that USP33

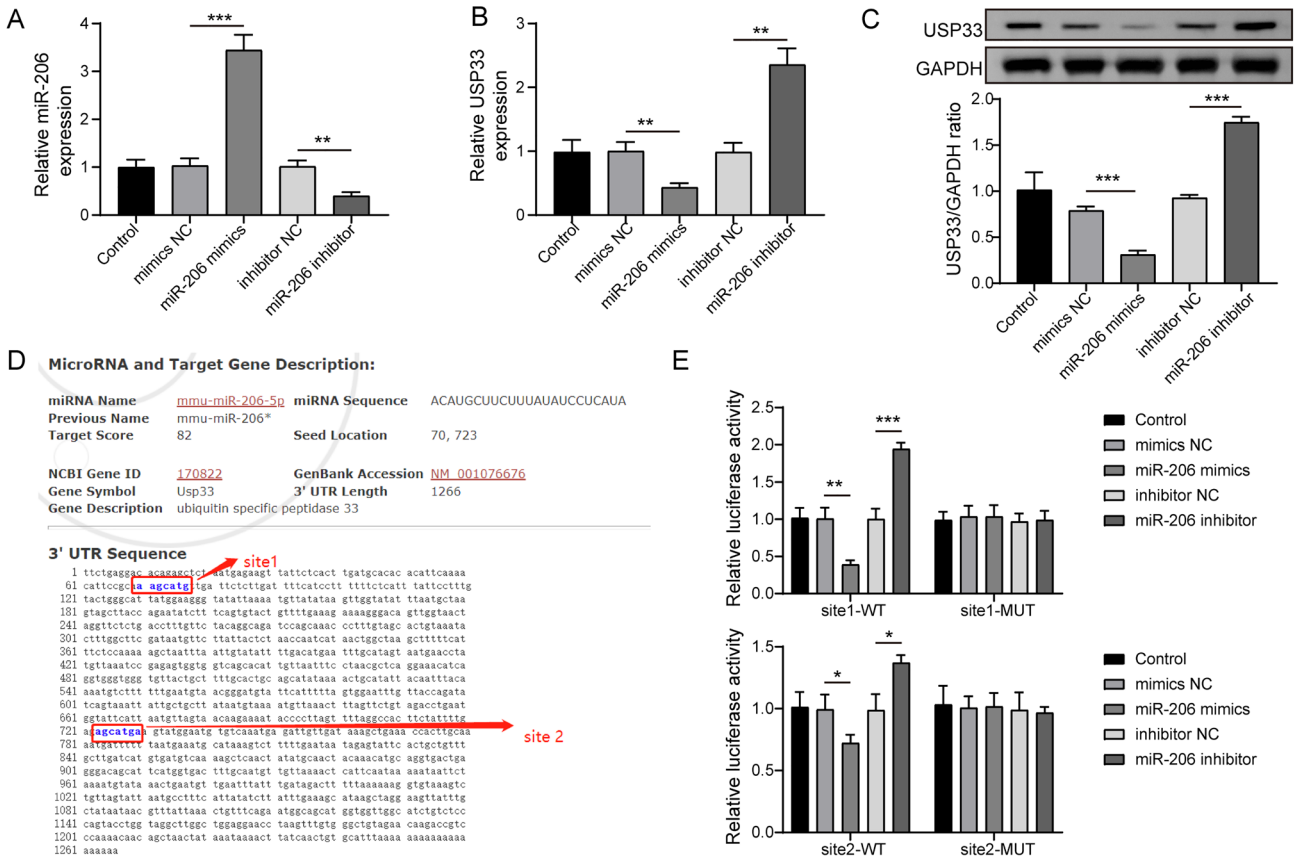

Fig. 3 USP33 was a direct target of miR-206. Primary mouse cardiomyocytes were transfected with mimics NC, miR-206 mimics, inhibitor NC or miR-206 inhibitor, respectively. **A** RT-qPCR measured the expressions of miR-206. **B, C** RT-qPCR and western blot detected the mRNA and protein levels of USP33. **D** miRDB exhibited the potential binding sequences between miR-206 and USP33. **E** Dual luciferase reporter assay evaluated the interactions between miR-206 and USP33. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$. Data are representative of three independent experiments

probably participated in the deubiquitination of JAK2 protein (Fig. 5A). Then, we further determined the interaction between USP33 and JAK2. Co-IP results revealed that Flag-labeled USP33 significantly precipitated JAK2 protein (Fig. 5B). Further in vivo ubiquitination assay verified that USP33 overexpression significantly decreased JAK2 ubiquitination compared with the control group (Fig. 5C). Moreover, western blot results showed that USP33 overexpression increased phosphorylation of STAT3 (p-STAT3) relative to the control group (Fig. 5D). All those results demonstrated that USP33 overexpression activated JAK2/STAT3 signaling pathway through deubiquitinating JAK2.

Activation of JAK2/STAT3 antagonized the biological roles of miR-206 in LPS-induced cardiomyocytes

As shown in Fig. 6A, our results showed that miR-206 overexpression significantly reversed the promoting roles of LPS on the phosphorylation of the JAK2 and STAT3. However, when cells were exposed to coumermycin A1, the expressions of p-JAK2 and p-STAT3 were further upregulated, suggesting Coumermycin A1 reversed miR-206 overexpression-induced inhibition of JAK2/STAT3 (Fig. 6A). CCK-8 assay showed that miR-206 overexpression enormously increased cell viability in LPS-treated cardiomyocyte, and this effect was antagonized after Coumermycin A1 treatment (Fig. 6B). Additionally, ELISA and flow cytometry assay pointed out that miR-206 overexpression significantly inhibited LPS-induced secretions of cytokines (TNF-α, IL-1β, and IL-6) and cell apoptosis, and these roles were further reversed by Coumermycin A1 administration (Fig. 6C, D). Similarly, western blot results revealed that Coumermycin A1 restrained miR-206 mediated the promoting role on Bcl2 expression and the inhibitory role on Bax expression (Fig. 6E). Our results suggested that the activation of the JAK2/STAT3 pathway by Coumermycin A1 significantly attenuated the protective role of miR-206 in LPS-stimulated cardiomyocytes.

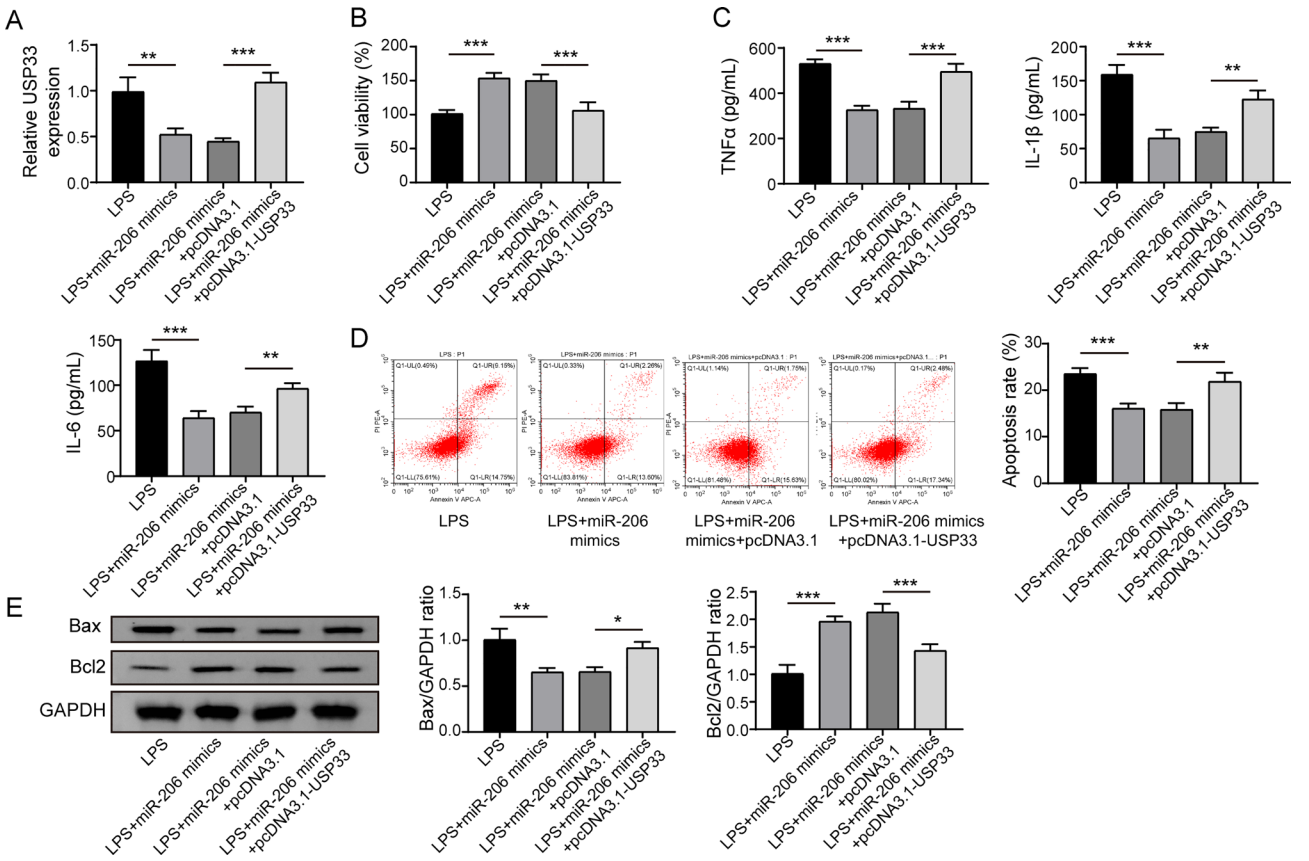

Fig. 4 miR-206 overexpression alleviated LPS-induced inflammatory injuries via inhibiting USP33. Primary mouse cardiomyocytes were transfected with miR-206 mimics, miR-206 mimics + pcDNA3.1 empty vector, or miR-206 mimics + pcDNA3.1-USP33 for 48 h, then followed by the treatment with LPS (5 μg/ml) for 24 h. **A** RT-qPCR was performed to detect the mRNA levels of USP33. **B** CCK8 was used to assess cell viability. **C** ELISA was used to measure the levels of cytokines TNF-α, IL-1β, and IL-6. **D** Flow cytometry was used to estimate cell apoptosis. **E** The protein levels of Bax and Bcl-2 were measured by Western blot. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$. Data are representative of three independent experiments

Discussion

Septic cardiomyopathy (SCM) is a reversible and severe myocardial complication mainly induced by infection-induced imbalanced systematic inflammation. In SCM, cardiomyocytes were drastically injured at functional and structural levels due to the overloading of pro-inflammatory cytokines [20]. Several reports confirmed that miRNAs were involved in the pathogenesis of SCM and might be potential targets for SCM treatment[21, 22]. In this study, we developed LPS-stimulated primary cardiomyocytes as an in vitro sepsis model, and the experimental results showed that miR-206 was significantly downregulated, but USP33 was upregulated in LPS-induced cardiomyocytes. Gain-of-function of miR-206 promoted the proliferation but suppressed the inflammatory responses and apoptosis of LPS-induced cardiomyocytes by targeting USP33 and suppressing the JAK2/STAT3 signaling pathway. These results implied miR-206 might be a new candidate for SCM therapy.

MiR-206 is one of the members of the myomir family, an essential regulator for mitochondrial and muscle function[23]. A previous experiment confirmed that miR-206 could directly target TIMP-3 to alleviate cardiac remodeling after acute myocardial infarction [24]. Yang et al. also showed that miR-206 contributed to cardiomyocyte hypertrophy and survival by regulating YAP [25]. Moreover, Zhang et al.'s also highlighted that a decrease in miR-206 could impair cardiac function and promote oxidative stress by removing the inhibitory role of TLR4[26]. These data implied that miR-206 was most likely a heart-protective factor. In sepsis, Zhou et al. revealed that miR-206 was reduced in CLP septic mouse model, and its overexpression could alleviate inflammatory responses and improve the permeability of the alveolar air-blood barrier in sepsis-induced acute lung injury[27].

Similarly, miR-206 was also proved to suppress NLRP3 inflammasome activation in sepsis-induced acute kidney injury[28]. All the above results further hinted at the biological function of miR-206 in SCM. As expected, our data

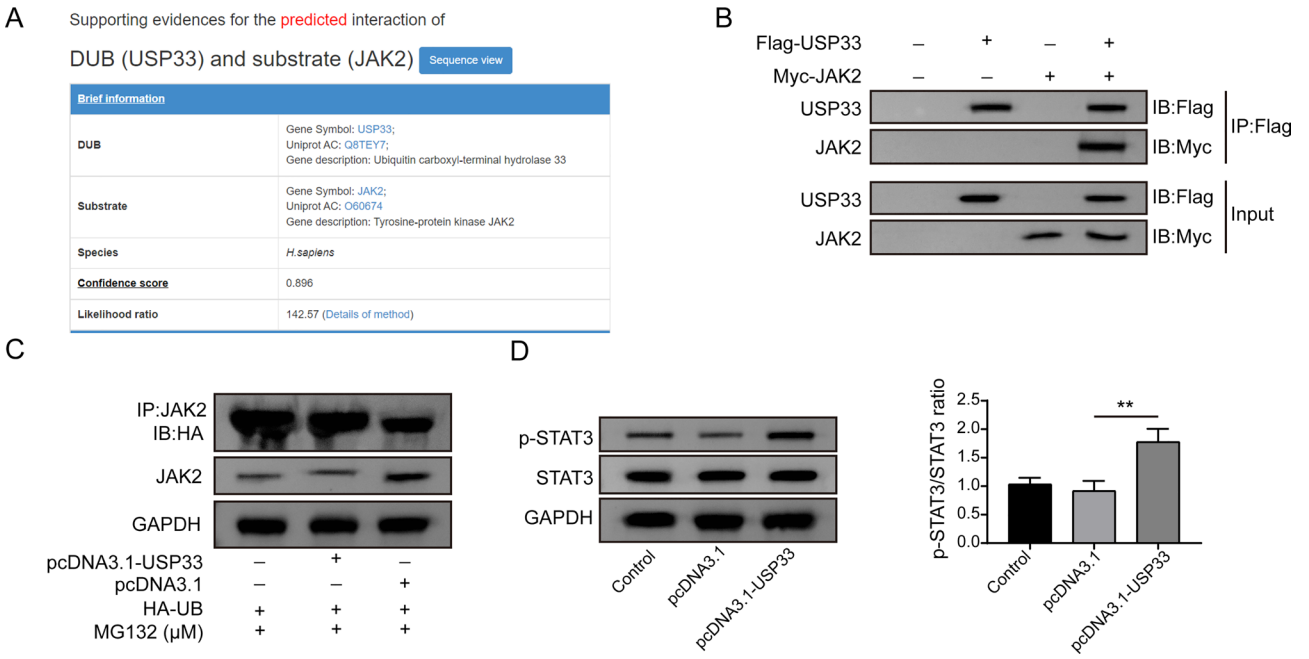

Fig. 5 USP33 activated JAK2/STAT3 pathway via deubiquitinating JAK2. **A** The potential role of USP33 in the ubiquitination of JAK2 was predicted by the online software UbiBrowser 2.0 (http://ubibrowser.ncpsb.org.cn/ubibrowser_v3/home/index). **B** Co-IP assay analyzed the binding of USP33 to JAK2. **C** In vitro ubiquitination experiment evaluated the ubiquitination level of JAK2. **D** The protein levels of total STAT3 and phosphorylated-STAT3 were detected by Western blot. $*P<0.05$, $**P<0.01$, $***P<0.001$. Data are representative of three independent experiments

showed that miR-206 was lowly expressed in a septic cardiomyocytes model in vitro, and gain-of-function experiments exhibited the suppressive roles on LPS-triggered inflammation and apoptosis. All those results showed that miR-206 was protective factor in LPS-induced cellular injuries in cardiomyocytes.

Deubiquitinating enzymes (DUBs) are essential modulators in the ubiquitin–proteasome system (UPS), which can remove ubiquitin (Ub) from the ubiquitinated substrates [29]. Increasing evidence showed that bacteria regulate the host immune response by intervening in the substate proteins' ubiquitination [30]. USP33 is a deubiquitinating enzyme. Previous studies confirmed that USP33 participated in many diseases, such as cancers [16, 31] and neuroinflammation[18, 19]. For instance, USP33 overexpression deubiquitinated ATF3 to control the production of cytokines, such as TNF-α, NF-κB, and IFN-β, etc. [18]. Moreover, previous reports confirmed that USP33 was highly expressed in patients with advanced acute respiratory distress syndrome and was associated with inflammation and fibrosis[32]. From these data, it could be considered that USP33 might be involved in the inflammatory response during sepsis, but this hypothesis still needs to be confirmed. In the current study, our data elucidated for the first time that USP33 was upregulated in a septic cardiomyocytes model in vitro and acted as a direct target of miR-206. More importantly, USP33 upregulation exerted a pro-inflammatory and pro-apoptotic role in cardiomyocytes, which markedly reversed the protective functions of miR-206, implying USP33 was an effector of miR-206 in SCM.

JAK2/STAT3 pathway is a significant signaling controlling inflammatory cytokine secretion. In sepsis, the activated JAK2/STAT3 by cytokines such as IL-6 contributed significantly to cardiomyocyte atrophy [33]. Increasing evidence exhibited that the inhibition of JAK2/STAT3 showed an obvious protective role in sepsis-induced organ injuries [34-36]. Notably, there were several reports pointed out that the expression of JAK2 was closely related to K63-linked poly-ubiquitination [37, 38]. Whereas, the ubiquitination mechanism of JAK2 is not clear in SCM. In the present experiment, we found for the first time that USP33 could directly interact with JAK2 and maintain its protein stability by reducing its ubiquitination modification, thus activating JAK2/STAT3 signaling pathway. In addition, the activation of JAK2/STAT3 by coumermycin A1 remarkably reversed the protective role of miR-206 in LPS-induced inflammatory injuries, suggesting JAK2/STAT3 signaling was a key downstream pathway of miR-206/USP33 in SCM.

In this study, our data revealed that miR-206 alleviated LPS-induced inflammatory injuries by directly targeting USP33 and suppressing the activation of JAK2/STAT3

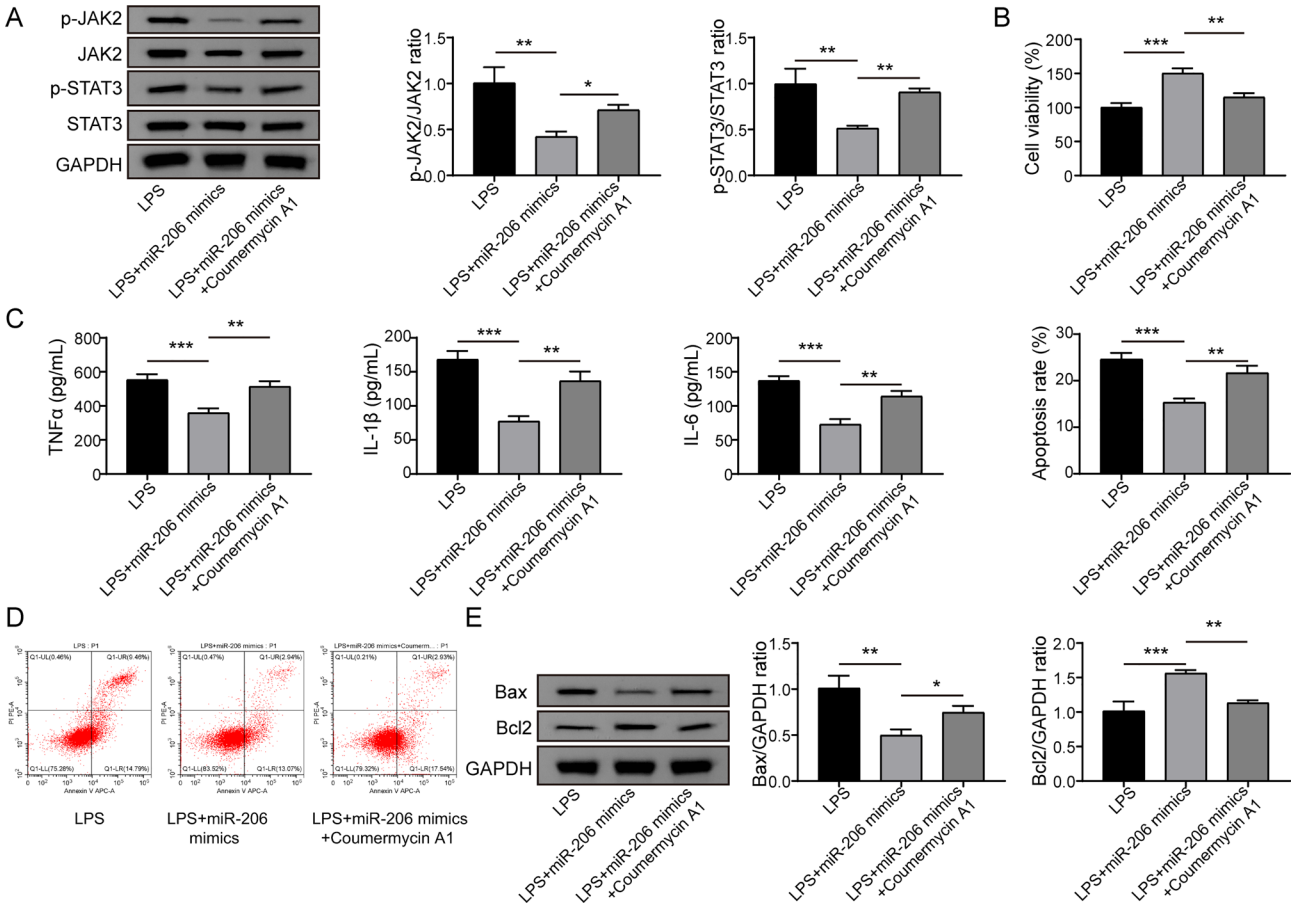

Fig. 6 Activation of JAK2/STAT3 antagonized the biological roles of miR-206 in LPS-induced cardiomyocytes. **A** The expressions of p-JAK2, JAK2, p-STAT3, and STAT3 were measured by Western blot. **B** Cell viability was detected by CCK-8 assay. **C** ELISA measured the protein levels of cytokines TNF-α, IL-1β, and IL-6. **D** Cell apoptosis was analyzed by flow cytometry. **E** The expressions of Bax and Bcl-2 were evaluated by Western blot. $*P<0.05$, $**P<0.01$, $***P<0.001$. Data are representative of three independent experiments

signaling pathway. This is the first time to validate the roles and mechanism of miR-206/USP33/JAK2/STAT3 axis in SCM. SCM is a severe complication of Sepsis that contributes to a high mortality rate. Until now, there are limited effective therapies for SCM, and identifying miR-206 as a potential therapeutic target opens new avenues for treating SCM. In the future, the validation of the miR-206/USP33/JAK2/STAT3 axis in animal models will be crucial for advancing clinical translation.

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Data availability The data underlying this article will be shared on reasonable request to the corresponding author.

Declarations

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval All animal experiments were performed according to the Guidelines for the Care and Use of Laboratory Animals and the protocols approved by Laboratory Animals of the First Affiliated Hospital of Nanchang University Ethics Committee.

Consent for publication All participants were informed and gave written consent.

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