Pretreatment with interleukin-15 attenuates inflammation and apoptosis by inhibiting NF-κB signaling in sepsis-induced myocardial dysfunction

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ABSTRACT

Sepsis-induced myocardial dysfunction (SIMD) is associated with poor prognosis and increased mortality in patients with sepsis. Cytokines are important regulators of both the initiation and progression of sepsis. Interleukin-15 (IL-15), a pro-inflammatory cytokine, has been linked to protective effects against myocardial infarction and myocarditis. However, the role of IL-15 in SIMD remains unclear. We established a mouse model of SIMD *via* cecal ligation puncture (CLP) surgery and a cell model of myocardial injury *via* lipopolysaccharide (LPS) stimulation. IL-15 expression was prominently upregulated in septic hearts as well as cardiomyocytes challenged with LPS. IL-15 pretreatment attenuated cardiac inflammation and cell apoptosis and improved cardiac function in the CLP model. Similar cardioprotective effects of IL-15 pretreatment were observed *in vitro*. As expected, IL-15 knockdown had the opposite effect on LPS-stimulated cardiomyocytes. Mechanistically, we found that IL-15 pretreatment reduced the expression of the pro-apoptotic proteins cleaved caspase-3 and Bax and upregulated the anti-apoptotic protein Bcl-2. RNA sequencing and Western blotting further confirmed that IL-15 pretreatment suppressed the activation of nuclear factor kappa B (NF-κB) signaling in mice with sepsis. Besides, the addition of NF-κB inhibitor can significantly attenuate cardiomyocyte apoptosis compared to the control findings. Our results suggest that IL-15 pretreatment attenuated the cardiac inflammatory responses and reduced cardiomyocyte apoptosis by partially inhibiting NF-κB signaling *in vivo* and *in vitro*, thereby improving cardiac function in mice with sepsis. These findings highlight a promising therapeutic strategy for SIMD.

Key words: IL-15; sepsis-induced myocardial dysfunction; inflammation; apoptosis; NF-κB.

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Introduction

Sepsis is characterized as a deregulated host inflammatory response leading to multiple-organ dysfunction, and it is the main cause of death in intensive care units.[1,2] It has been estimated that sepsis occurs in approximately 31.5 million patients worldwide each year, and 19.4 million patients develop severe sepsis, resulting in up to 5.3 million deaths per year.[3] Sepsis-induced myocardial dysfunction (SIMD), which occurs in approximately 40-60% of patients diagnosed with sepsis, results in a greater financial burden and increased mortality than in patients with sepsis but without cardiac dysfunction (70-90% *vs* 20%).[4] Although several therapeutic strategies are available for this condition, including wide-spectrum antibiotic treatment and fluid resuscitation, there is no specific therapy for this lethal disease because the molecular mechanism of SIMD pathogenesis has not been completely elucidated.[5] Interleukin-15 (IL-15) is a pleiotropic cytokine belonging to the IL-2 family that plays a crucial role in the development of inflammatory reactions, immune responses, and cell apoptosis.[6,7] It is widely distributed in various tissues such as muscle, the heart, bone marrow, and immune cells.[8] IL-15 performs multiple immunoregulatory functions, including regulating tissue repair and modulating inflammation, and it is essential for the proliferation and maintenance of NK and T cells.[9,10] Accumulating evidence indicates that IL-15 slows the progression of cardiovascular diseases in animal experiments.[11-13] One recent study reported that IL-15 supplementation reduces cell death, promotes vascularity, and improves heart function after myocardial infarction in a mouse model.[11] Similar results were recorded in a viral myocarditis model, in which IL-15 exerted a positive effect on the clinical course and improved cardiac function and survival rates in mice.[12] In addition, IL-15 is upregulated in both human and animal atherosclerotic lesions, and it might promote the recruitment of T cells during atherogenesis.[14] However, the role of IL-15 in SIMD remains obscure. Therefore, it is reasonable to hypothesize that IL-15 exerts a protective effect on cardiac function in sepsis and prevents the deterioration of this life-threatening disease. In the present study, we established a sepsis model *via* cecal ligation puncture (CLP) surgery and an inflammatory cell model *via* lipopolysaccharide (LPS) treatment to investigate changes in inflammation, cardiac function, and cell apoptosis *in vivo* and *in vitro*. Our results demonstrated that IL-15 plays a cardioprotective role in SIMD by ameliorating myocardial dysfunction and cardiomyocyte apoptosis through the nuclear factor kappa B (NF-κB) signaling pathway.

Materials and Methods

Animals

Male C57BL/6 mice (Experimental Animal Center of Hangzhou Medical College, Zhejiang, China) aged 8-10 weeks and weighing 18-22 g were housed in a temperature- and humidity-controlled environment with a natural light-dark cycle and free access to food and water. The animal experiments were conducted according to protocols approved by the Animal Ethics Committee of the Second Affiliated Hospital of Zhejiang University School of Medicine (2022-LY-470).

CLP model and treatment

The mice were randomly divided into four groups: sham group, sham+IL-15 group, CLP group, and CLP+IL-15 group. CLP surgery was performed as described in previous studies.[15] Briefly, mice were anesthetized with sodium pentobarbital (50 mg/kg) *via* an intraperitoneal injection after fasting for 8 h. A small midline incision (1-2 cm) was made on the anterior abdomen to expose the cecum and ligated with 4-0 silk approximately in the middle of the cecum. An 18G needle was then used to puncture the ligated part of the cecum, and a small amount of fecal content was extruded gently through the puncture site. Afterward, the intestines were returned to the abdominal cavity, and the incision was sutured in two layers. Each animal received a subcutaneous injection of 100 μL of normal saline to compensate for fluid loss after the operation. Sham-operated mice were subjected to a similar procedure without cecal ligation and puncture. For the intervention groups, mice received daily injections of IL-15 (250 ng, intraperitoneally) for 3 days prior to CLP surgery. The dosage of IL-15 was based on the report by Bigalke *et al*., who found that this dose could confer a positive effect on the clinical course of coxsackievirus B3-induced murine myocarditis.[12]

Cardiomyocyte isolation, culture, and treatment

Primary cardiomyocytes were isolated from neonatal mice and cultured as previously described. Briefly, the hearts of 1-3-day-old neonatal mice were digested with 0.125% trypsin and 0.05% collagenase type I (Thermo Fisher Scientific Inc., Waltham, MA, USA) solution.[16] After centrifugation and resuspension, cardiomyocytes were cultured in DMEM containing 10% fetal bovine serum and 1% penicillin-streptomycin in a 5% CO_2, 37°C humidified atmosphere, and 0.1 mM BrdU (B9285; Sigma-Aldrich, St. Louis, MO, USA) was added to inhibit the proliferation of fibroblasts.

Serum-free DMEM was used to pretreat cardiomyocytes overnight before the intervention. Primary cardiomyocytes were seeded in a six-well plate and transfected with IL-15-specific small interfering RNA (siRNA) or scrambled siRNA using Lipofectamine RNAiMax (13778075; Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. After 24 h of transfection, the knockdown efficiency was examined by Western blotting. IL-15–specific siRNAs were synthesized by Tsingke Biotech (Beijing, China). The sequences of the siRNAs are presented in Table 1. Cardiomyocytes were pretreated with IL-15 siRNA or IL-15 (50 ng/mL) for 24 h, followed by LPS (1 μg/mL) stimulation for 12 h to mimic the process of sepsis-induced myocardial damage *in vitro*.[17] Thus, seven groups of cells were created as follows: PBS group, LPS+PBS group, LPS+IL-15 group, PBS+scrambled siRNA (si-NC) group, PBS+si-IL15 group, LPS+si-NC group, and LPS+si-IL15 group. We also used NF-κB inhibitor SN50 (18 μM) in IL-15 silenced cardiomyocytes to confirm the effect of IL-15 is partly attributable to the attenuation of cardiomyocyte apoptosis *via* the NF-κB signaling pathway, four groups of cells were created as follows: LPS+si-NC group, LPS+si-IL15 group, LPS+si-NC+SN50 group, and LPS+si-IL15+SN50 group.

Table 1. Sequences of siRNA.

siRNA name	Sequence (5' to 3')
siIL-15 1 sense	GCAAAGUUACUGCAAUGAA
siIL-15 1 antisense	UUCAUUGCAGUAACUUUGC
siIL-15 2 sense	GCACUCUGUCUUCUAACAA
siIL-15 2 antisense	UUGUUAGAAGACAGAGUGC
siIL-15 3 sense	CAGUGUAGGUCUCCCUAAA
siIL-15 3 antisense	UUUAGGGAGACCUACACUG
Scramble sense	GCAUCAUAGCACAUCUATT
Scramble antisense	UUGACCUGUGCUAAUGCTT

Echocardiography

Transthoracic echocardiography was performed 24 h after CLP surgery. The mice were anesthetized *via* inhalation of 1-2% isoflurane, followed by echocardiography using a Vevo 2100 ultrasound imaging system (VisualSonics, Toronto, Canada). Subsequently, the left ventricular end-systolic diameter (LVEDs) and left ventricular end-diastolic diameter (LVEDd) were measured on the parasternal long axis and short axis views. The ejection fraction (EF) and fractional shortening (FS) were calculated on the basis of LVEDs and LVEDd.

Histology and immunohistochemistry

Heart tissues were harvested from mice, fixed in 10% phosphate-buffered formalin, and then embedded in paraffin. Tissue sections of the myocardium were sectioned (4 μm) and stained with hematoxylin and eosin (H&E) to analyze histological features in the myocardium. Immunohistochemistry was performed with an IL-15 rabbit antibody (1:200, ab273625; Abcam, Carlsbad, CA, USA) to assess the expression of IL-15. In brief, the tissue slices were incubated with 3% H_2O_2 for 10 min and treated with 10% donkey serum for 30 min at room temperature, following by incubation with relative antibodies (1:200, ab97046; Abcam) overnight at 4°C. The following day, the tissue was managed with the corresponding secondary antibody for 60 min. Negative controls were performed by omitting the primary antibody and replacing it with PBS. Morphological changes of cells were observed by optical microscopy (Leica, Wetzlar, Germany) at ×200 magnification.

Western blotting

Heart tissues homogenates and cardiomyocytes were solubilized in RIPA lysis buffer (Beyotime Biotechnology, Haimen, China) containing protease inhibitors for 30 min on ice. Subsequently, the protein concentration was quantified using the BCA Protein Assay kit (Thermo Fisher Scientific), and equal amounts of proteins (50 μg) were separated by SDS-PAGE and transferred to polyvinylidene fluoride membranes. After blocking with 5% nonfat milk in PBST for 1 h, membranes were incubated overnight at 4°C with primary antibodies, including IL-15 monoclonal antibody, IL-15 receptor alpha (IL-15Rα) antibody (1:1000, PA5-114215; Thermo Fisher Scientific), cleaved caspase-3 antibody (1:1000, ab184787; Abcam), Bax antibody (1:1000, ab182733; Abcam), Bcl-2 antibody (1:2000, AMAB91492, Sigma-Aldrich), phosphorylated-p65 (p-p65) antibody (1:1000, SAB4502610; Sigma-Aldrich), phosphorylated inhibitor of kappa B alpha (p-IκBα) antibody (1:500, SAB5700926; Sigma-Aldrich), GAPDH (1:2000, 60004-1-Ig; Proteintech, Rosemont, IL, USA), and tubulin antibody (1:2000, 11224-1-AP; Proteintech). Finally, blots were incubated with the appropriate goat anti-rabbit (1:1000, ab205718; Abcam) or goat anti-mouse (1:1000, ab205719; Abcam) secondary antibodies for 60 min at room temperature for target probing. The immunoblots were subjected to enhanced chemiluminescence, and chemiluminescence signals were visualized using Image Quant 800 Software (GE HealthCare, Chicago, IL, USA).

RNA extraction and RT-PCR

Total RNA was extracted using TRIzol reagent (15596026CN; Invitrogen), and then 2 μg of RNA were reverse-transcribed into cDNA using PrimeScript RT Master Mix kits (TaKaRa Biotechnology Co., Ltd. Kusatsu, Japan). RT-PCR was amplified using TB Green Premix Ex Taq kits (TaKaRa Biotechnology Co., Ltd.) on a LightCycler 480 machine (Roche, Basel, Switzerland), and the expression was normalized to that of GAPDH. All primers were designed by Primer Premier 5.0 software (Premier, San Francisco, USA), and the sequences are listed in Table 2.

Immunofluorescence analysis

Hearts were isolated from mice, and frozen sections were sliced (7 μm). The samples were incubated with primary antibodies against IL-15 (1:200), troponin T (Tn-T, 1:200, ab193546; Abcam), vimentin (1:200, 60330-1-Ig; Proteintech), CD31 (1:200, 550274, BD Biosciences, Franklin Lakes, NJ, USA), and CD68 (1:200, MCA1957GA; Bio-Rad Lab Inc., Hercules, CA, USA) overnight at 4°C. Cy3-conjugated goat anti-rabbit IgG (1:500; Invitrogen) or FITC-conjugated goat anti-mouse IgG (1:300; Invitrogen) were applied as secondary antibodies. Omission of the primary antibody was used as a negative control. DAPI staining was used to identify nuclei (D9542; Sigma-Aldrich). All slides were photographed under a fluorescence microscope (Leica Microsystems, Inc., Buffalo Grove, IL, USA).

Table 2. Primers of quantitative polymerase chain reaction.

Gene	Species	Sequence (5' to 3')
IL-15	Mouse	F: CAGCAGATAACCAGCCTACAG R: GCCCAGGTAAGAGCTTCAAT
IL-15 Rα	Mouse	F: ACAGAGCACGGACAGTCAAG R: TCTGGCTCTTTTGCAGAGGG
Cleaved caspase-3	Mouse	F: CCGACTTCCTGTATGCTTACTCTA R: ATGTGCATGAATTCCAGCTTGT
Bax	Mouse	F: GAGCACATCATGAAGACAGGGG R: ATTCGCTTGAGACACTCGCC
Bcl-2	Mouse	F: CTGGTGGACAACATCGCTCT R: GCATGCTGGGGCCATATAGT
P-p65	Mouse	F: ACTATGGATTTCCTGCTTACGG R: GCACAATCTCTAGGCTCGTT
P-IκBα	Mouse	F: TACGCCCCAGCATCTCCACTCCG R: CTCCACGATGCCCAGGTAGCCAT
GAPDH	Mouse	F: GTCAAGCTCATTTCCTGGTAT R: TCTCTTGCTCAGTGTCCTTGC
β-actin	Mouse	F: TGTGATGGTGGGAATGGGTCAGAA R: TGTGGTGCCAGATCTTCTCCATGT

TUNEL assay

TUNEL staining was performed to calculate the apoptosis index both *in vivo* and *in vitro* using *in situ* cell death detection kits (12156792910; Roche) following the manufacturer's instructions. Tissues or cells were fixed and permeated, followed by the addition of 50 μL of TUNEL reaction mixture to each sample for 1 h at 37°C in the dark. Then, the samples were stained with DAPI for 5 min and finally captured using a Leica confocal microscope system (Leica SP5 Confocal Microscope, Leica). The quantification of TUNEL-positive cells was performed using ImageJ software (US National Institutes of Health, Bethesda, MD, USA).

ELISA

The serum levels of creatine kinase-MB (CK-MB, ab285231; Abcam) and pro-inflammatory cytokines, including IL-6 (ab222503; Abcam) and tumor necrosis factor-alpha (TNF-α, EPX01A-20607-901; Invitrogen) were assayed using commercial ELISA kits adhering to the manufacturer's instructions.

Flow cytometric detection of cell apoptosis

Apoptosis was analyzed by flow cytometry using annexin V-FITC/propidium iodide (PI) apoptosis detection kits (C1062M, Beyotime, Shanghai, China). The cells received different interventions according to the experimental design, and they were stimulated with LPS for 12 h. Then, the cells were harvested, resuspendedin binding buffer (200 μL), and then labeled with annexin V (5 μL) and PI (10 μL) for 5 min in the dark. The apoptotic cells were analyzed using CytoFLEX LX (Beckman Coulter, Brea, CA, USA).

RNA sequencing

Total RNA was isolated with TRIzol reagent following the

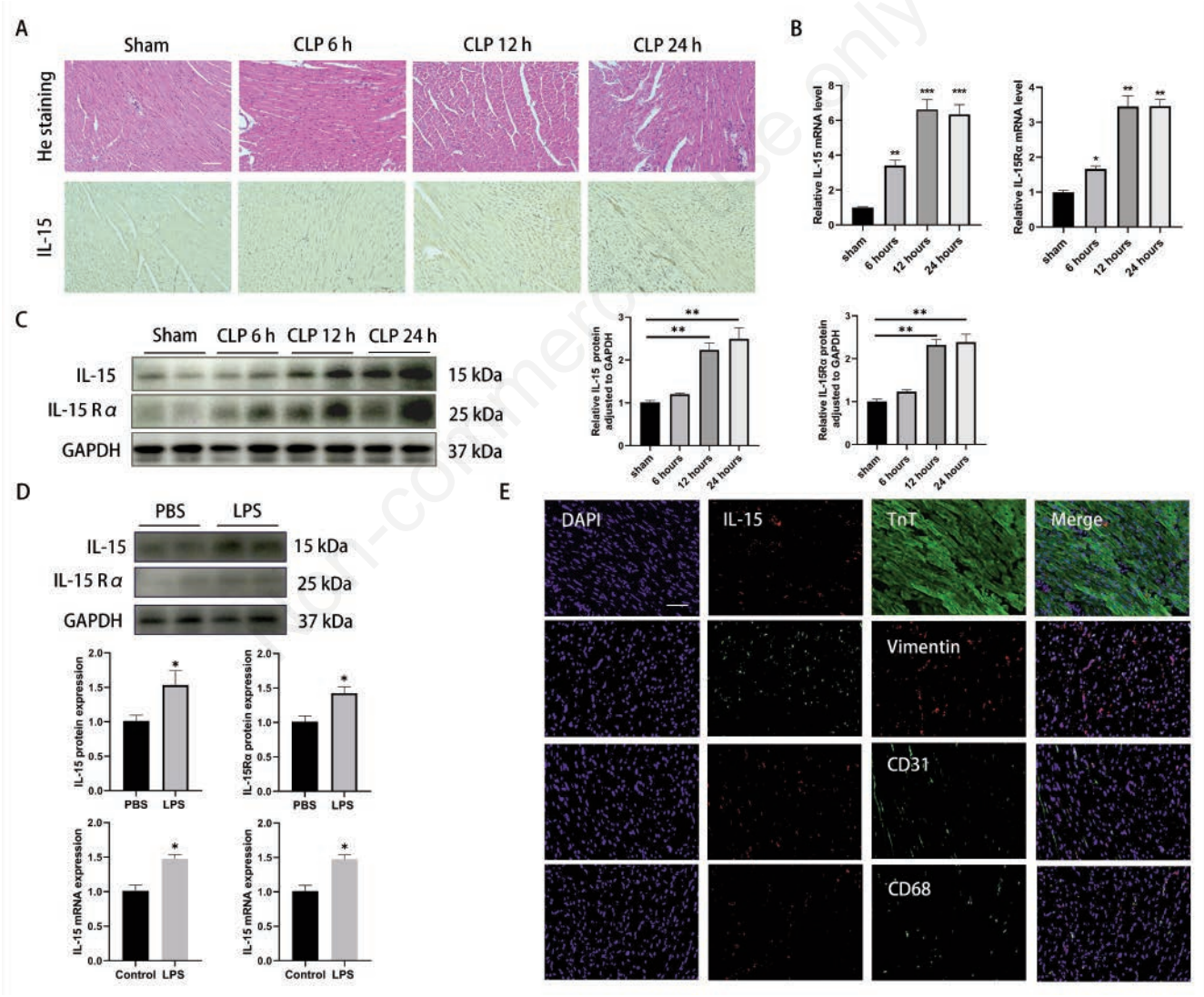

Figure 1. IL-15 upregulated in septic hearts and cardiomyocytes. **A**) Representative HE staining and immunohistochemistry of septic and control hearts at 6 h, 12 h and 24 h after CLP surgery (n=6); scale bar: 50 μm. **B,C**) IL-15 and IL-15Rα expression at 6 h, 12 h and 24 h after CLP surgery detected by Western blotting and RT-PCR (n=6). **D**) IL-15 and IL-15Rα expression in cardiomyocytes with or without LPS stimulation detected by Western blotting and RT-PCR (n=3). **E**) Immunofluorescence labeling of IL-15, Tn-T, vimentin, CD31, and CD68 in septic hearts at 24 h; scale bar: 50 μm. *p<0.05; **p<0.01; ***p<0.001.

manufacturer's procedure, and the samples were sent to Hangzhou LC-Bio Technology Corporation (Hangzhou, China) for RNA-Seq analysis. The total RNA quantity and purity were measured at 260/280 using an RNA 6000 Nano LabChip Kit (Agilent, Santa Clara, CA, USA) and evaluated using a 2100 Bioanalyzer (Agilent). Differentially expressed genes (DEGs), as indicated by false discovery rate <0.05 and greater than two-fold changes in expression, were identified between different samples using DESeq2 software (https://www.bioconductor.org/) and then subjected to Gene Ontology and Kyoto Encyclopedia of Genes and Genome (KEGG) analyses.

Statistical analysis

All statistical analyses were conducted using GraphPad Prism 8.0 (GraphPad, San Diego, CA, USA). Data are presented as the mean ± SD. If variables passed the tests for normality and equality of variances, we further evaluated differences between two groups using Student's t-test, and for comparisons of three or more groups, we employed one-way ANOVA followed by Tukey's *post-hoc* test. A *p*-value <0.05 was considered statistically significant.

Results

IL-15 was upregulated in septic hearts and cardiomyocytes

To explore the protein expression of IL-15, mouse hearts were harvested 24 h after CLP surgery. H&E staining showed obvious edema, disordered cell arrangement, and inflammatory infiltration in septic hearts, and immunohistochemistry revealed a gradual elevation of IL-15 expression after the CLP procedure (Figure 1A). Similarly, the protein and mRNA expression of IL-15 and IL-15Rα was significantly upregulated in the hearts of mice with sepsis compared to that in healthy control mice (Figure 1 B,C). In addition, we further measured IL-15 expression in neonatal mouse cardiomyocytes stimulated with LPS by Western blotting and RT-PCR. Consistent with our *in vivo* findings, IL-15 expression apparently increased in cardiomyocytes after LPS treatment compared to the control level (Figure 1D). Immunofluorescence staining indicated that IL-15 was mainly localized in cardiomyocytes, whereas it displayed weak co-staining with fibroblasts, endothelial cells, and macrophages (Figure 1E).

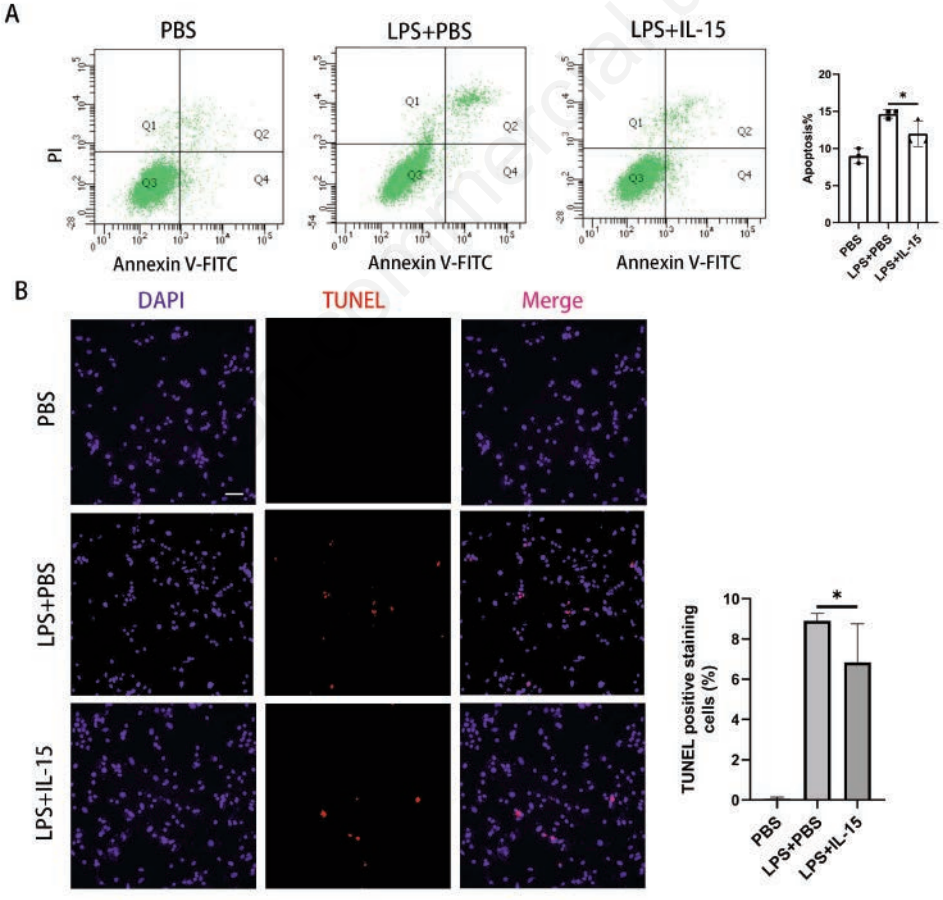

Figure 2. IL-15 reduced LPS-induced cardiomyocyte apoptosis. **A**) Cardiomyocyte apoptosis rate after IL-15 pretreatment and LPS stimulation measured by flow cytometry (n=3). **B**) TUNEL staining revealing apoptosis in cardiomyocytes after IL-15 pretreatment and LPS stimulation (n=3), scale bar: 50 μm. *p<0.05.

IL-15 reduced LPS-induced cardiomyocyte apoptosis

Because the alteration in IL-15 expression in septic hearts was mainly observed in cardiomyocytes, we further examined whether IL-15 pretreatment modulated the fate of cardiomyocytes during LPS stimulation. Primary cardiomyocytes from neonatal mice were isolated and challenged with LPS, and we found that IL-15 pretreatment could attenuate LPS-induced cellular apoptosis (Figure 2A). In addition, TUNEL staining was employed to assess the effect of IL-15 on apoptosis in cardiomyocytes. Consistent with the results of flow cytometry, IL-15 pretreatment triggered a remarkable decrease in cardiomyocyte apoptosis compared to that in the control group (Figure 2B).

IL-15 attenuated inflammation and apoptosis and improved cardiac function in mice

The upregulation of TNF-α expression induced by sepsis in mice was partially alleviated by IL-15 pretreatment, whereas IL-6 expression did not differ between the groups. In addition, CK-MB expression was significantly lower in the treatment group than in the control group (Figure 3A).

Inflammation induced by CLP resulted in the deterioration in cardiac dysfunction and cardiomyocyte apoptosis; therefore, we investigated the effect of IL-15 on CLP-induced cardiac dysfunction. IL-15 pretreatment significantly improved EF and FS compared to those in the control group (Figure 3 B,C). As expected, IL-15 pretreatment also suppressed CLP-induced cellular apoptosis (Figure 3D). We further detected the protein expression of apoptotic markers in septic hearts. In the CLP group, the expression of pro-apoptotic proteins, including cleaved caspase-3 and Bax, was significantly increased, whereas the anti-apoptotic protein Bcl-2 was downregulated compared to its expression in the sham group. Meanwhile, IL-15 pretreatment had opposite effects on these alterations (Figure 3E).

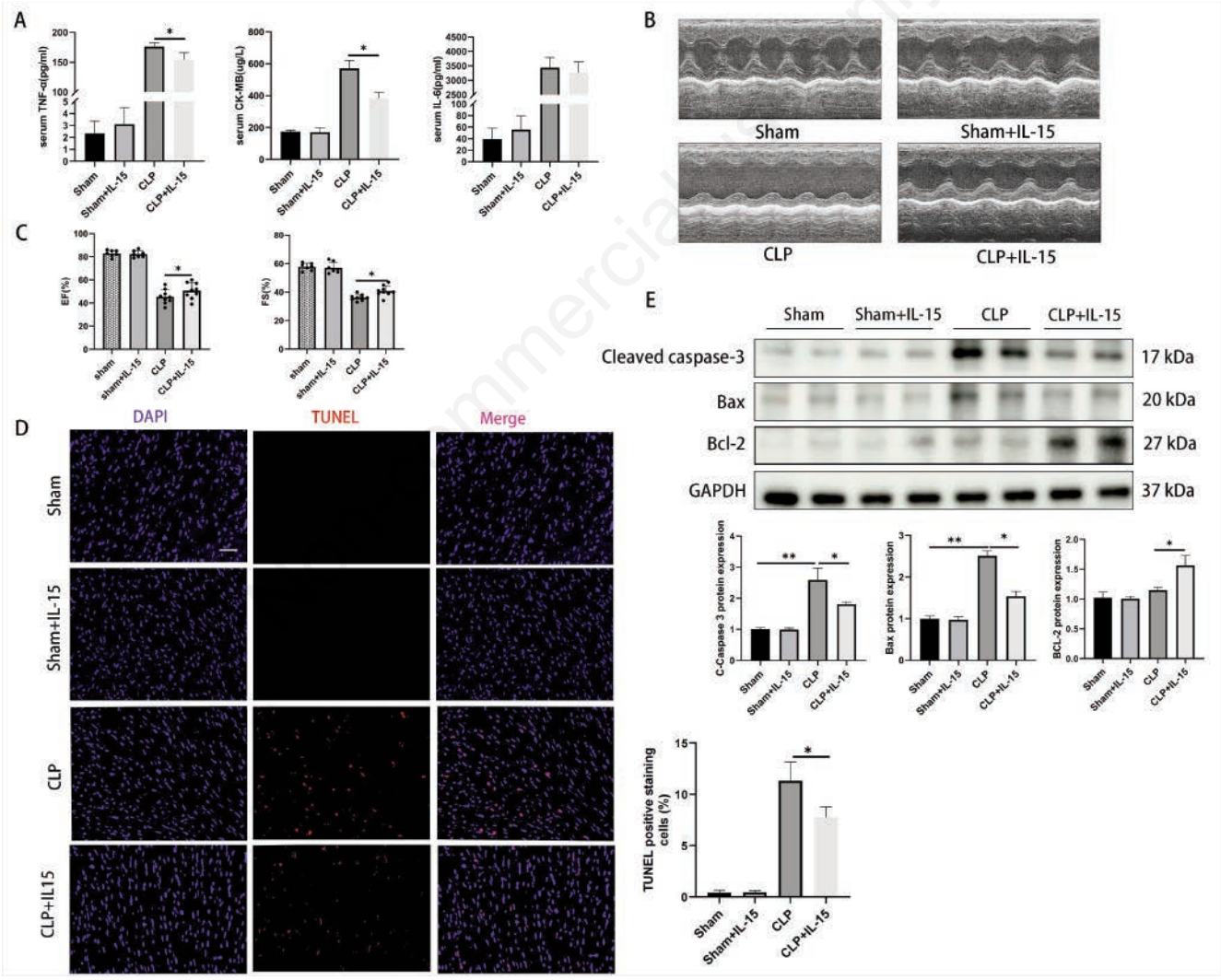

Figure 3. IL-15 attenuated inflammation and apoptosis and improved cardiac function in mice. **A**) ELISA used to detect the concentrations of TNF-α, IL-6, and CK-MB (n=3). **B,C**) Cardiac function measured by M-mode echocardiography (n = 8). (**D**) Cellular apoptosis in heart tissue after IL-15 pretreatment and CLP surgery as detected by TUNEL staining (n=6); scale bar: 50 μm. **E**) Western blotting of apoptosis-related proteins, including cleaved caspase-3, Bax, and Bcl-2 (n = 3). *p<0.05; **p<0.01.

IL-15 knockdown aggravated cardiomyocyte apoptosis

Given that the administration of IL-15 can attenuate sepsis-induced inflammation, apoptosis, and cardiac dysfunction, we hypothesized that IL-15 knockdown would aggravate LPS-induced apoptosis. Cardiomyocytes were transfected with siRNA to down-regulate IL-15 expression in cardiomyocytes. Western blotting and RT-PCR confirmed that both siIL-15 2 and siIL-15 3 effectively decreased IL-15 protein expression (Figure 4A).

Then, cardiomyocytes were incubated with 1 μg/mL LPS, and apoptosis rates were subsequently assessed by flow cytometry and TUNEL staining. As shown in Figure 4B, IL-15 knockdown exacerbated cell apoptosis compared to the control findings. Moreover, the number of TUNEL-positive cells was significantly increased following IL-15 knockdown, as expected (Figure 4C). Additionally, consistent with findings in the CLP mouse model, the expression of cleaved caspase-3 and Bax was significantly increased, and that of Bcl-2 was decreased in cardiomyocytes subjected to IL-15 inhibition and LPS stimulation (Figure 4 D,E).

IL-15 reduced cardiomyocyte apoptosis by inhibiting the NF-κB signaling pathway

To investigate the potential mechanism of the effects of IL-15 in sepsis, we conducted comprehensive RNA-Seq to identify DEGs in cardiomyocytes after IL-15 knockdown and LPS challenge. DEGseq was applied to identify DEGs and clustered according to their expression profiles. As presented in Figure 5A, the volcano map revealed that 679 DEGs were identified, including 368 upregulated and 311 downregulated genes. KEGG pathway enrichment analysis was performed, revealing that cell apoptosis and NF-κB signaling pathways were enriched in the LPS+si-IL15 group (Figure 5B). Heatmap of apoptotic-related gene expression levels between two groups are shown in Figure 5C. In summary, RNA-seq data indicated that IL-15 knockdown promoted cardiomyocyte apoptosis by inhibiting the NF-κB signaling pathway.

To verify the possible molecular mechanisms of IL-15 in our sepsis model suggested by RNA-Seq, we assessed components of the NF-κB signaling pathway, including p-p65 and p-IκBα, by Western blotting and RT-PCR. The results showed that the expres-

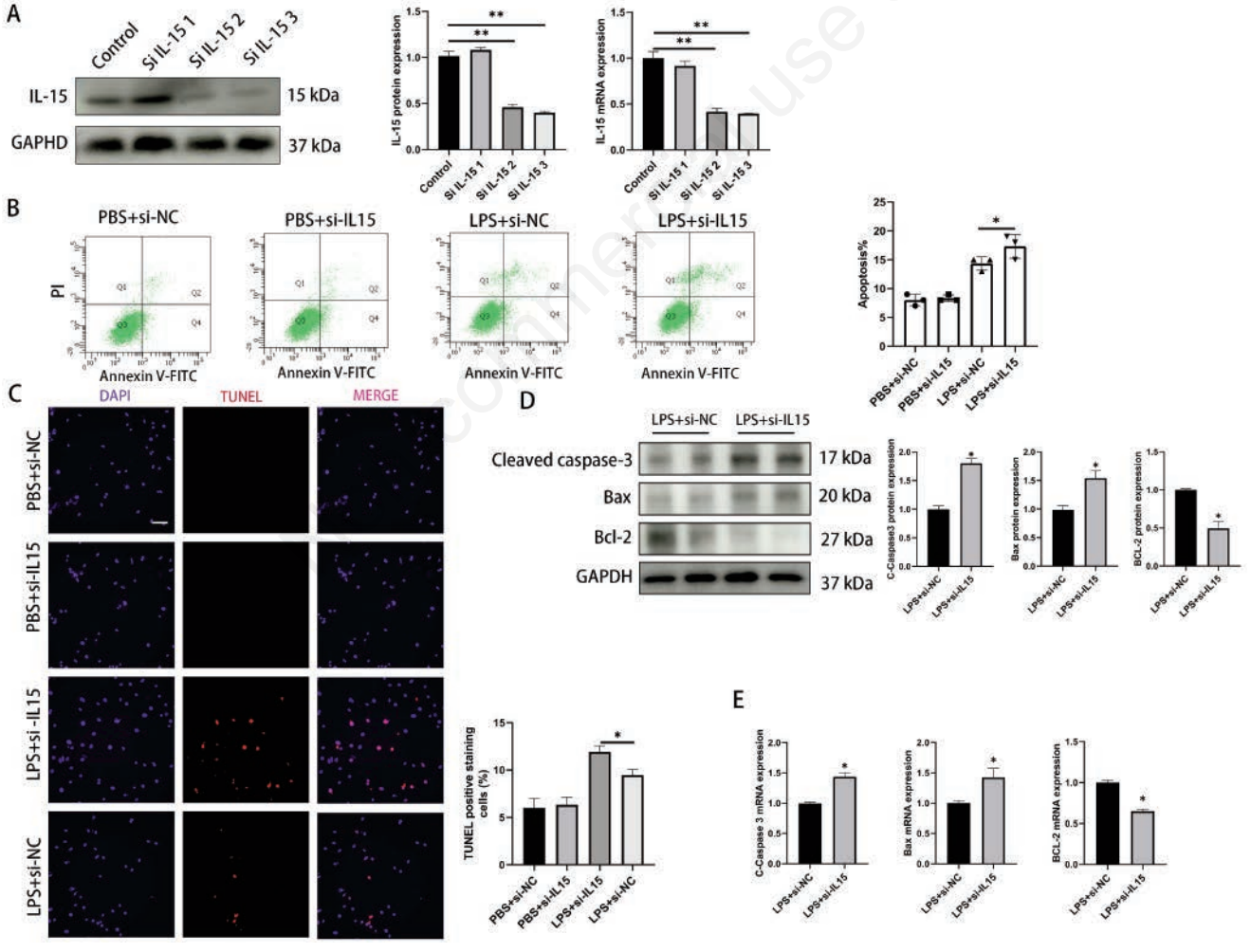

Figure 4. IL-15 knockdown aggravated cardiomyocyte apoptosis. **A**) Western blotting and RT-PCR measurements of IL-15 expression after siRNA knockdown (n=3). **B**) Cardiomyocyte apoptosis rate after IL-15 knockdown and LPS stimulation as by flow cytometry (n=3). **C**) TUNEL staining revealing apoptosis in cardiomyocytes after IL-15 knockdown (n=3); scale bar: 50 μm. **D,E**) Western blotting and RT-PCR of apoptosis-related proteins, including cleaved caspase-3, Bax, and Bcl-2 (n=3). *p<0.05; **p<0.01.

sion of p-p65 and p-IκBα was decreased in mice treated with IL-15 before CLP surgery (Figure 6A). Besides, the expression of p-p65 and p-IκBα was increased on LPS treatment in cardiomyocytes. This downregulation was antagonized when cardiomyocytes were transfected with IL-15 siRNA and stimulated with LPS (Figure 6 B,C). Taken together, our findings suggested that the NF-κB signaling pathway is involved in the regulation of cardiomyocyte apoptosis in SIMD.

To confirm the effect of IL-15 is partly attributable to the attenuation of cardiomyocyte apoptosis *via* the NF-κB signaling pathway, we used NF-κB inhibitor SN50 in IL-15 silenced cells and detected the apoptosis levels. As shown in Figure 7 A-D, the addition of NF-κB inhibitor can significantly attenuate cardiomyocyte apoptosis compared to the control findings as expected.

Discussion

Sepsis is a fatal condition often accompanied by cardiac dysfunction. In the present study, we established a CLP-induced sepsis mice model and an LPS-induced cell model to observe cardiac function, inflammatory responses, and cardiomyocyte apoptosis *in vivo* and *in vitro*. The results illustrated that IL-15 protein expression was prominently increased in septic hearts and inflammatory cardiomyocytes. Furthermore, exogenous supplementation with recombinant IL-15 could alleviate inflammatory responses and improve cardiac function in septic mice. We confirmed that the cardioprotective effect of IL-15 is partly attributable to the attenuation of cardiomyocyte apoptosis *via* the NF-κB signaling pathway. Conversely, IL-15 knockdown had the opposite effect. Based

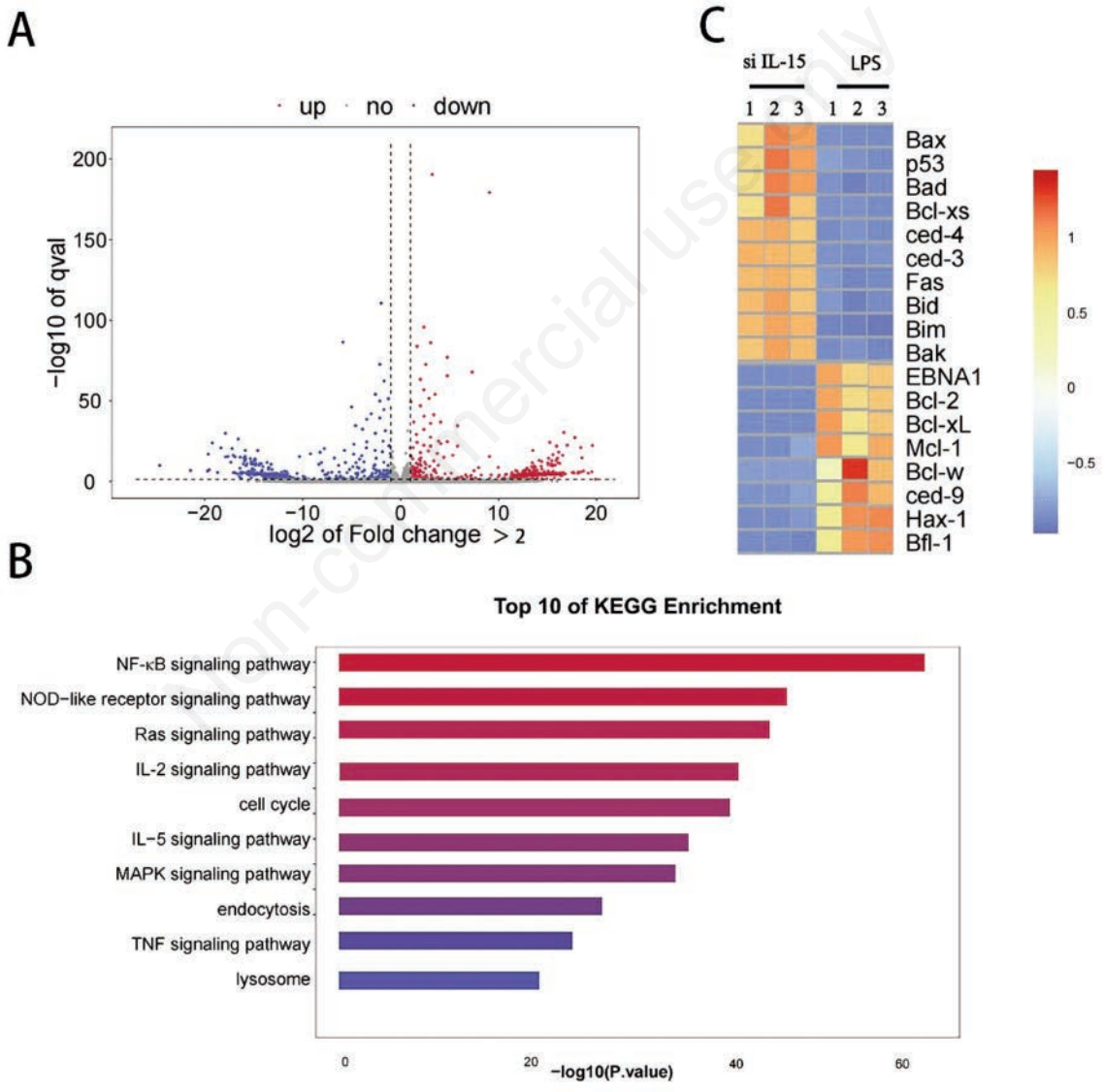

Figure 5. RNA-Seq to identify DEGs in cardiomyocytes after IL-15 knockdown and LPS stimulation. **A**) Volcano plot of DEGs. DEGs were defined by |log2 fold change| >2 and adjusted p<0.05; red dots represent upregulated DEGs, and blue dots represent downregulated DEGs. **B**) KEGG pathway enrichment analysis of DEGs was conducted to identify functionally related gene pathways between the IL-15 knockdown and control groups. **C**) Heatmap of apoptotic-related gene expression levels between two groups; downregulated genes are represented in blue, and upregulated genes are represented in red.

on these findings, we conclude that IL-15 might act as a promising therapeutic target in cardiac injury induced by sepsis.

Sepsis leads to multiple-organ dysfunction with high mortality rates, in which SIMD is a well-recognized manifestation that is primarily attributable to circulatory and microvascular abnormalities, cardiovascular autonomic dysregulation, mitochondrial dysfunction, inflammation, and myocardial cell apoptosis.[18] Numerous studies have demonstrated that the heart exhibits reduced EF accompanied by excessive inflammatory reactions and increased apoptosis in the myocardium during sepsis.[19,20] SIMD is a complex process that is closely associated with myocardial apoptosis. In our previous work, the ablation of heme oxygenase-1 in myeloid cells alleviated myocardial injury partly by reducing cardiomyocyte apoptosis in septic hearts.[21] In addition, a growing number of studies have reported that microRNAs attenuate cardiac dysfunction and cell apoptosis in sepsis by the NF-κB signaling pathway.[22-24] Intriguingly, one recent study reported that IL-5 knockout exacerbates sepsis-induced cardiac injury *via* the NF-κB p65 pathway.[25]

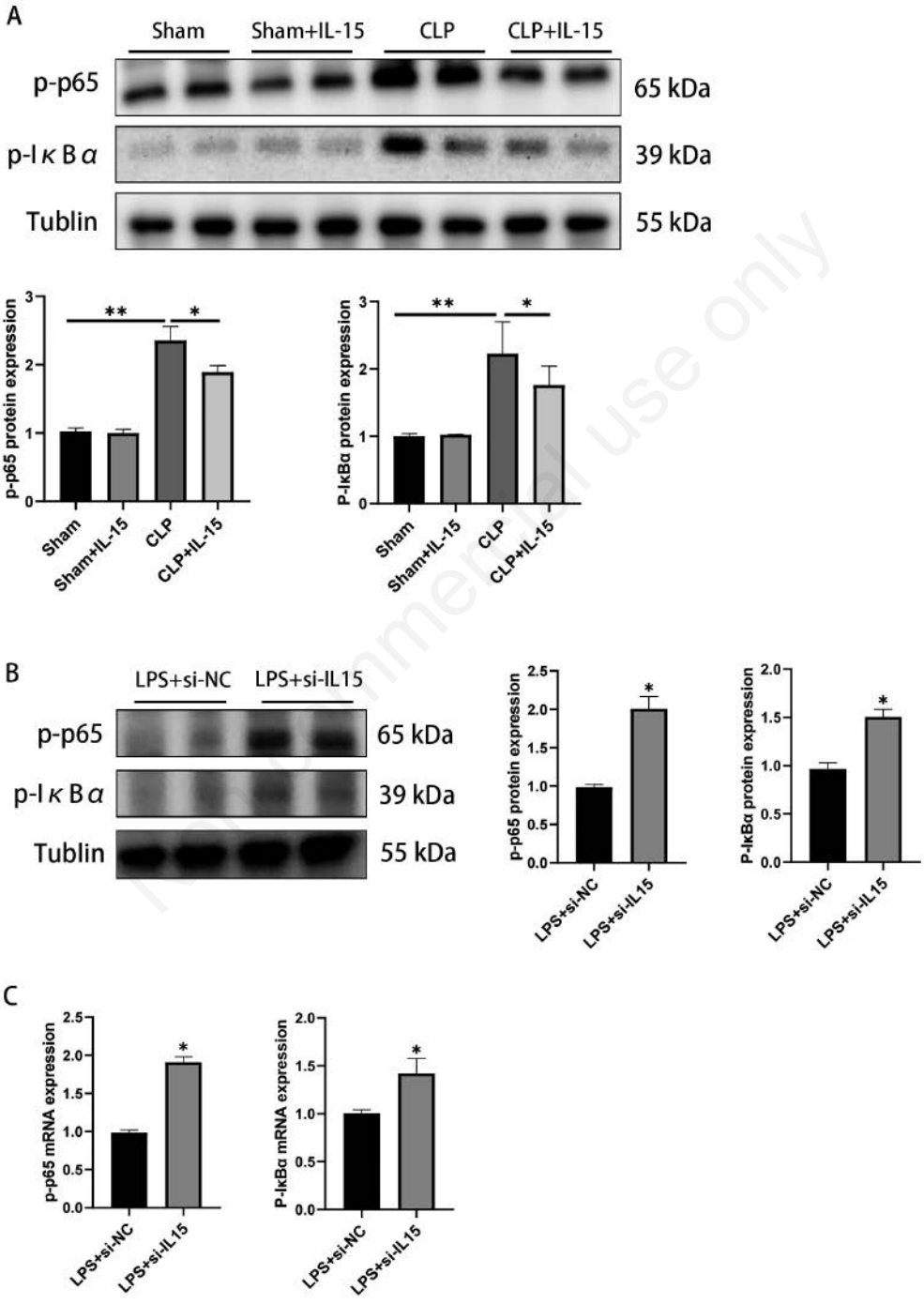

Figure 6. IL-15 reduced cardiomyocyte apoptosis by inhibiting the NF-κB signaling pathway. **A**) Western blotting of p-p65 and p-IκBα expression in heart tissue after CLP surgery with or without IL-15 pretreatment (n=3). **B,C**) Western blotting and RT-PCR of p-p65 and p-IκBα expression in cardiomyocytes after IL-15 knockdown and LPS stimulation (n=3). *p<0.05; **p<0.01.

Importantly, IL-5 and IL-15 are both members of the IL-2 superfamily, which is also known as the γ-chain family, owing to their ability to bind IL-2Rγ. Therefore, we speculated that IL-15 plays a vital role in cardioprotection in sepsis.

IL-15 is a four α-helix bundle cytokine with pleiotropic functions in the immune system and anti-tumor immunity.[26] The expression of IL-15 and its receptor has been found in many cell types, including cardiomyocytes, fibroblasts, and immune cells. Hiromatsu *et al*. found that IL-15 can relieve Escherichia coli-induced septic injury by inhibiting TNF-α–induced cell apoptosis in the peritoneal cavity, liver, and lungs.[27] In a mouse model of sepsis, IL-15 supplementation significantly prevented the apoptosis of NK cells and CD8+ T cells, reversed immune dysfunction, and improved survival.[28] A growing body of evidence has revealed that IL-15 is involved in the development and progression of various cardiovascular diseases. In recent years, several studies have reported that IL-15 is upregulated in myocardial infarction and atherosclerotic lesions, and it acts as a biomarker in patients with atrial fibrillation.[12,14,29] Although prior studies revealed that IL-15 exerts protective effects in myocardial infarction and myocarditis by attenuating cardiomyocyte apoptosis and cardiac dysfunction in mice, the precise mechanisms remain unclear.[11,12] Furthermore, IL-15 administration increased the survival of cardiomyocytes under oxidative stress.[13] In this study, we detected an increase in IL-15 expression in septic hearts and further demonstrated that it was mainly colocalized with cardiomyocytes. This observation agreed with previous findings that cardiomyocytes secrete IL-15.

IL-15 is a multifaceted regulator and involved in numerous signaling pathways and molecular interaction. Upregulation of IL-15 and IL-15Rα is found during inflammatory responses and both human and animal atherosclerotic lesions.[14] Therefore, IL-15 was upregulated in septic mice to participate in inflammation. We also found that IL-15 pretreatment attenuated inflammatory responses in septic mice along with improving cardiac function and reduced cardiomyocyte apoptosis, whereas IL-15 knockdown had the opposite effects. Taken together, these results suggest that IL-15 plays a cardioprotective effect in SIMD.

Numerous studies suggested that extensive inflammatory responses are harmful and that they ultimately contribute to cardiomyocyte apoptosis and impaired repair processes in SIMD.[30,31] The NF-κB pathway plays crucial roles in various physiological processes, including immune regulation, inflammatory responses, and sepsis-related organ failure. As reported previously, the NF-κB pathway is associated with the promotion of pro-inflammatory cytokine production and cell apoptosis in sepsis.[32-34] Given the results of RNA-Seq, we hypothesized that IL-15 inhibits cell apoptosis through the NF-κB pathway. As expected, exogenous IL-15 supplementation significantly decreased p-p65 expression and inhibited NF-κB signaling in mice with sepsis by promoting IκBα phosphorylation. Besides, to investigate the effect of IL-15 is part-

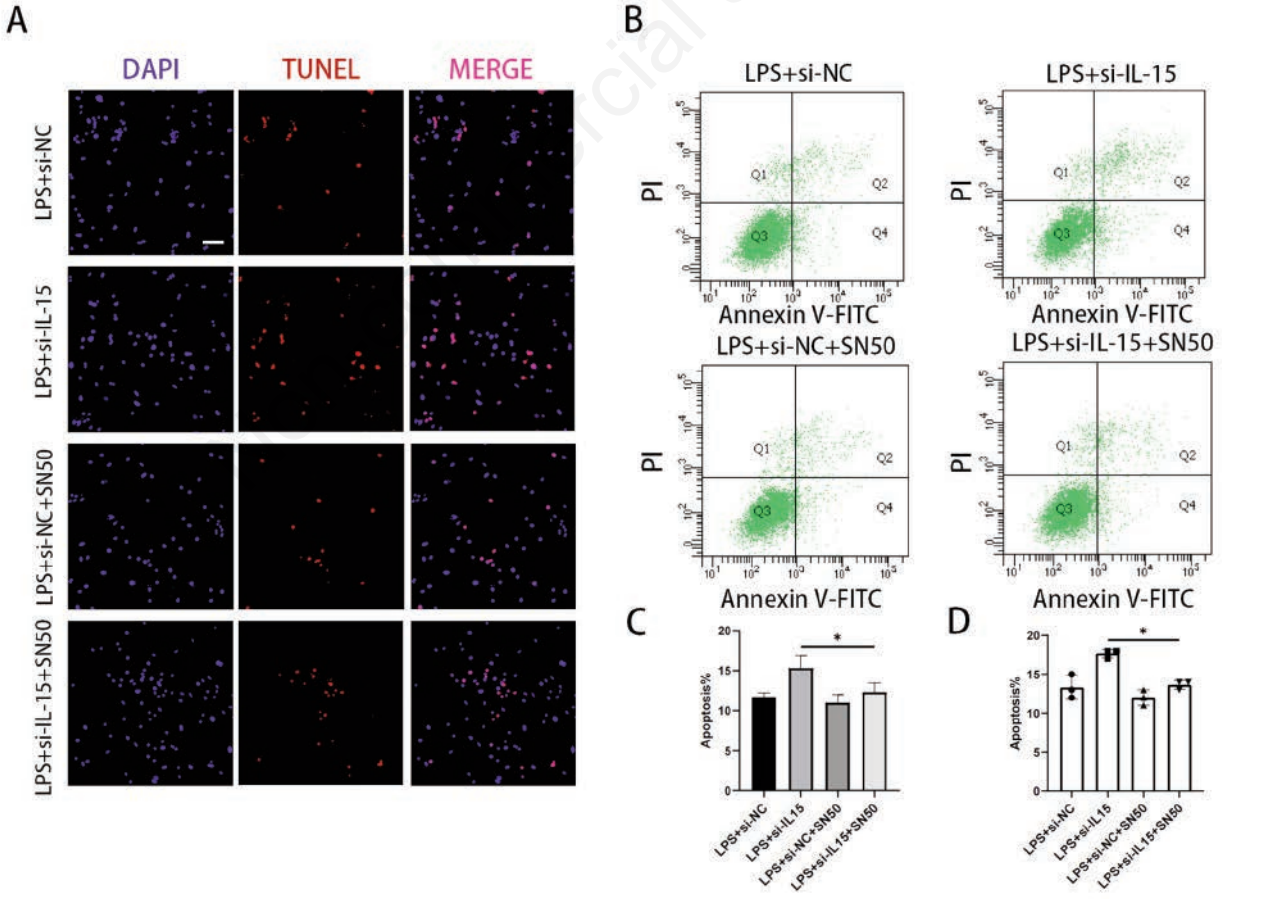

Figure 7. SN50 can significantly attenuate cardiomyocyte apoptosis after LPS stimulation and IL-15 knockdown. **A,C**) TUNEL staining revealing apoptosis in IL-15 silenced cells after addition of SN50 (n=3); scale bar: 50 μm. **B,D**) Flow cytometry revealing apoptosis in IL-15 silenced cells after addition of SN50 (n=3). *p<0.05.

ly attributable to the attenuation of cardiomyocyte apoptosis *via* the NF-κB signaling pathway, we used NF-κB inhibitor SN50 in IL-15 silenced cells and found the addition of SN50 can significantly attenuate cell apoptosis compared to the control group. Furthermore, we observed that IL-15 pretreatment attenuated apoptosis by reducing cleaved caspase-3 and Bax expression and increasing Bcl-2 expression in septic hearts, which in turn improved cardiac function after CLP surgery. Knockdown of IL-15 had the opposite effect. Our findings are consistent with previous results that excessive cardiomyocyte apoptosis is tightly related to cardiac dysfunction in the CLP-induced sepsis model.[35,36] In addition, one study showed that IL-15 protected hypoxia-induced cardiomyocyte death through activation of STAT3 and PI3K-ERK1/2 pathways,[13] which maybe other possible mechanisms that contribute to the cardioprotective effect of IL-15. Finally, Inoue *et al*. found that IL-15 prevent immunosuppression and apoptosis, and improved survival in sepsis.[28]

In summary, we have provided the first evidence that IL-15 pretreatment attenuates inflammatory responses in septic mice, along with improving cardiac function and reducing cardiomyocyte apoptosis. Mechanistically, our results reveal that the NF-κB signaling pathway is involved in the cardioprotective effects of IL-15. These findings provide a potential therapeutic target for SIMD.

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