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Ubiquitin-specific protease 11 Aggravates Ischemia-reperfusion-induced Cardiomyocyte Pyroptosis and Injury by Promoting TRAF3 Deubiquitination

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Background: In myocardial ischemia-reperfusion injury, myocardial damage is aggravated when blood perfusion is restored in myocardial infarction. Ubiquitin-specific protease 11 (USP11), a deubiquitinating enzyme, could remove the ubiquitination of substrate proteins and regulate protein stability, thereby affecting multiple pathological processes.

Aims: To investigate the potential function of USP11 in myocardial ischemia-reperfusion injury and its underlying mechanisms.

Study Design: In vivo and in vitro experimental study.

Methods: The ischemia-reperfusion rat model in vivo was evolved, wherein the left anterior descending coronary artery was ligated for 30 min, followed by ligature release for 120 min. Meanwhile, H9C2 cells were brought to hypoxia for 6 h and then reoxygenated for 18 h to establish a cell hypoxia-reoxygenation (H/R) injury in vitro. Then, the loss-of-function experiments of USP11 were performed. Triphenyltetrazolium chloride and hematoxylin and eosin staining were performed to observe myocardial injury. The MTT assay was utilized to detect H9C2 cell viability. Pyroptosis was analyzed by TUNEL staining and flow cytometry. Pyroptosis-related protein expression and TRAF3 were analyzed via Western blot. The content of inflammatory factors was examined by enzyme-linked immunoassay. Co-immunoprecipitation and ubiquitination assays were performed to analyze for USP11 interacting with TRAF3.

Results: USP11 was upregulated in the ischemic heart tissue. Ischemia-reperfusion and H/R injuries increased USP11 expression. USP11 loss-of-function assays showed that USP11 knockdown alleviated ischemia-reperfusion- and H/R-induced myocardial cell damage, pyroptosis, pro-inflammatory factor secretion, and IKKβ/NF-κB pathway activation. In H9C2 cells, USP11 stabilized TRAF3 by deubiquitination. Furthermore, rescue experiments revealed that TRAF3 overexpression reversed the protection of silencing USP11 on H/R-induced H9C2 cell injury.

Conclusion: This study confirmed that USP11 knockdown ameliorated myocardial ischemia-reperfusion injury by downregulating TRAF3, suggesting that USP11 silencing can be a novel target of myocardial infarction.

INTRODUCTION

Myocardial infarction (MI) is a vital class of cardiovascular diseases with high disability and mortality worldwide.[1,2] Currently, the most effective treatment for MI is early restoration of tissue blood supply; however, the recovery of blood supply often caused further myocardial damage, which is known as ischemia-reperfusion (I/R) injury.[3,4] Myocardial I/R injury (MIRI) is a vital factor in the prognosis of patients with MI.[5] Therefore, the mechanism of MIRI has drawn much attention. Recently, pyroptosis, a highly pro-inflammatory programmed cell death, was reported to be tightly related to MIRI.[6,7] Herein, the regulatory mechanism of pyroptosis was explored deeply to determine an effective intervention target for myocardial I/R damage.

Ubiquitin-specific protease 11 (USP11), a deubiquitinating enzyme, belongs to the USP family of cysteine protease.[8] A previous study indicated that USP11 aggravated spinal I/R injury by deubiquitinating

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Beclin1 and mediating autophagy-dependent ferroptosis.[9] Moreover, USP11 could stabilize p53 via deubiquitination, thereby activating the NF-kB pathway, which contributes to neurological inflammatory injury following intracerebral hemorrhage.[10] In this study, USP11 was found to be upregulated in the left ventricle tissue of ischemic cardiomyopathy by analyzing the GEO database (GSE116250). However, its role in myocardial reperfusion injury requires further investigation.

Tumor necrosis factor receptor-associated factor 3 (TRAF3) is a component of the TRAF protein group that regulates multiple biological processes, such as cell apoptosis, and inflammation, through binding to receptors, enzymes, and regulators.[11,12] TRAF3 was expressed both in ischemic stroke and spinal cord injury and promoted neuronal apoptosis by stimulating the MAPK and NF-kB pathways.[13,14] In addition, TRAF3 was reported to be involved in I/R-induced myocardial injury and may serve as a target for ameliorating myocardial injury.[15] In this study, we predicted that USP11, as a histone deubiquitinase, may catalyze the deubiquitination of TRAF3. Accordingly, we explored the possibility that USP11 stabilized TRAF3 by deubiquitination to regulate MIRI.

MATERIALS AND METHODS

Adenoviral Vector Construction

Adenovirus encoding a specific short-hairpin RNA targeting USP11 (Ad-shUSP11) and a nonsense shRNA (Ad-shNC) were designed and transducted into cardiomyocytes as described previously.[16]

Ischemia-Reperfusion Animal Model Creation

All 24 adult SD rats (male, 200-250 g) were taken from Shanghai Laboratory Animal Company (China) and raised in the same pathogen-free room. The rats were randomized to four groups of six rats each: sham+Ad-shNC, sham+Ad-shUSP11, I/R+Ad-shNC, and I/R+Ad-shUSP11. For the I/R model, rats were anesthetized with 10% chloral hydrate intraperitoneally. Then, a chest median incision was made. Following tracheostomy, the rats were intubated with a vein puncture needle and connected to a small animal-specific breathing machine. Then, for 30 min, the left anterior descending coronary artery was blocked, and reperfusion was performed for 120 min. Meanwhile, rats that underwent thoracotomy without coronary artery ligation were considered as the sham group. For Ad-shNC and Ad-shUSP11 transduction, before 48 h of coronary ligation, 100 µl of adenovirus solutions were injected intramyocardially. In all cases, the Ethics Committee of The First Affiliated Hospital of Xinjiang Medical University Hospital approved the experiments.

Establishing the Cellular Hypoxia-Reoxygenation (H/R) Model

H9C2 cells were cultured for simulating MIRI in vitro through H/R exposure. The cells were randomly classified into four groups: control+Ad-shNC, control+Ad-shUSP11, H/R+Ad-shNC, and H/R+Ad-shUSP11. Before H/R treatment, H9C2 cells were transfected with Ad-shNC or Ad-shUSP11 adenovirus vector for 48 h. Later, the cells at 80% confluence of the H/R group were maintained in a glucose-free medium and kept for 6 h in an anaerobic chamber with 95% nitrogen and 5% carbon dioxide and then reoxygenation for 18 h at 95% O_2 and 5% CO_2. The control group was maintained in an incubator with 95% O_2 and 5% CO_2.

RNA Extraction and Quantitation Analysis

Total RNA was isolated using a Trizol kit (Invitrogen, Carlsbad, CA, USA), and reverse transcriptase was employed for reverse transcription to cDNA (TaKara, Kusatsu, Japan). Then, a SYBR Green kit (TaKara) was employed to perform reverse-transcription polymerase chain reaction (RT-PCR) on an ABI7500 system. The mRNA expression of USP11 relative to β-actin was calculated by utilizing the $2^{-\Delta\Delta ct}$ method. The primer sequences were as follows: USP11: Fwd-5'TTCCACGGCCTCTTCAAGTC3', Rev-5'CGCGGATCCATGGGGATAAA3'; β-actin: Fwd-5'TGAGCTGCGTTTTACACCCT3', Rev-5'GCCT TCACCGTTC CAGTTTT3'.

Western Blot

RIPA lysis buffer (Sigma, Shanghai, China) was applied to extract total proteins. A BCA kit (Sigma) was employed to measure the concentration of protein in the supernatant. Sodium dodecyl-sulfate polyacrylamide gel electrophoresis was used to separate proteins, followed by their electrical transfer to a polyvinylidene fluoride membrane. Post blocking with 5% BSA, membranes were kept overnight at 4 °C alongside diluted primary antibodies: anti-USP11 (1:5000, ab109232, Abcam), anti-NLRP3 (1:1000, ab263899, Abcam, Cambridge, UK), anti-GSDMD-N (1:1000, ab215203, Abcam), anti-caspase-1 (1:1000, ab207802, Abcam), anti-IL-1β (1:1000, ab300501, Abcam), anti-IL-18 (1:1000, ab207323, Abcam), anti-p-IKKβ (1:1000, ab194528, Abcam), anti-IKKβ (1:500, ab32135, Abcam), anti-p-NF-kB (1:1000, ab76302, Abcam), anti-NF-kB (1:1000, ab16502, Abcam), anti-TRAF3 (1:1000, ab155298, Abcam), and anti-β-actin (1:1000, ab8227, Abcam) and then incubation for 1 h by secondary antibody. An ECL chromogenic substrate was used to visualize the bands.

Triphenyltetrazolium Chloride (TTC) Staining and Histopathological Examinations

The myocardial tissue slices of rats were stained using 1% TTC (Sigma) solution for 20 min at 37 °C. After washing with phosphate-buffered saline, tissue slices were fixed in 10% formaldehyde for 6 h. Finally, slices were evaluated and photographed, and the infarct size was estimated based on the amount of necrosis in the myocardium as a percentage of the myocardial area. For histopathological examinations, 2-µm tissue sections were dehydrated, followed by hematoxylin and eosin (HE) staining, sealing with a neutral gum, and observing under an optical microscope.

Lactate Dehydrogenase (LDH) Activity Assay

A LDH assay kit (Dojindo, Kumamoto, Japan) was applied to measure the LDH level in the blood samples of rats and the supernatant of H9C2 cells.

MTT Assay

For the MTT assay, 2 x 10^3 cells/well were cultured in 96-well plates overnight. Then, 20 µl of the MTT solution (Sigma) was mixed and then co-incubated for 4 h. A microplate reader (BioTek, Winooski, VT, USA) was employed to record the absorbance at 490 nm, and cell viability was calculated.

TUNEL Staining

At 30 min after paraformaldehyde (4%) fixation, tissue sections were treated with 0.1% TritonX-100 for 15 min. Then, a TUNEL kit was employed to perform TUNEL staining. Briefly, the sections were incubated for 2 h with the TdT enzyme reaction mixture at 37 °C. Thereafter, DAPI was added, and incubated for 10 min. A total of five randomly selected fields in each section were viewed to determine the percentage of TUNEL-positive cells under a fluorescent microscope (Nikon, Japan).

Flow Cytometry

Caspase-1 was measured by flow cytometry to assess H9C2 cell pyroptosis. Briefly, according to the FAM-FLICA Caspase-1 Assay Kit (Immunochemistry Technologies, USA) instructions, H9C2 cells were mixed with FLICA and PI, hatched in the dark for 1 h at 37 °C, and then analyzed with the FACSlyric flow cytometry (BD Pharmingen, San Diego, CA).

Enzyme-linked Immunosorbent Assay (ELISA)

Rat blood samples and H9C2 cell supernatants were collected, and interleukin (IL)-18, IL-1β, IL-6, and TNF-α levels were examined using an ELISA kit (Abcam) following the manufacturer's protocol.

Co-immunoprecipitation (Co-IP) Assay and Ubiquitination Assay

Co-IP and ubiquitination assays were carried out as described previously.[10] Briefly, H9C2 cells were lysed with an IP buffer solution. Cell lysates were incubated with anti-USP11 and anti-TRAF3 overnight and then incubated with pretreated magnetic beads for 4 h. Western blot was used to analyze the immunoprecipitated proteins. Cells were treated with MG132 or cycloheximide (CHX) for ubiquitination before incubating with antibodies.

Statistical Analysis

GraphPad Prism 8.0 was used for statistical data analysis, and values are presented as mean ± standard deviation. Comparisons between the two groups were performed by a t-test. Multiple groups were compared for the differences through multi-group analysis of variance. The p-value was considered significant when it was < 0.05.

RESULTS

USP11 was Highly Expressed in I/R Rats and Cell Model

As shown in Figure 1a, USP11 expression in the human left ventricular tissue was derived from the GEO database (GSE116250) and was upregulated in ischemic cardiomyopathy in comparison with normal tissues. Then, we established the H/R-induced H9C2 cell model and the I/R rat model. The results of qRT-PCR and Western blot showed increased levels of USP11 mRNA and protein for I/R rat myocardial tissues compared with

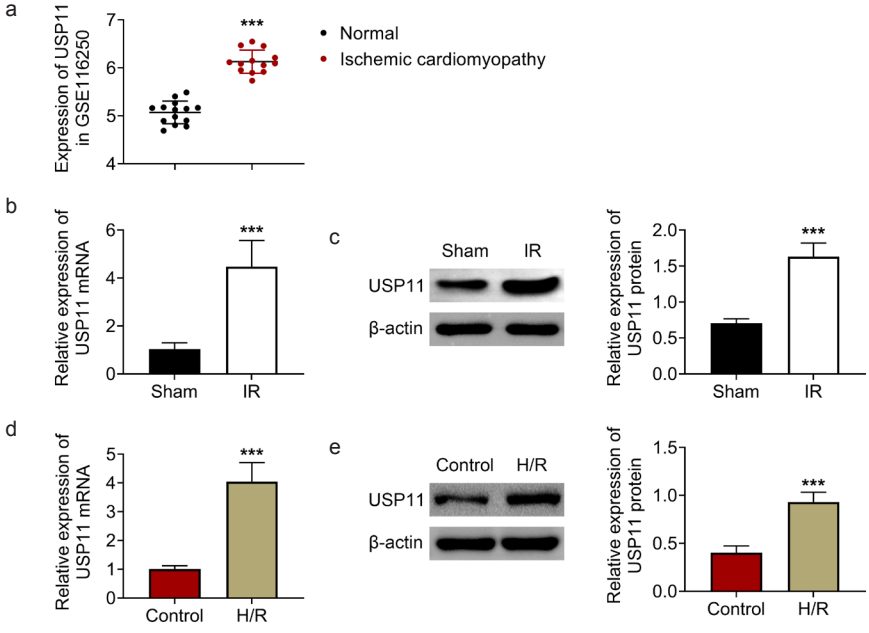

FIG. 1. USP11 was highly expressed in the ischemia–reperfusion rat model and cell model. a) The expression level of USP11 was obtained from the GEO database (GSE116250). ***$p < 0.001$, compared with the control group, b, c) qPCR and Western blot evaluation of rat myocardial tissue on mRNA and protein expression of USP11. ***$p < 0.001$ compared with the sham group, d, e) qPCR and Western blot analysis of H9C2 cells on mRNA and protein expression of USP11. ***$p < 0.001$ compared with the control group.
qPCR, real-time polymerase chain reaction.

those of sham rats (Figure 1b, 1c). Consistently, USP11 mRNA and protein expressions were boosted for H9C2 cells with H/R injury compared with the control group (Figure 1d, 1e).

USP11 Silencing Alleviated Myocardial Injury Caused by I/R

To substantiate USP11 function in myocardial I/R injury, USP11 loss-of-function experiments were performed in rat and cell models. For the I/R rat model, the Western blot assay showed that compared with the sham+Ad-shNC group, the sham+Ad-shUSP11 group had decreased USP11 protein expression, whereas it increased in the I/R+Ad-shNC group. In addition, the protein level of USP11 in I/R+Ad-shUSP11 was significantly lower than that in I/R+Ad-shNC (Figure 2a). Subsequently, the myocardial infarct size following USP11 knockdown was assessed by TTC staining. Compared with the sham+Ad-shNC and sham+Ad-shUSP11 groups, the percentage of the infarct size was increased in the I/R+Ad-shNC group, whereas this size was reduced after knocking down of USP11 (Figure 2b). The HE-stained cardiac sections showed that the cardiomyocytes were disorganized and enlarged after I/R treatment, whereas USP11 silencing ameliorated this damage (Figure 2c). Moreover, the LDH contents in the serum of rats were elevated after I/R injury, but reduced after USP11 silencing (Figure 2d). Then, we observed the effect of USP11 on H/R-induced H9C2 cell injury. Compared with control+Ad-shNC, control+Ad-shUSP11 downregulated USP11 protein expression and H/R+Ad-shNC upregulated it (Figure 2e). The USP11 level was reduced in H9C2 cells treated with H/R and transfected with shUSP11 (Figure 2e). Furthermore, we observed a declined cell activity and increased LDH levels in H/R-induced H9C2 cells, whereas these effects were reversed via USP11 silencing (Figure 2f, 2g). In summary, the data validate that USP11 knockdown alleviated I/R injury in vivo and H/R injury in vitro in rats.

USP11 Silencing Suppressed the Pyroptosis of Myocardial Cells During Alleviated I/R Damage

Evidence proves that programmed cell death by pyroptosis was tightly related to the myocardial injury caused by I/R.[17,18] Herein, to verify that USP11 knockdown affected myocardial cell

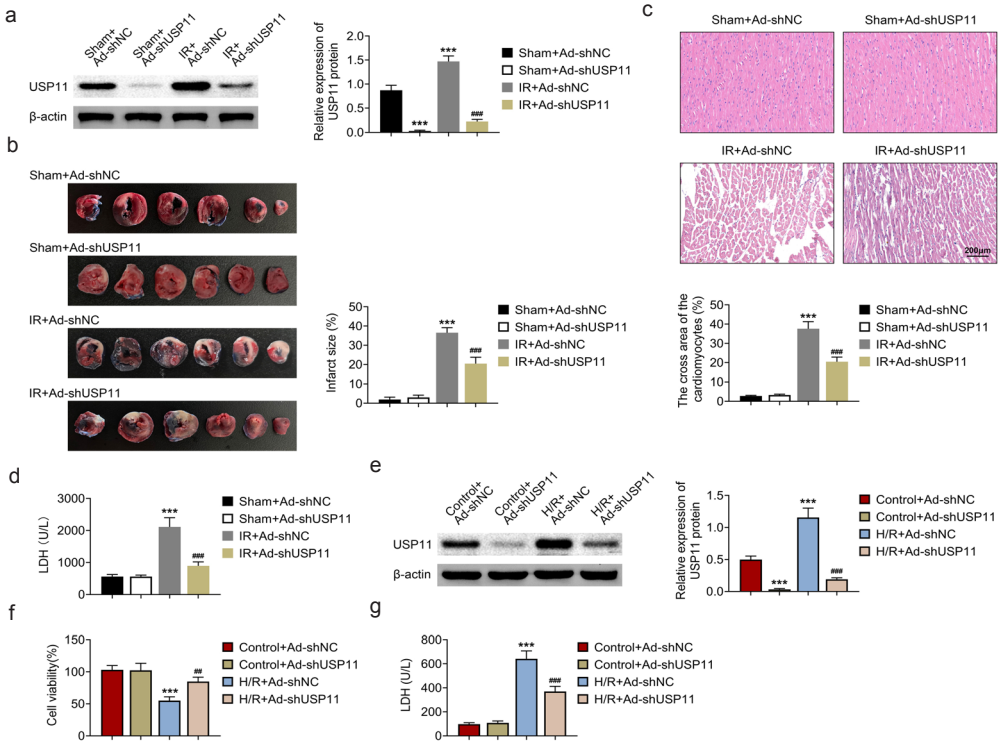

FIG. 2. USP11 knockdown protected against myocardial injury in vivo and in vitro. a) The protein level of USP11 in myocardial tissue was examined by Western blot. $^{***}p < 0.001$ when compare with the sham + Ad-shNC group. $^{###}p < 0.001$ compared with the I/R+Ad-shNC group, b) Representative images of myocardial tissue section by TCC staining (left) and averaged data of the percentage of the infarction size to the total area (right). $^{***}p < 0.001$ compared with the sham + Ad-shNC group. $^{###}p < 0.001$ compared with the I/R+Ad-shNC group, c) Typical pictures of myocardial tissue by HE staining, d) The LDH level in the blood of rat was detected by ELISA. $^{***}p < 0.001$ compared with the sham + Ad-shNC group. $^{###}p < 0.001$ compared with the I/R + Ad-shNC group, e) The protein level of USP11 in H9C2 cells was analyzed by Western blot. $^{***}p < 0.001$ compared with the control + Ad-shNC group. $^{###}p < 0.001$ compared with the H/R + Ad-shNC group, f) Viability of H9C2 cells was detected by the MTT assay. $^{***}p < 0.001$ compared with the control + Ad-shNC group. $^{##}p < 0.01$ compared with the H/R + Ad-shNC group, g) The level of LDH in the H9C2 cell supernatant was detected by ELISA. $^{***}p < 0.001$ compared with the control + Ad-shNC group. $^{###}p < 0.001$ compared with the H/R+Ad-shNC group.

USP11, ubiquitin-specific protease 11; LDH, lactate dehydrogenase; HE, hematoxylin and eosin.

pyroptosis during MIRI, TUNEL staining of myocardial tissue was performed after I/R, and myocardial NLRP3, IL-18, IL-1β, caspase-1, and GSDMD-N levels were determined via Western blot. The percentage of TUNEL-positive cells and NLRP3, IL-18, IL-1β, caspase-1, and GSDMD-N levels were also increased in the I/R+Ad-shNC group in comparison with the sham group (Figure 3a, 3b). Moreover, compared with the I/R+Ad-shNC group, these indices in the I/R+Ad-shUSP11 group decreased. In addition, after H/R damage, the H9C2 cell pyroptosis rate was enhanced by flow cytometry; however, the H9C2 cell pyroptosis rate after transfection with Ad-shUSP11 decreased (Figure 3c). Western blot revealed similar results for NLRP3, IL-18, IL-1β, caspase-1, and GSDMD-N levels in H9C2 cells after H/R treatment (Figure 3d). Altogether, these data indicated that USP11 silencing could relieve I/R-induced myocardial pyroptosis.

USP11 Silencing Inhibited the I/R-induced Inflammatory Damage

The leading feature of pyroptosis is the rapid rupture of the plasma membrane, resulting in the release of proinflammatory intracellular contents.[19] To gain further insight into the effect of USP11 function loss on myocardial inflammation, we monitored the content of inflammatory factors by ELISA and the activity of the signaling pathway for IKKβ/NF-κB by Western blot. Consequently, the concentrations of IL-6, IL-18, TNF-α, and IL-1β were significantly higher in rat myocardial tissue that suffered from I/R injury than that in the sham group, all of which were lower in the I/R+Ad-shUSP11 group relative to the I/R+Ad-shNC group (Figure 4a). Similarly, Western blot illustrated higher phosphorylation levels of IKKβ and NF-kB in the I/R group than in the sham group, which were partially abolished by USP11 knockdown (Figure 4b). Like in the I/R rat model, consistent results were observed in the H/R

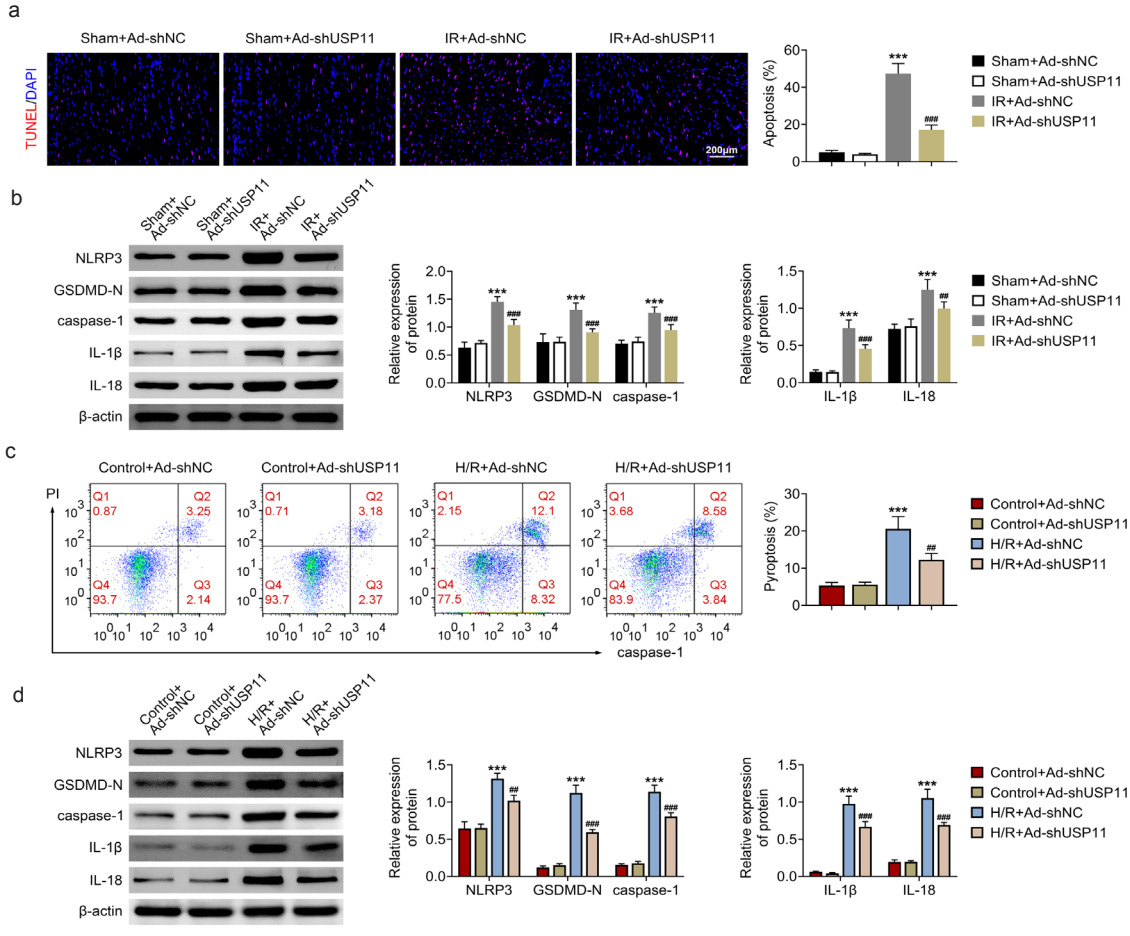

FIG. 3. USP11 knockdown alleviated the pyroptosis of myocardial cells during I/R injury. a) TUNEL staining of myocardial tissues (left) and the TUNEL-positive cell rate was counted (right), $^{***}p < 0.001$ compared with the sham + Ad-shNC group. $^{###}p < 0.001$ compared with the I/R + Ad-shNC group, b) The pyroptosis-related protein levels in myocardial tissues were analyzed by Western blot. $^{***}p < 0.001$ compared with the sham + Ad-shNC group. $^{###}p < 0.001$, $^{##}p < 0.01$ compared with the I/R + Ad-shNC group, c) The pyroptosis rate of H9C2 cells was analyzed by flow cytometry. $^{***}p < 0.001$ compared with the control + Ad-shNC group. $^{##}p < 0.01$ compared with the H/R + Ad-shNC group, d) Pyroptosis-related protein levels in H9C2 cells were found by Western blot. $^{***}p < 0.001$ compared with the control+Ad-shNC group. $^{###}p < 0.001$, $^{##}p < 0.01$ compared with the H/R + Ad-shNC group.

H9C2 cell model (Figure 4c, 4d). These data indicate that USP11 silencing could inhibit I/R-induced myocardial inflammation.

USP11 Stabilized TRAF3 Expression by Deubiquitination

UbiBrowser (http://ubibrowser.ncpsb.org) was used to further predict E3 ubiquitin ligase for USP11 and found that TRAF3 was predicted as a candidate for USP11. The binding of USP11 to TRAF3 was then determined. The results of the Western blot showed that USP11 and TRAF3 protein levels were downregulated in the Ad-shUSP11 group of the sham group, whereas they were upregulated in the Ad-shNC group of rats with I/R, but reduced once with USP11 loss (Figure 5a). Exactly as observed in the rat models, the expressions levels of USP11 and TRAF3 increased in the Ad-shNC group of H/R-treated H9C2 cells, but decreased after USP11 knockdown (Figure 5b). Furthermore, USP11 and TRAF3 binding was verified by the co-IP assay in H9C2 cells (Figure 5c). Meanwhile, USP11 silencing decreased the TRAF3 ubiquitination extent (Figure 5d). Then, in the presence of USP11, CHX was used to treat H9C2 cells to confirm the effect of USP11 on TRAF3 stability. As expected, to some extent, the inhibition of TRAF3 degradation by USP11 notably stabilized TRAF3 (Figure 5e). These data suggest that USP11 could stabilize TRAF3 through deubiquitination.

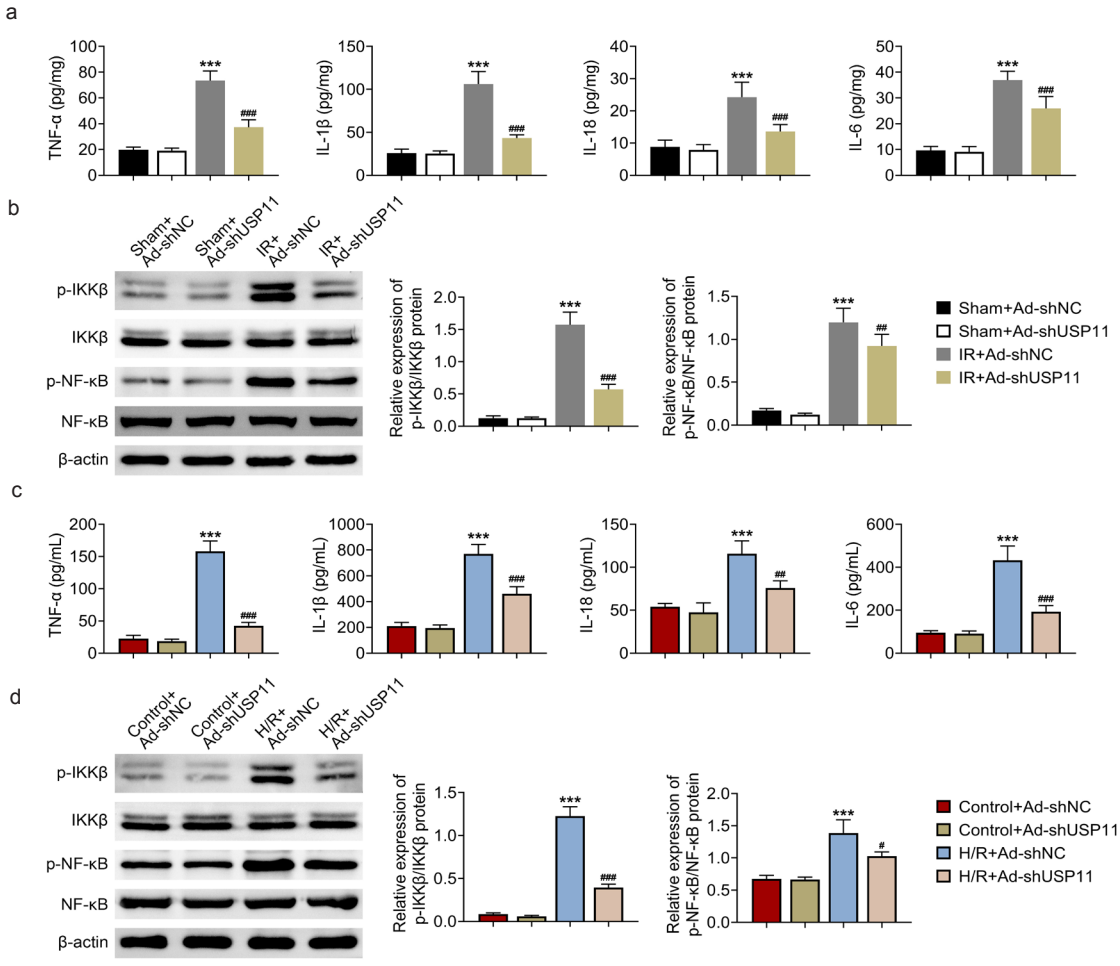

FIG. 4. USP11 silencing mitigated the I/R-induced myocardial inflammatory damage.
a) ELISA quantification of IL-6, IL-18, IL-1β, and TNF-α expressions in rat blood samples. $^{***}p < 0.001$ compared with the sham+Ad-shNC group. $^{###}p < 0.001$ compared with the I/R + Ad-shNC group, b) Western blotting of the total and phosphorylated IKKβ and NF-kB in myocardial tissues. $^{***}p < 0.001$ compared with the sham+Ad-shNC group. $^{###}p < 0.001$ compared with the I/R + Ad-shNC group, c) ELISA detection of the levels of IL-6, IL-18, IL-1β, and TNF-α in the H9C2 cell supernatant. $^{***}p < 0.001$ compared with the control + Ad-shNC group. $^{###}p < 0.001$ and $^{##}p < 0.01$ compared with the H/R+Ad-shNC group, d) Western blotting of total and phosphorylated IKKβ and NF-kB in H9C2 cells. $^{***}p < 0.001$ compared with the control + Ad-shNC group. $^{###}p < 0.001$ and $^{#}p < 0.05$ compared with the H/R + Ad-shNC group.

IL, interleukin; TNF-α, tumor necrosis factor α; USP11, ubiquitin-specific protease 11.

TRAF3 Overexpression Abrogated the Protection of USP11 Knockdown on H9C2 Cells

Then, to explore the possibility that USP11 functioned by stabilizing TRAF3, we co-transfected Ad-shUSP11 with NC or TRAF3 into H9C2 cells. As shown in Figure 6a, the level of TRAF3 protein was significantly upregulated in H/R-treated H9C2 cells with TRAF3 overexpression. Importantly, the cell viability improved in the H/R+shUSP11+NC group, whereas it decreased after TRAF3 overexpression (Figure 6b). Moreover, TRAF3 overexpression significantly recovered the LDH, pyroptosis, and inflammatory-inhibiting effects caused by Ad-shUSP11 in H/R-induced H9C2 cells (Figure 6c-6e). Taken together, these data demonstrated that USP11 silencing could protect myocardial cells from H/R-induced injury by TRAF3 degradation.

DISCUSSION

MIRI is a serious complication after restoring the blood supply in patients with MI.[20] The pathological process of MIRI involves inflammation, autophagy, and reactive oxygen and ultimately leads to irreversible myocardial cell death.[21,22] Pyroptosis as a novel

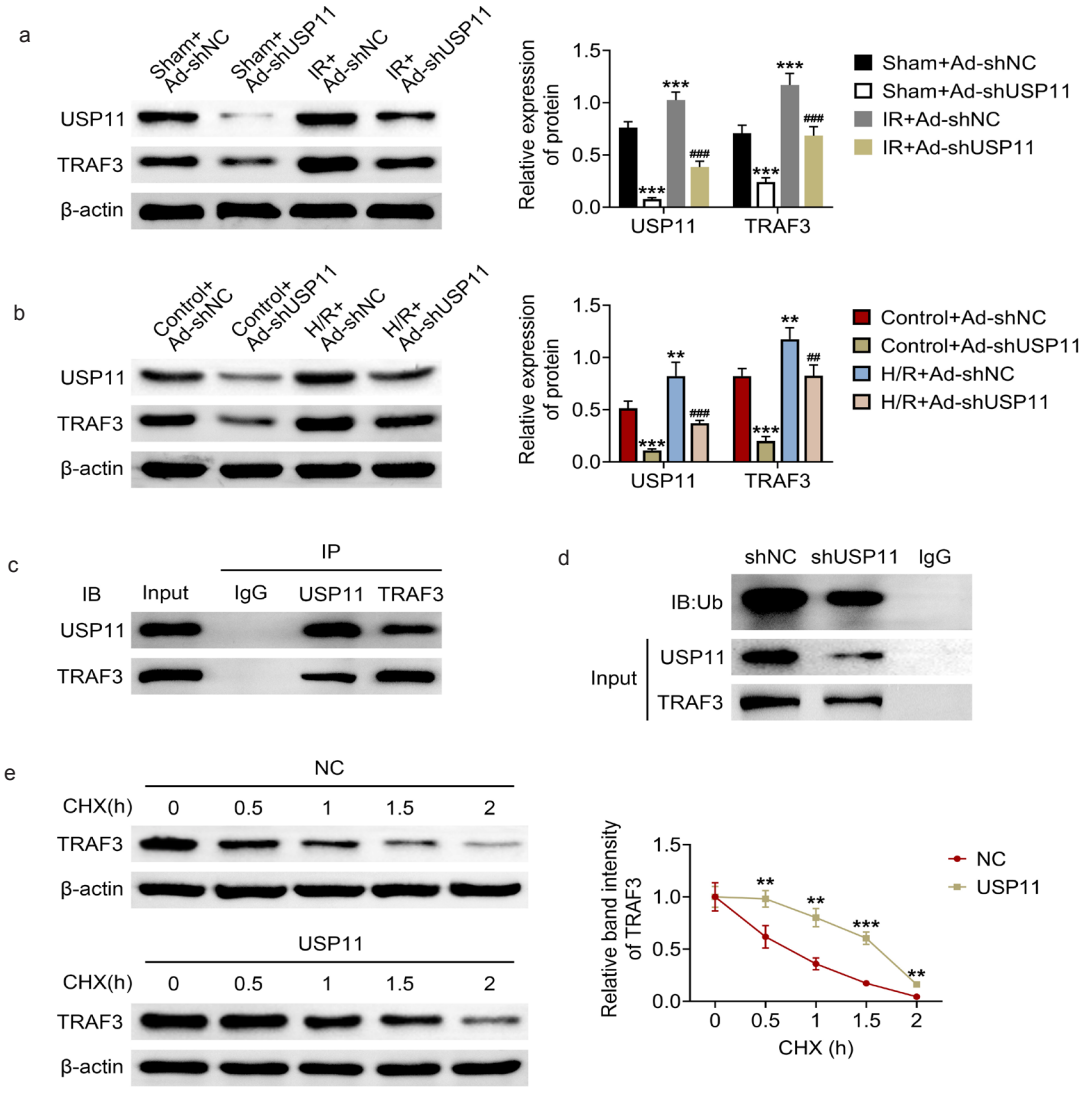

FIG. 5. USP11 stabilized TRAF3 by deubiquitination. a, b) Western blot assay for the determination of USP11 and TRAF3 protein levels in the myocardial tissues of rat and H9C2 cells. $^{***}p < 0.001$ and $^{**}p < 0.01$ compared with the sham + Ad-shNC group. $^{###}p < 0.001$ and $^{##}p < 0.01$ compared with the /IR + Ad-shNC group, c) Co-IP revealing USP11 and TRAF3 interaction, d) TRAF3 ubiquitination level shown by immunoprecipitation after USP11 overexpression, e) TRAF3 stability after getting treated with CHX in the presence of USP11. $^{***}p < 0.001$ and $^{**}p < 0.01$ compared with the NC group.

USP11, ubiquitin-specific protease 11.

programmed cell death type, is marked by cysteine asparaginase -1 (caspase-1) dependence and accompanied by the release of numerous pro-inflammatory factors.[23] Recent findings revealed the involvement of pyroptosis in MIRI, and targeting pyroptosis may be a new therapeutic means for MIRI.[24] In this study, we illuminate USP11 silencing protected MIRI and cell pyroptosis through the reduction of TRAF3 levels and revealed USP11 as a promising target for MIRI cure.

In this study, USP11 was upregulated after I/R and H/R injury, which is consistent with the result from the GEO database (GSE116250). Furthermore, USP11 knockdown was found to mitigate myocardial damage in rats from post-IR and promoted the activity of H9C2 cells from post-H/R. In I/R models, evidence indicated that the activated NLRP3 contributed to myocardial injury by activating caspase-1-dependent cell pyroptosis.[25] Caspase-1 activated other IL-18- and IL-1β-cleaved precursors, generating mature IL-18 and IL-1β forms, followed by their release from cells, thus promoting inflammatory response.[26] Moreover, caspase-1 activation also clicked gasdermine D (GSDMD) and generated GSDMD-N, forming pores in the cell membrane and causing cell pyroptosis.[27] Interestingly, our results verified that USP11 silencing repressed the pyroptotic cell death by inhibiting the activation of IL-18, caspase-1, NLRP3, IL-1β, and GSDND-N in in vivo and in vitro models, indicative of an anti-pyroptosis effect of USP11 knockdown. Moreover, USP11 knockdown resulted in the reduced production of pro-inflammatory factors and decreased activity of the IKKβ/NF-κB pathway. Of note, many deubiquitination enzymes, such as USP7, HSP47, USP49, and Abro1, were reported to play an important role in MIRI by controlling the ubiquitination of substrates.[28-31] Therefore, we explored the binding substrate of USP11 in depth.

Except for Beclin1 and p53, USP11 can also bind to PPP1CA, p21, NONO, and KLF4, thereby playing a role in cancer, DNA damage, and liver disease by mediating their deubiquitination and stabilization.[32-35] In this study, by analyzing UbiBrowser, we found that TRAF3 can act as a substrate for USP11. Importantly,

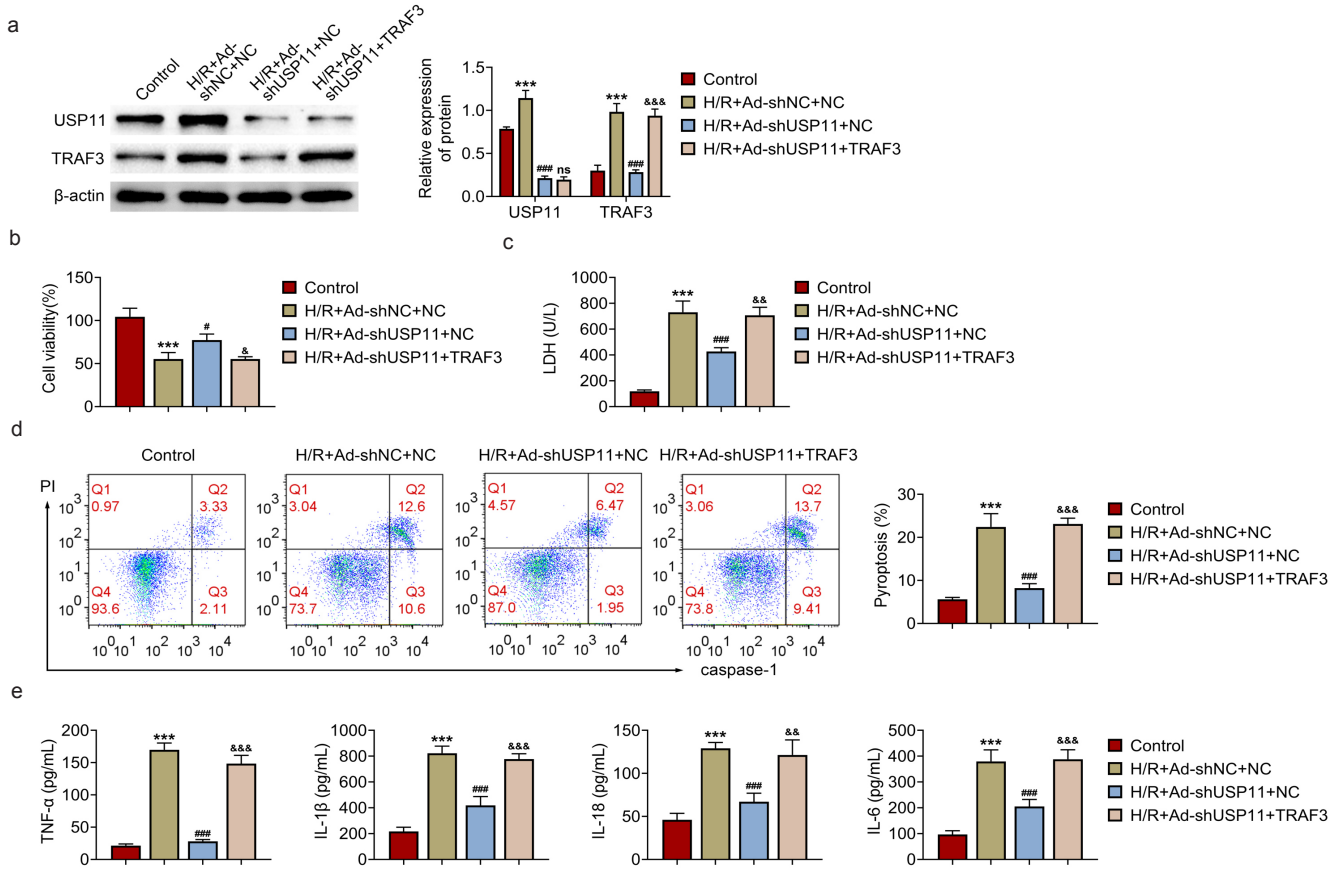

FIG. 6. TRAF3 overexpression abrogated the protection of USP11 knockdown on H9C2 cells. a) Protein expressions of USP11 and TRAF3 in cells were detected by Western blot, b) The MTT assay was used to assess cell viability, c) The level of LDH was quantified by ELISA, d) Flow cytometry analysis of cell pyroptosis, e) ELISA detection of the levels of IL-6, IL-18, IL-1β, and TNF-α in the supernatant of cells. $^{***}p < 0.001$ compared with the control group. $^{\#}p < 0.05$ and $^{\#\#\#}p < 0.001$ compared with the H/R+Ad-shNC + NC group. $^{\&}p < 0.05$, $^{\&\&}p < 0.001$, and $^{\&\&\&}p < 0.001$ compared with the H/R + Ad-shUSP11 + NC group.

LDH, lactate dehydrogenase; IL, interleukin; TNF-α, tumor necrosis factor α; USP11, ubiquitin-specific protease 11.

TRAF3 has been reported to promote LPS-induced pyroptosis of macrophages.[36,37] Study data provided evidence that USP11 stabilized TRAF3 expression by deubiquitination in H9C2 cells. Then, we observed that the anti-pyroptotic and anti-inflammation effects of USP11 silencing on H/R-treated H9C2 cells were largely abolished in the presence of TRAF3. These data suggested that USP11 silencing mitigated pyroptosis and MIRI through TRAF3 degradation. However, more evidence is needed to verify the role of USP11/TRAF3 axis in MIRI.

In conclusion, our study demonstrated that USP11 was upregulated in MIRI, and its inhibition could improve myocardial injury after MIRI and reduce pyroptosis and inflammation of myocardial cells by mediating TRAF3 deubiquitination. Our data highlight the potential of USP11 as a novel strategy for MIRI treatment.

Ethics Committee Approval: In all cases, the Ethics Committee of The First Affiliated Hospital of Xinjiang Medical University Hospital approved the experiments.

Data Sharing Statement: The data that support the findings of this study are available from the corresponding author upon reasonable request.

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REFERENCES

1. Jenča D, Melenovský V, Stehlik J, et al. Heart failure after myocardial infarction: incidence and predictors. *ESC Heart Fail.* 2021;8:222-237. [CrossRef]
2. Damluji AA, van Diepen S, Katz JN, et al. Mechanical Complications of Acute Myocardial Infarction: A Scientific Statement From the American Heart Association. *Circulation.* 2021;144:e16-e35. [CrossRef]
3. Frank A, Bonney M, Bonney S, Weitzel L, Koeppen M, Eckle T. Myocardial ischemia reperfusion injury: from basic science to clinical bedside. *Semin Cardiothorac Vasc Anesth.* 2012;16:123-132. [CrossRef]
4. Wang J, Toan S, Zhou H. New insights into the role of mitochondria in cardiac microvascular ischemia/reperfusion injury. *Angiogenesis.* 2020;23:299-314. [CrossRef]
5. Giustino G, Dangas GD. Ischemia-reperfusion injury and ischemic post-conditioning in acute myocardial infarction: Lost in translation. *Catheter Cardiovasc Interv.* 2017;90:1068-1069. [CrossRef]
6. Toldo S, Mauro AG, Cutter Z, Abbate A. Inflammasome, pyroptosis, and cytokines in myocardial ischemia-reperfusion injury. *Am J Physiol Heart Circ Physiol.* 2018;315:H1553-H1568. [CrossRef]
7. Zhang J, Huang L, Shi X, et al. Metformin protects against myocardial ischemia-reperfusion injury and cell pyroptosis via AMPK/NLRP3 inflammasome pathway. *Aging (Albany NY).* 2020;12:24270-24287. [CrossRef]
8. Jurga M, Abugable AA, Goldman ASH, El-Khamisy SF. USP11 controls R-loops by regulating senataxin proteostasis. *Nat Commun.* 2021;12:5156. [CrossRef]
9. Rong Y, Fan J, Ji C, et al. USP11 regulates autophagy-dependent ferroptosis after spinal cord ischemia-reperfusion injury by deubiquitinating Beclin 1. *Cell Death Differ.* 2022;29:1164-1175. [CrossRef]
10. Zhang X, Liu T, Xu S, et al. A pro-inflammatory mediator USP11 enhances the stability of p53 and inhibits KLF2 in intracerebral hemorrhage. *Mol Ther Methods Clin Dev.* 2021;21:681-692. [CrossRef]
11. Bishop GA, Stunz LL, Hostager BS. TRAF3 as a Multifaceted Regulator of B Lymphocyte Survival and Activation. *Front Immunol.* 2018;9:2161. [CrossRef]
12. Li H, Hostager BS, Arkee T, Bishop GA. Multiple mechanisms for TRAF3-mediated regulation of the T cell costimulatory receptor GITR. *J Biol Chem.* 2021;297:101097. [CrossRef]
13. Dai Q, Ma Y, Xu Z, et al. Downregulation of circular RNA HECTD1 induces neuroprotection against ischemic stroke through the microRNA-133b/TRAF3 pathway. *Life Sci.* 2021;264:118626. [CrossRef]
14. Wu Y, Zheng M, Wang S, et al. Spatiotemporal pattern of TRAF3 expression after rat spinal cord injury. *J Mol Histol.* 2014;45:541-553. [CrossRef]
15. Liu X, Zhang L, Qin H, et al. Inhibition of TRAF3 expression alleviates cardiac ischemia reperfusion (IR) injury: A mechanism involving in apoptosis, inflammation and oxidative stress. *Biochem Biophys Res Commun*. 2018;506:298-305. [CrossRef]
16. Guo X, Hu S, Liu JJ, et al. Piperine protects against pyroptosis in myocardial ischaemia/reperfusion injury by regulating the miR-383/RP105/AKT signalling pathway. *J Cell Mol Med.* 2021;25:244-258. [CrossRef]
17. Shi H, Gao Y, Dong Z, et al. GSDMD-Mediated Cardiomyocyte Pyroptosis Promotes Myocardial I/R Injury. *Circ Res.* 2021;129:383-396. [CrossRef]
18. Qiu Z, Lei S, Zhao B, et al. NLRP3 Inflammasome Activation-Mediated Pyroptosis Aggravates Myocardial Ischemia/Reperfusion Injury in Diabetic Rats. *Oxid Med Cell Longev.* 2017;2017:9743280. [CrossRef]
19. Shi J, Gao W, Shao F. Pyroptosis: Gasdermin-Mediated Programmed Necrotic Cell Death. *Trends Biochem Sci.* 2017;42:245-254. [CrossRef]
20. DeFilippis AP, Chapman AR, Mills NL, et al. Assessment and Treatment of Patients With Type 2 Myocardial Infarction and Acute Nonischemic Myocardial Injury. *Circulation.* 2019;140:1661-1678. [CrossRef]
21. Yao Y, Li F, Zhang M, et al. Targeting CaMKII-δ9 Ameliorates Cardiac Ischemia/Reperfusion Injury by Inhibiting Myocardial Inflammation. *Circ Res.* 2022;130:887-903. [CrossRef]
22. Marin W, Marin D, Ao X, Liu Y. Mitochondria as a therapeutic target for cardiac ischemia-reperfusion injury (Review). *Int J Mol Med.* 2021;47:485-499. [CrossRef]
23. Bertheloot D, Latz E, Franklin BS. Necroptosis, pyroptosis and apoptosis: an intricate game of cell death. *Cell Mol Immunol.* 2021;18:1106-1121. [CrossRef]
24. Zhaolin Z, Guohua L, Shiyuan W, Zuo W. Role of pyroptosis in cardiovascular disease. *Cell Prolif.* 2019;52:e12563. [CrossRef]
25. Li S, Sun Y, Song M, et al. NLRP3/caspase-1/GSDMD-mediated pyroptosis exerts a crucial role in astrocyte pathological injury in mouse model of depression. *JCI Insight.* 2021;6:e146852. [CrossRef]
26. Fu Q, Wu J, Zhou XY, et al. NLRP3/Caspase-1 Pathway-Induced Pyroptosis Mediated Cognitive Deficits in a Mouse Model of Sepsis-Associated Encephalopathy. *Inflammation.* 2019;42:306-318. [CrossRef]
27. Yao F, Jin Z, Zheng Z, et al. HDAC11 promotes both NLRP3/caspase-1/GSDMD and caspase-3/GSDME pathways causing pyroptosis via ERG in vascular endothelial cells. *Cell Death Discov.* 2022;8:112. [CrossRef]
28. Tang LJ, Zhou YJ, Xiong XM, et al. Ubiquitin-specific protease 7 promotes ferroptosis via activation of the p53/TfR1 pathway in the rat hearts after ischemia/reperfusion. *Free Radic Biol Med.* 2021;162:339-352. [CrossRef]
29. Xie S, Xing Y, Shi W, et al. Cardiac fibroblast heat shock protein 47 aggravates cardiac fibrosis post myocardial ischemia-reperfusion injury by encouraging ubiquitin specific peptidase 10 dependent Smad4 deubiquitination. *Acta Pharm Sin B.* 2022;12:4138-4153. [CrossRef]
30. Zhang W, Zhang Y, Zhang H, Zhao Q, Liu Z, Xu Y. USP49 inhibits ischemia-reperfusion-induced cell viability suppression and apoptosis in human AC16 cardiomyocytes through DUSP1-JNK1/2 signaling. *J Cell Physiol.* 2019;234:6529-6538. [CrossRef]

31. Cilenti L, Balakrishnan MP, Wang XL, et al. Regulation of Abro1/KIAA0157 during myocardial infarction and cell death reveals a novel cardioprotective mechanism for Lys63-specific deubiquitination. *J Mol Cell Cardiol.* 2011;50:652-661. **[CrossRef]**
32. Sun H, Ou B, Zhao S, et al. USP11 promotes growth and metastasis of colorectal cancer via PPP1CA-mediated activation of ERK/MAPK signaling pathway. *EBioMedicine.* 2019;48:236-247. **[CrossRef]**
33. Deng T, Yan G, Song X, et al. Deubiquitylation and stabilization of p21 by USP11 is critical for cell-cycle progression and DNA damage responses. *Proc Natl Acad Sci U S A.* 2018;115:4678-4683. **[CrossRef]**
34. Feng P, Li L, Dai J, et al. The regulation of NONO by USP11 via deubiquitination is linked to the proliferation of melanoma cells. *J Cell Mol Med.* 2021;25:1507-1517. **[CrossRef]**
35. Yang H, Park D, Ryu J, Park T. USP11 degrades KLF4 via its deubiquitinase activity in liver diseases. *J Cell Mol Med.* 2021;25:6976-6987. **[CrossRef]**
36. Shen Y, Liu WW, Zhang X, et al. TRAF3 promotes ROS production and pyroptosis by targeting ULK1 ubiquitination in macrophages. *FASEB J.* 2020;34:7144-7159. **[CrossRef]**
37. Shi JH, Ling C, Wang TT, et al. TRK-fused gene (TFG) regulates ULK1 stability via TRAF3-mediated ubiquitination and protects macrophages from LPS-induced pyroptosis. *Cell Death Dis.* 2022;13:93. **[CrossRef]**