RESEARCH ARTICLE

Inhibition of Stimulator of Interferon Genes Protects Against Myocardial Ischemia-Reperfusion Injury in Diabetic Mice

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Abstract

Background: Although the past decade has witnessed substantial scientific progress with the advent of cardioprotective pharmacological agents, most have failed to protect against myocardial ischemia/reperfusion (I/R) injury in diabetic hearts. This study was aimed at investigating the role of stimulator of interferon genes (STING) in I/R injury in diabetic mice and further exploring the underlying mechanisms.

Methods: Type 2 diabetic mice were subjected to I/R or sham operation to investigate the role of STING. STING knockout mice were subjected to 30 minutes of ischemia followed by reperfusion for 24 hours. Finally, myocardial injury, cardiac function, and inflammation levels were assessed.

Results: STING pathway activation was observed in diabetic I/R hearts, as evidenced by increased p-TBK and p-IRF3 expression. STING knockout significantly decreased the ischemic area and improved cardiac function after I/R in diabetic mice. STING knockout also elicited cardio-protective effects by decreasing serum cardiac troponin T and lactate dehydrogenase levels, thus diminishing the inflammatory response in the heart after I/R in diabetic mice. *In vitro*, STING inhibition decreased the expression of hypoxia-re-oxygenation-induced inflammatory cytokines.

Conclusions: Targeting STING inhibits inflammation and prevents I/R injury in diabetic mice. Thus, STING may be a potential novel therapeutic target against myocardial I/R injury in diabetes.

Keywords: STING; Myocardial I/R; Diabetes; Inflammation

Background

Many diseases are widely acknowledged to induce cardiovascular disease, and diabetes is a high-risk factor associated with cardiovascular disease. After myocardial ischemia, myocardial reperfusion remains the optimal method to save the ischemic myocardium. However, it also causes ischemia/reperfusion (I/R) injury, thereby leading to changes in the ultrastructure, function, metabolism, electrophysiology, and other aspects of myocardial cells, or even more serious irreversible damage [1, 2].

The most common cause of death in people with diabetes is cardiovascular complications [3]. In this respect, people with diabetes are at elevated

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risk of coronary artery damage and myocardial infarction, thus accounting for their poor prognosis. Current reperfusion strategies are often ineffective when applied to treat myocardial infarction in patients with diabetes and can potentially exacerbate their condition [4]. Moreover, the incidence of I/R injury is elevated in patients with diabetes, and most cardio-protective treatments are ineffective [5].

Inflammation has been associated with I/R injury and diabetes. An increasing body of evidence suggests that inflammation during I/R diminishes myocardial function, and induces ventricular remodeling and further development of heart failure [6–8]. However, how inflammation is initiated in I/R and diabetes remains unclear. The innate immune system plays a crucial role in damage repair. Hosts use pattern recognition receptors to recognize pathogen-associated molecular pattern molecules released by pathogenic microorganisms, and subsequently activate immune responses against pathogens and maintain homeostasis [9]. The cyclic GMP-AMP synthase (cGAS)-stimulator of interferon genes (STING) pathway has recently been reported to induce sterile inflammation. cGAS binds abnormal or exogenous DNA and then transforms guanosine triphosphate and adenosine triphosphate to cyclic GMP-AMP [10], a second messenger molecule that acts as a STING ligand [11, 12]. STING mediates the recruitment of TANK binding kinase 1 (TBK1), thus resulting in phosphorylation of Interferon Regulatory Factor 3 (IRF3), which is transported and subsequently regulates the secretion of type I interferon and other cytokines downstream in the nucleus, thus triggering the innate immune response and the production of inflammatory factors [13, 14]. According to a growing consensus, the cGAS-STING signaling pathway is closely associated with non-infectious immune inflammation in multiple systems, including myocardial I/R injury and diabetes, thus suggesting the extensive role of this pathway in the cardiovascular system [15, 16]. Therefore, we hypothesized that STING might play a role in I/R in diabetic conditions, and inhibiting STING may be effective in alleviating I/R injury in diabetic mice.

Methods

Diabetic Mouse Model

Our experiments were approved by the Chongqing Medical University Animal Ethics Research Committee. Adult healthy male C57BL/6J and STING knockout mice were used in our study and housed at the Chongqing Medical University laboratory animal center under controlled conditions (temperature 26 °C ± 2 °C, relative humidity 50% ± 15%). All mice were given ad libitum access to food and water. During the initial phase, the mice were assigned to two dietary regimens and fed a normal diet or high-fat diet (HFD; 34.9% fat, 26.2% protein, and 26.3% carbohydrate, as a percentage of total kcal) ad libitum, according to the literature [17]. After 4 weeks of continuous feeding, mice in the HFD group were given daily intraperitoneal injections of STZ (30 mg/kg) for 7 days after an overnight fast, whereas mice in the normal diet group were given a dose volume of Vehicle Lemon (30 mg/kg) in saline buffer (pH 4.4). Blood glucose was assessed after the 7 days of STZ injection (Figure S1). Mice with 12-h fasting blood glucose levels above 11.1 mmol/L were used in the following experiments.

Experimental Group and Myocardial Ischemic-Reperfusion Injury Surgery

We divided the mice into four groups: (1) sham group; (2) I/R group; (3) diabetes mellitus and I/R group (D-I/R); and (4) diabetes mellitus, I/R, and STING-knockout group (D-I/R+STING$^{-/-}$). For induction of I/R injury, the mice were fixed on a wooden board and anesthetized with isoflurane inhalation (3 min), and their tracheas were connected to a ventilator. Electrodes were inserted from the limbs into the subcutaneous layer of the mouse skin, and an electrophysiological apparatus (BIOPAC, USA) was connected to measure electrocardiograms. The thoracic skin of the mice was shaved and sterilized, and the skin was incised at the point of the apex of the heart (the fourth intercostal section of the left sternal region). The muscles were bluntly separated, and the ribs were opened with a retractor. We used a 7–0 suture to ligate the left anterior descending coronary artery (LAD) and a polyethylene tube with a straight length of 1 mm. The procedure was considered successful when the

anterior heart wall became cyanosed, and myocardial infarction (the ST-segment elevation) was observed for 30 min through electrocardiography. Thirty minutes later, the ligature was released to restore the blood flow, and the reperfusion was maintained for 24 h. For the sham group, the procedure was the same, except that the LAD was not ligated. Echocardiography was performed to assess cardiac function after 24 h of reperfusion, and the mice were then sacrificed.

Echocardiographic Assessment

In the present study, echocardiography was performed to assess cardiac function with an ultrasound probe (L8-18i-D PROBE, GE Healthcare, Boston, MA, USA). Briefly, mice were anesthetized with 2% isoflurane and placed on a handling platform, and the fur was carefully removed from their chests with a depilatory cream. The ultrasound probe was placed at the level of the parasternal mitral valve to image the heart in the transverse plane. Subsequently, the end-systolic volume, end-diastolic volume, systolic left ventricular internal diameter, and diastolic left ventricular internal diameter were measured with M-mode. Left ventricular ejection fraction (LVEF) and left ventricular fractional shortening values, converted and calculated with systolic and diastolic left ventricular internal diameters, were used as cardiac function indexes. The echocardiographic assessment was performed three times, and the average value was taken. All data were collected by investigators blinded to the experimental groups.

Myocardial Enzyme Determination

Blood samples were collected and centrifuged for 15 minutes. Myocardial injury was evaluated on the basis of the serum expression of troponin (cTn-T) and lactate dehydrogenase (LDH). These biochemical parameters were assessed with an ELISA kit (Solarbio, Beijing, China) according to the manufacturer's instructions.

Morphological Analysis

After myocardial I/R, the mice were sacrificed, and their hearts were harvested and immersed in 4% paraformaldehyde for at least 24 hours at room temperature, then dehydrated in ethanol gradients,

cut transversely into 5 µm thick sections, and deparaffinized with xylene. After hematoxylin and eosin staining, the myocardial structure was observed under a Leica DM4B microscope.

For assessment of the myocardial I/R area, at the end of myocardial I/R, the mouse hearts were rapidly harvested and frozen at −20 °C for 30 min, then sequentially cut into 1 mm thick sections from the ligature, which were then incubated in 1.0% 2,3,5-triphenyl tetrazolium chloride (TTC), pH 7.4, for 30 minutes away from light at 37 °C. The sizes of the total area and the myocardial infarction area were evaluated in ImageJ software, and the results were calculated as myocardial infarction area/total area $\times$ 100%.

For examination of the effects of STING on apoptosis, slides were permeabilized with 0.1% Triton X-100. Subsequently, dT-UTP nick end labeling (TUNEL) assays were performed with a one-step TUNEL kit purchased from Beyotime Institute of Biotechnology, according to the manufacturer's instructions.

For visualizing the cardiac expression of p-IRF3, slides were permeabilized with 0.1% Triton X-100, and 10% goat serum in PBS was used to block nonspecific antibody binding sites. Subsequently, slides were incubated with primary antibodies to phospho-IRF3 (1:100) in PBS with 10% goat serum overnight at 4 °C, then incubated with the corresponding CY3-conjugated secondary antibodies for 1 hour at room temperature. Images were taken with a fluorescence microscope (Leica DM4 B).

Cell Culture and Treatment

The HL-1 mouse cardiomyocyte cell line was cultured in HL-1 cell culture medium at 37 °C with the following air conditions: 94% N_2, 5% CO_2, and 1% O_2. After establishing a hypoxia-re-oxygenation injury and hyperglycemia model, and administering the STING inhibitor H-151, we analyzed the protein changes at the cellular level. Briefly, the cells were cultured in medium with a glucose concentration of 33 mmol/L for 48 h to establish a high glucose model; the STING inhibitor H151 (2UM, 6 h) was then administered to inhibit the expression of STING; simultaneously, the cells were cultured in a hypoxic chamber (95% N_2 and 5% CO_2, 37 °C) and glucose-free DMEM for 2 h to simulate myocardial ischemia, then subjected to reoxygenation in

normal medium in a standard incubator (5% CO_2, 37 °C) for 3 h to establish the hypoxia-re-oxygenation injury and hyperglycemia model. The cells in the sham group were cultured for the same time at 37 °C under 5% CO_2, in 5.5 mmol/L glucose and 27.5 mmol/L mannitol. After culturing, the proteins were analyzed by western blotting.

Western Blot Assays

Sample tissues and HL-1 cells were lysed in RIPA lysis buffer for 30 min and centrifuged at $12,000 \times g$ for 15 min. Bradford assays were used to determine protein concentrations. Western blotting was conducted to analyze proteins. Briefly, the samples were separated and transferred to polyvinylidene fluoride membranes (Invitrogen), which were then blocked with 5% albumin in TBST and incubated with primary antibodies against STING (19,851; Proteintech, USA; 1:1000), IRF-3 (11,904; Cell Signaling Technology, USA; 1:1000), p-IRF3 (AF3438, Affinity, USA; 1:1000), IL-1β (12,703, Cell Signaling Technology, USA; 1:1000), TBK (DF7026, Affinity, USA; 1:1000), p-TBK (AF8190, Affinity, USA; 1:1000), GAPDH (60,004-1-Ig, Proteintech, USA; 1:10,000), or TNF-α (AF7014, Affinity; 1:1000) overnight at 4 °C and then with secondary antibodies for 1.5 h. Western blots were quantified in Quantity One Software.

Statistical Analysis

Data from at least three experiments are presented as means ± standard deviation (SD). All analyses were performed in GraphPad Prism 9.0 software. One-way analysis of variance was used to analyze the differences among three or more groups. A P value < 0.05 was considered statistically significant.

Results

Diabetes Exacerbates STING-Mediated Cardiac Inflammation

We first measured the activation of the STING cascade and inflammation. As shown in Figure 1A–C, STING and p-IRF3 were significantly upregulated in I/R hearts. The immunofluorescence assays consistently revealed elevated p-IRF3 expression in I/R hearts (Figure 1D), thus indicating activation of the STING cascade. In addition, diabetes further potentiated activation of the STING cascade, as indicated by significantly upregulated STING and p-IRF3 expression (Figure 1A–D). Next, we evaluated the expression of inflammatory cytokines. Diabetes was found to aggravate the inflammatory response caused by I/R, and to promote the expression of IL-1β, TNF-α, and IFNβ (Figure 1A and E–G).

STING Knockout Improves Cardiac Function in Diabetic I/R Mice

The cardiac function in mice after I/R injury was evaluated through echocardiography. As shown in Figure 2, LVEF and left ventricular fractional shortening decreased after I/R treatment in diabetic mice, and silencing of STING improved cardiac function. These results indicated the protective effects of STING knockout in diabetic mice subjected to I/R injury.

STING Knockout Alleviates I/R Injury in Diabetic Mice

TTC staining was conducted to visualize myocardial infarct sizes. Compared with control mice, the infarct sizes were significantly larger in diabetic mice subjected to I/R. However, STING knockout was associated with a significantly diminished myocardial infarct size in diabetic mice (Figure 3A, B). In accordance with the TTS staining results, the increase in serum cTn-T and LDH levels was diminished by STING knockout (Figure 3C, D).

Hematoxylin and eosin (HE) and TUNEL staining (Figure 3E) were conducted to visualize the severity of myocardial tissue damage and apoptosis. Substantial inflammatory cell infiltration was observed with increased TUNEL-positive cells in mice undergoing I/R injury, as compared with sham mice. Furthermore, this finding was pronounced in diabetic mice and was attenuated in STING^{-/-} diabetic mice (Figure 3E).

STING Knockout Mitigates the Inflammatory Response in Diabetic Mice Subjected to I/R Injury

The diabetic mice exhibited upregulated p-IRF3 and p-TBK expression, thus suggesting the activation
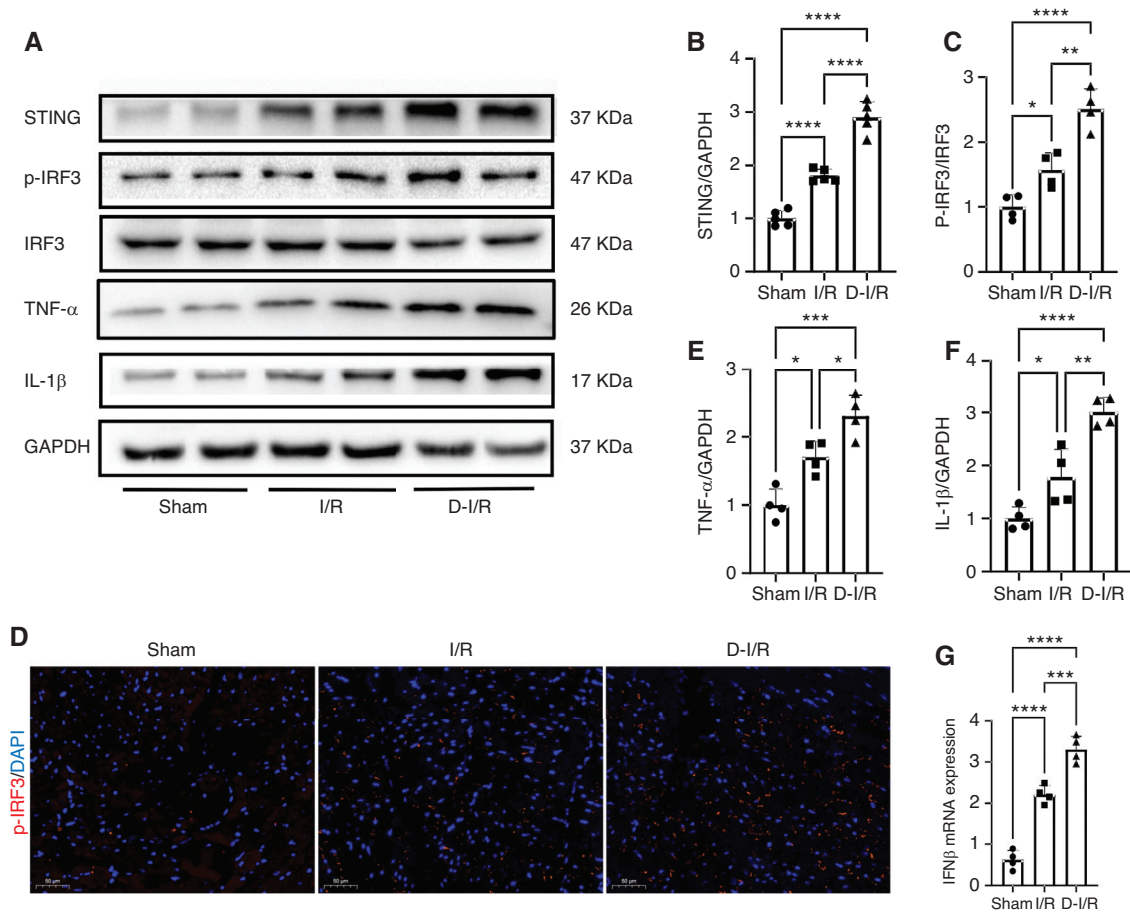
Figure 1 Diabetes Aggravates STING-Mediated Inflammation in Hearts.
(A) Expression of STING, p-IRF3, TNF-α, and IL-1β, measured by western blotting. (B, C) Greater expression of STING and p-IRF3 in the D-I/R group than the I/R group. (D) Expression of p-IRF3, measured by immunofluorescence assays. (E, F) Greater expression of TNF-α and IL-1β in the D-I/R group than the I/R group. (G) Greater mRNA expression of IFNβ in the D-I/R group than the I/R group. Data are expressed as means ± SD, n = 4. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$.

of STING-mediated inflammation, and exhibited upregulated IL-1β expression, thus indicating elevated inflammation (Figure 4A–D). However, STING knockout decreased STING-mediated inflammation and was associated with decreased expression of IL-1β and TNF-α (Figure 4E). These results suggest that silencing of STING mitigates I/R-induced inflammatory reaction in the diabetic mouse model.

Inhibition of STING Alleviates the Inflammatory Response *In Vitro*

High glucose and hypoxia-reoxygenation induced the activation of inflammation and the STING pathway *in vitro*, as evidenced by an increase in p-TBK, p-IRF3 and IL-1β expression (Figure 5A–D). However, inhibition of STING decreased the expression of IL-1β (Figure 5D), thus suggesting that STING inhibition directly alleviates inflammation *in vitro*.

Discussion

The present study demonstrated that the STING pathway is activated in the I/R myocardium under diabetic conditions, thereby leading to inflammation. STING knockout inhibits inflammatory response and decreases I/R injury, thus improving cardiac function in diabetic mice. These results provide preliminary evidence of the critical role of the STING pathway in diabetic I/R injury and indicate that targeting STING may be
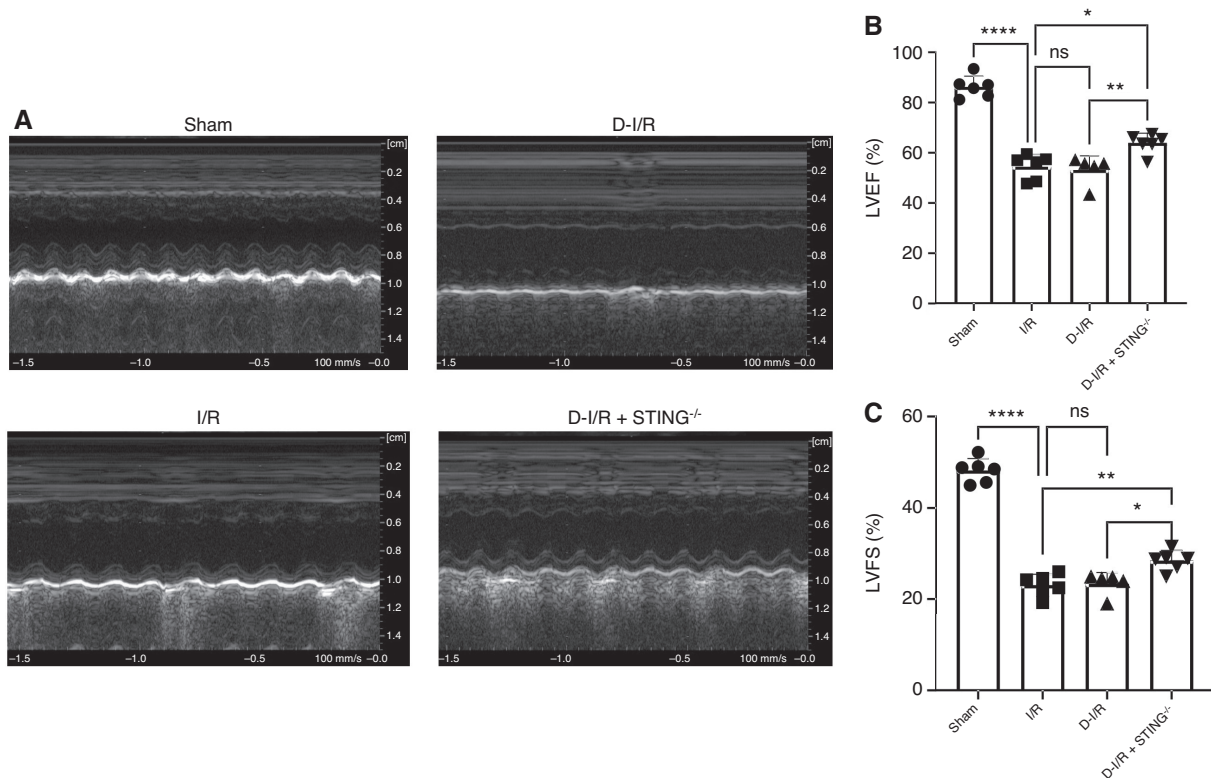
Figure 2 STING Knockout Improves Cardiac Function in Diabetic I/R Mice.
Mice were divided into sham, I/R, diabetic I/R, and diabetic I/R + STING knockout groups. Twenty-four hours after reperfusion, the mice were lightly anesthetized with isoflurane (2%), and echocardiography was performed immediately to assess cardiac function. (A) Representative M-mode images of echocardiography for each group. (B) Statistical analysis of the cardiac function index LVEF (%). (C) Statistical analysis of the cardiac function index FS. Data are expressed as means ± SD, n = 6. *P < 0.05, **P < 0.01, ****P < 0.0001 compared with each other.

a promising potential therapy for diabetic cardiac complications.

Type 2 diabetes is well-established as the most common type of diabetes [18]. Current evidence suggests that a high dose of STZ leads to pancreatic β cell necrosis and induces type I diabetes in mice. In contrast, low-dose STZ injection reduced insulin secretion, and consumption of an HFD results in insulin resistance, which simulates the metabolic characteristics of human type 2 diabetes [19]. The present study established a mouse diabetes model mimicking human type 2 diabetes by feeding of an HFD for 4 weeks, followed by a low-dose injection of STZ for seven consecutive days [20]. In addition, we ligated and released the LAD to induce an I/R model in diabetic mice. Greater serum levels of LDH and cTn-T, and myocardial damage, were observed in the I/R diabetic mouse model than the sham group, thus suggesting that the heart was more vulnerable to I/R injury in the diabetic mice.

STING, the stimulator of interferon genes, is an endoplasmic reticulum membrane localized adaptor protein. An increasing body of evidence suggests that activating the STING pathway is a crucial step in the inflammatory response. STING can be activated by endoplasmic reticulum stress, mitochondrial DNA, or viral infection [21, 22]. TANK binding kinase (TBK) directly binds STING, thereby forming the STING signalosome, which phosphorylates interferon regulatory factor 3 [23]. P-IRF3 is translocated to the nucleus, where it regulates transcription of type 1 interferons and decreases inflammation [24–26]. Inflammation has recently been reported to play important roles in I/R injury, and inhibition of inflammation has been reported
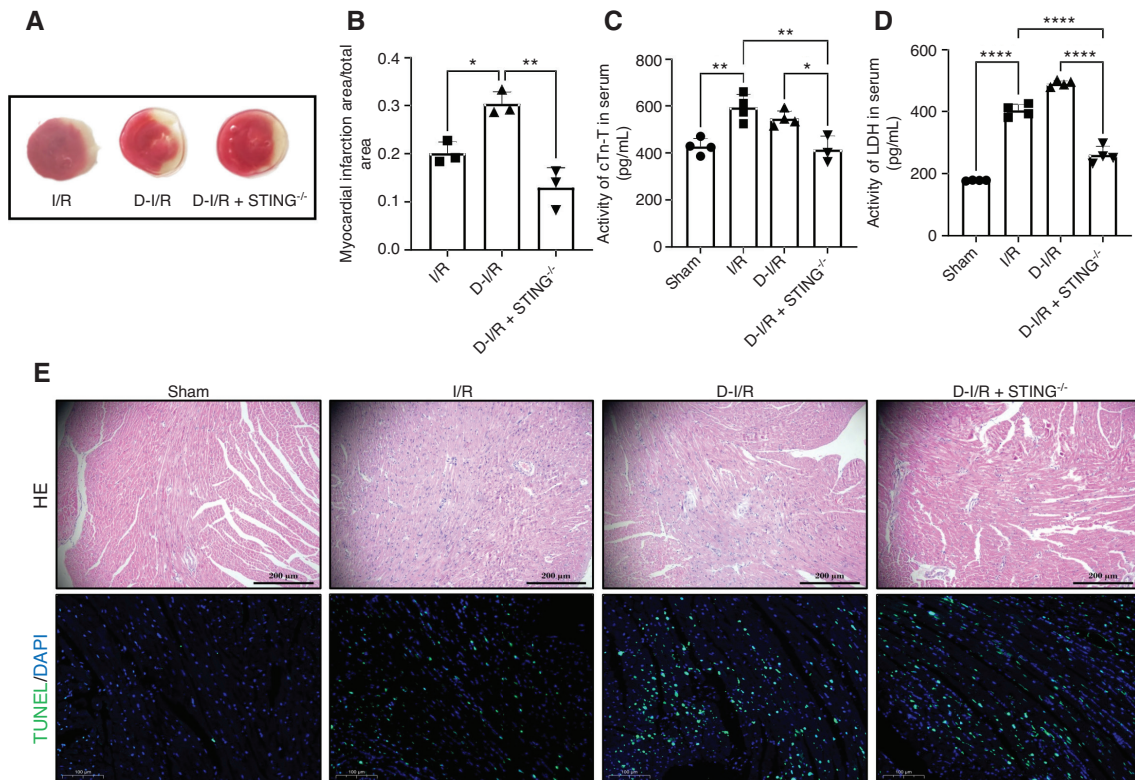
Figure 3 STING Knockout Alleviates I/R Injury in Diabetic Mice.
(A) Area of TTC staining. (Representative images of cardiac sections stained by TTC. The non-infarcted region is red, and the infarcted region is white.) (B) Statistical analysis of myocardial infarct size. (C) Statistical analysis of LDH in the serum. (D) Statistical analysis of cTn-T in the serum. (E) Representative images of HE staining and TUNEL staining for each group. Scale bars: 100 μm and 200 μm. Data are expressed as means ± SD, n = 6. *P < 0.05, **P < 0.01, ****P < 0.0001.

to significantly alleviate I/R injury in the myocardium [27, 28]. The STING-IRF3 pathway has been demonstrated to play a pathogenic role in pressure overload-induced heart failure [29], and STING knockout has been observed to provide some protection against inflammation in diabetic mice [30]. In myocardial infarction, blocking the STING-IRF3 signaling pathway decreases the cell infiltration level of inflammatory factors in myocardial tissue and improves cardiac function [31]. Another study has consistently indicated that 3 weeks of pharmacological STING inhibition decreases myocardial hypertrophy and improves cardiac function in I/R mice [32]. In the present study, we observed an increase in cardiac STING activation in diabetic mice after I/R injury, thus suggesting the important role of STING in mediating I/R injury in diabetes. STING knockout significantly decreased the inflammatory response and inhibited the cardiac levels of LDH and cTn-T in diabetic mice with I/R injury. Moreover, STING knockout significantly mitigated myocardial injury and apoptosis, and alleviated cardiac dysfunction caused by I/R in diabetic mice. These results substantiate the protective effects of STING knockout in diabetic mice subjected to I/R injury.

Our findings demonstrated that myocardial I/R led to more severe heart muscle damage and inflammation in diabetic than control mice. Knockout of STING decreased IL-1β and TNF-α expression in the myocardium and attenuated myocardial damage in diabetic mice. These results suggested that STING-mediated inflammation plays a crucial role in I/R injury in diabetes. Consequently, targeting the STING pathway and anti-inflammatory treatment may improve cardiac function in diabetes with I/R injury. However, the present study has several limitations. First, we used HE staining to visualize
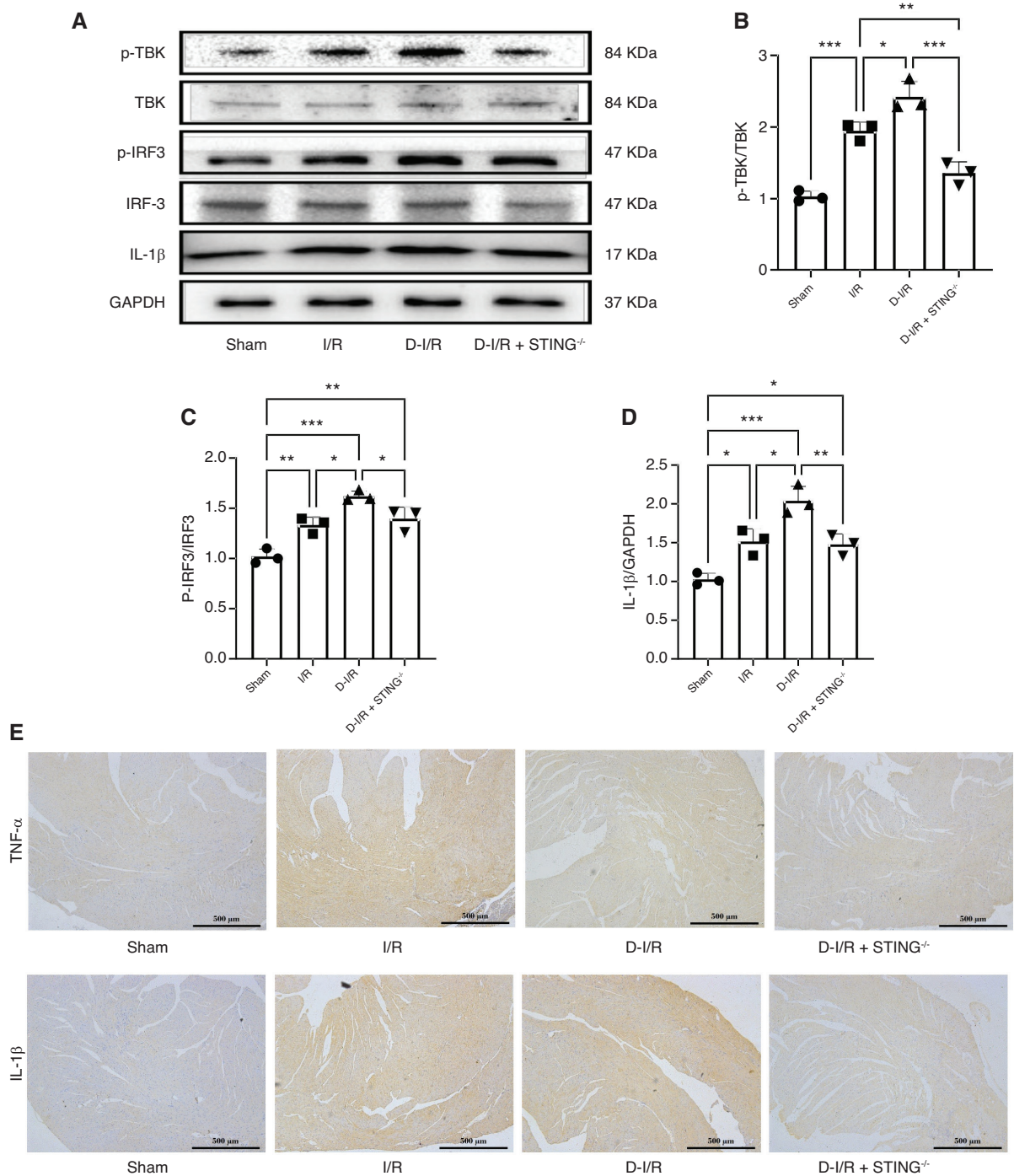
Figure 4 STING Knockout Mitigates the Inflammatory Response in Diabetic Mice Subjected to I/R Injury.
Mice were treated as described in Figure 2. (A) Representative western blotting results of p-TBK, p-IRF3, and IL-1β.
(B) Statistical analysis of p-TBK/TBK in each group. (C) Statistical analysis of p-IRF3/IRF3 in each group. (D) Statistical
analysis of IL-1β/GAPDH in each group. (E) Immunohistochemical results of TNF-α and IL-1β. Data are expressed as means
± SD, n = 3. *P < 0.05, **P < 0.01, ***P < 0.001.

the myocardial injury induced by I/R injury, as validated by transmission electron microscopy. Furthermore, we focused on the effects of STING knockout on inflammation in the present study. However, the potential mechanisms responsible for the protective role of STING knockout warrant further study. Moreover, we used mice with global STING knockout to investigate the role of STING. Whether STING affects both cardiomyocytes and non-cardiomyocytes remains unclear.
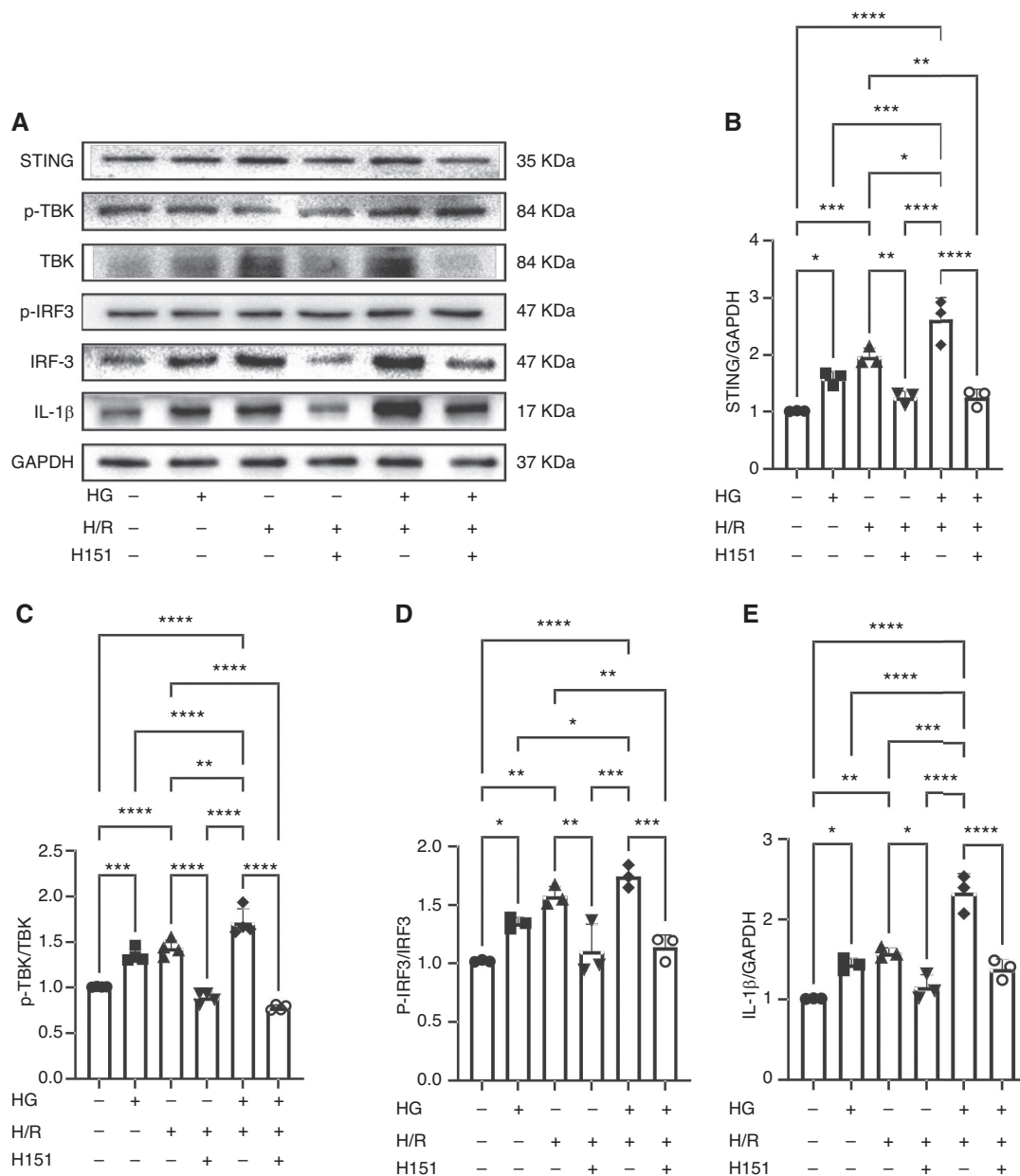
Figure 5 Inhibition of STING Alleviates the Inflammatory Response *In Vitro*.
(A) Representative western blotting results of p-TBK, p-IRF3, STING, and IL-1β. (B) Statistical analysis of STING/GAPDH in each group. (C) Statistical analysis of p-TBK/TBK in each group. (D) Statistical analysis of p-IRF3/IRF3 in each group. Data are expressed as means ± SD, n = 3. *P < 0.05, **P < 0.01, ***P < 0.001. (E) Statistical analysis of IL-1β/GAPDH in each group. Data are expressed as means ± SD, n = 4. *P < 0.05, **P < 0.01, ***P < 0.001,****P < 0.0001.

Conclusions

Our research revealed the essential role of STING in regulating myocardial I/R injury and inflammation in diabetic mice. STING knock-out prevents inflammation and alleviates I/R injury in diabetic mice. Overall, our findings suggest that targeting STING may be a promising therapeutic approach for myocardial I/R in diabetes.

Declarations

Ethics approval and consent to participate

The animal study was reviewed and approved by the Animal Ethic Committee of Chongqing Medical University.

Consent for publication

Not applicable.

Availability of data and materials

All authors confirm that the data supporting the findings of the study are available on reasonable request.

Competing interests

The authors declare that they have no conflicts of interest.

Funding

Authors' contributions

Yuce Peng, Guoxiang Zhou, and Mingyu Guo performed experiments. Yuce Peng performed echocardiography and analyzed related indexes. Guoxiang Zhou and Mingyu Guo assisted in western blot experiments. Yuce Peng assisted in statistical analyses. Mingyu Guo assisted in data analysis. Yuce Peng, Guoxiang Zhou, and Yongzheng Guo wrote the manuscript. Yongzheng Guo conceptualized and designed the study. Suxin Luo and Yongzheng Guo contributed to supervision of the study. The final version of this manuscript was reviewed and revised by all authors, and all authors approved its submission.

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Supplementary material: Supplementary material is available online at the following link: https://cvia-journal.org/wp-content/uploads/2023/04/supplementary_Inhibition-of-stimulator-of-interferon-genes-protects-against-myocardial-.pdf