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Original Article

The downregulation of CD47 attenuates apoptosis and inflammation by inhibiting iNOS activity in a rat model of myocardial ischemia/reperfusion injury

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Abstract: Objective: Apoptosis and inflammation play critical roles in myocardial ischemia/reperfusion (I/R) injuries (MIRI). Various studies have demonstrated that CD47 can aggravate apoptosis and inflammation in various pathological processes. The purpose of the present study was to verify whether the downregulation of CD47 exerts a protective effect against myocardial apoptosis and inflammation during MIRI. Methods: Adult SD rats (n=60) were divided into 4 groups as follows: The sham group, the I/R group, the Ad-Control group, and the Ad-siCD47 group. A myocardial I/R model was established three days after the gene delivery. CK, The LDH levels were measured and the myocardial histomorphology was assessed to evaluate the myocardial damage. The infarct area was assessed using Evans Blue/TTC staining. A TUNEL assay was performed to measure the apoptosis. ELISA kits were used to measure the inflammatory mediator levels, such as IL-1β, IL-6, and TNF-α. The NO expressions and the iNOS activity were also measured. The iNOS, Bax, and caspase-3 protein expression levels were evaluated using western blot. The related mRNA levels were evaluated using RT-qPCR. Results: The results revealed that Ad-siCD47 significantly decreased the CD47 expression. In addition, with the downregulation of CD47, the infarct size, the CK and LDH levels, and the Bax, caspase-3, IL-1β, IL-6, and TNF-α expressions, as well as the iNOS activity and the NO content were significantly reduced. Conclusions: The downregulation of CD47 reduces apoptosis and inflammation by inhibiting the expression and activity of iNOS, so it may be a therapeutic target for MIRI.

Keywords: CD47, siRNA, myocardial ischemia/reperfusion injury, apoptosis, inflammation, inducible nitric oxide synthase

Introduction

Although timely revascularization for acute myocardial infarction (AMI) has significantly reduced its morbidity and mortality, myocardial ischemia/reperfusion (I/R) injury (MIRI) still remains a major obstacle that contributes to the further deterioration of heart function post-AMI, including arrhythmia, heart failure, etc. [1, 2]. Hence, the identification of effective strategies for minimizing MIRI to achieve the maximal cardioprotective effects is essential [3]. MIRI is a complicated process, and it has been proven that apoptosis and inflammation play central roles [4]. Although the precise mechanisms involved in MIRI are not yet fully understood, exploring new approaches to reduce apoptosis and inflammation may provide a novel therapeutic approach in the treatment of MIRI.

CD47 is a cell surface receptor of the immunoglobulin superfamily that can interact with the matricellular protein ligand thrombospondin-1 (TSP-1) [5]. CD47 is closely related to cell survival and death by influencing different cellular pathways. The disrupted expression of CD47 can regulate oxidative stress, autophagy, apoptosis and inflammation in multiple diseases, such as cancer [6, 7], immune diseases [8], atherosclerosis [9], pulmonary hypertension [10, 11], etc. Recently published studies have highlighted the potential of CD47 blocking strategies in renal and liver I/R injuries by inhibiting oxidative stress, apoptosis and inflammation [12-15]. In addition, Wang *et al.* [16] and Li *et al.* [17] also indicated that CD47 blockades significantly reduce MIRI by attenuating oxidative stress or by rescuing autophagic clearance. Hence, an acute CD47 blockade

during myocardial I/R may also enhance cardiac repair. However, the effects of CD47 blockades on MIRI have not been fully elucidated so further research is required to elucidate the physiological functions and underlying mechanisms of CD47 blockade in MIRI.

Studies have indicated that inducible NOS (iNOS) expressions are upregulated following I/R injury. The elevated levels of iNOS then lead to the production of NO and the formation of peroxynitrite, thereby causing tissue damage [15]. In addition, iNOS has also been confirmed to be a driver of inflammation and apoptosis, as it can induce the expression of pro-inflammatory cytokines and apoptosis-related proteins in response to environmental stimuli [18, 19]. Notably, unpublished data from the authors' laboratory have confirmed that iNOS expression is significantly increased in the I/R-injured myocardium but is reduced following the downregulation of CD47. However, whether the downregulation of CD47 can decrease inflammation and apoptosis by suppressing iNOS during MIRI remains largely unknown.

The present study was undertaken to investigate whether the CD47 blockade attenuates the expression of the apoptosis-related proteins, Bax and caspase-3, and the expressions of the inflammatory mediators, IL-1β, IL-6, and TNF-α, in MIRI, and the role of iNOS in this process.

Materials and methods

Animals and animal care

Adult SD rats (male, weighing 220-250 g) were purchased from the Medical School of Nanjing University. The procedures for the experiments and the animal care were approved by the Animal Care and Use Committee of the Medical School of Nanjing University and conformed to the *Guide for the Care and Use of Laboratory Animals* by the National Institutes of Health.

Short interference RNA (siRNA)

A lentiviral (RNA interference) vector against CD47 was synthesized and generated using the AdMax system (Microbix Biosystems). The acquired lentivirus was then packaged and amplified with 293 cells. The final virus concentration was 1×10^{11} PFU.

Establishment of a rat model of MIRI

A total of 60 SD rats were divided into a sham-operated group (the sham group, n=15), an I/R group (the I/R group, n=15), a lentivirus expressing GFP group (the Ad-Control group, n=15), and a lentivirus expressing CD47-siRNA group (the Ad-siCD47 group, n=15). A rat model of myocardial I/R was then established. Briefly, pentobarbital (30 mg/kg; intraperitoneally) was used to anesthetize the rats and their chests were then opened. Ad-Control, Ad-siCD47, and saline (80 μl solution, respectively) were injected into the rats' heart walls. After 3 days, the rats' chests were re-opened, and ligations of the left anterior descending arteries (LAD) were performed under anesthesia. After 30 min, the rats' circulation was restored for approximately 4 h. At 4 h post-reperfusion, the rats were re-anesthetized with 3% sodium pentobarbital (30 mg/kg; intraperitoneally) and blood was collected from their jugular veins. Subsequently, 10% potassium chloride (75 mg/kg) was injected via the jugular vein for euthanasia (the rats were still under anesthesia at this time, and the rats weighed 300-320 g). Finally, the rats' hearts were harvested. The sham group was used as a control with no occlusion of the LAD.

Determination of the myocardial enzymes

The blood collected from the jugular veins (approximately 5 ml) was used to measure the myocardial enzyme levels, including creatine kinase (CK) and lactate dehydrogenase (LDH). We used commercially available biochemical kits and followed the instructions provided with the apparatus to measure the LDH and CK levels.

Measurement of the infarct areas (IA)/areas at risk (AAR)

The IAs and AARs were evaluated using Evans blue/TTC staining. Briefly, the rats' LADs were ligated, followed by 4 h of reperfusion. Approximately 1 ml Evans blue (Sigma-Aldrich; Merck KGaA) was then injected intravenously. Subsequently, the rats' hearts were removed and sectioned, followed by incubation with 1.5% TTC (Sigma-Aldrich; Merck KGaA) for a further 15 min. Finally, the risk areas were stained red, but the IA remained white. We used Image-Pro Plus 5.0 software to measure the IA and AAR.

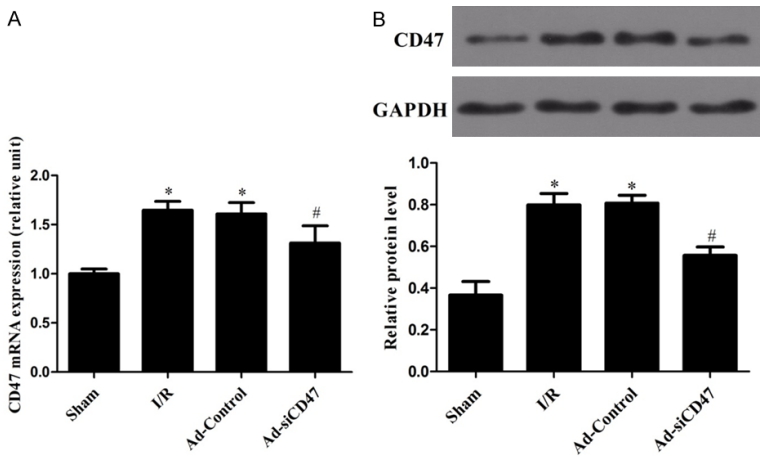

Figure 1. Delivery of the CD47-targeting RNA interference attenuated CD47 levels induced by I/R. A. RT-qPCR was used to measure the mRNA levels of CD47. B. Western blot was performed to measure the CD47 protein levels. *indicates in comparison with the sham group, $P<0.05$; #indicates in comparison with the I/R group or the Ad-Control group $P<0.05$.

Histologic examinations (H&E staining)

The harvested hearts were fixed in formalin and embedded in paraffin. The paraffin-embedded blocks were then cut into sections and stained with H&E. The morphological changes were observed under a lighted microscope.

Determination of the myocardial apoptosis

Following to the manufacturer's instructions, the apoptosis following the reperfusion was measured using TUNEL assays (Roche Applied Science).

Measurement of NO and iNOS activity

According to the manufacturer's instructions, the NO and iNOS activity levels were measured using commercially available kits (Nanjing Jiancheng Bioengineering Institute).

Reverse transcription-quantitative PCR (RT-qPCR)

The mRNA levels were measured as previously described. Briefly, TRIzol reagent was used to obtain total RNA. Subsequently, using the 7500 and ABI Prism systems, qPCR was performed using SYBR-Green Master Mix (Thermo Fisher Scientific, Inc.). The PCR conditions were as follows: 2 min at 50°C, 95°C for 10 min, and immediately following 40 cycles of 95°C for 30 sec and 60°C for 30 sec. The CD47 mRNA expression levels were normalized to those of GAPDH. The primers used in RT-qPCR were as follows: CD47 forward, 5'-AGAAGCCCGTGAAGAACGC-3' and reverse, 5'-CACATCCCGACCACAGCAA-3'; and GAPDH forward, 5'-TGGCCTTCCGTGTTCCTAC-3' and reverse, 5'-GAGTTGCTGTTGAAGTCGCA-3'.

Western blot analysis

The CD47, Bax, caspase-3, and iNOS protein expression levels were measured using western blot. In brief, protein was extracted, and the protein concentration was determined using BCA assays (Beyotime Institute of Biotechnology, Inc.). Subsequently, 10% SDS-PAGE was used to separate the proteins followed by protein transfer onto PVDF membranes electrophoretically (EMD Millipore). Thereafter, the PVDF membranes were incubated with antibodies against CD47 (1:2000, Abcam), Bax (1:1,000, Abcam) caspase-3 (1:1,000, Abcam), and iNOS (1:1,000, Cell Signaling Technology, Inc.) overnight. Finally, an enhanced chemiluminescence system (Thermo Fisher Scientific, Inc.) was used to visualize the protein bands.

Measuring the inflammatory mediators

Following to the manufacturer's instructions, ELISA was employed to measure the IL-1β, IL-6, and TNF-α levels in the heart samples.

Statistical analysis

SPSS 21.0 was used for the statistical analysis. The data are expressed as the means ± SD. Student's t-tests were used for the comparisons between two groups. One-way ANOVA was used for the comparisons between multiple groups, followed by Tukey's post hoc test. $P<0.05$ was considered to indicate a statistically significant difference.

Results

The targeted delivery of CD47-siRNA decreased the CD47 levels induced by I/R

Compared with the sham control group, the CD47 expressions were significantly increased in the I/R group. Of note, following the delivery of siRNA targeting CD47, the CD47 expressions were significantly decreased ($P<0.05$, **Figure 1**).

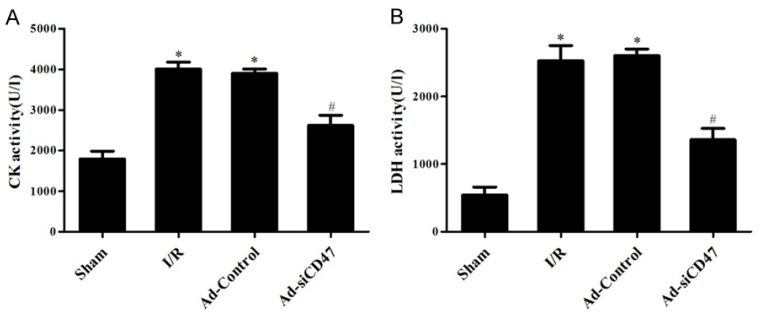

Figure 2. Delivery of the CD47-targeting RNA interference alleviated serum marker enzymes. CK (A) and LDH (B) were apparently reduced after the gene transfer of Ad-siCD47. *indicates in comparison with the sham group, *P*<0.05; #indicates in comparison with the I/R group or the Ad-Control group *P*<0.05.

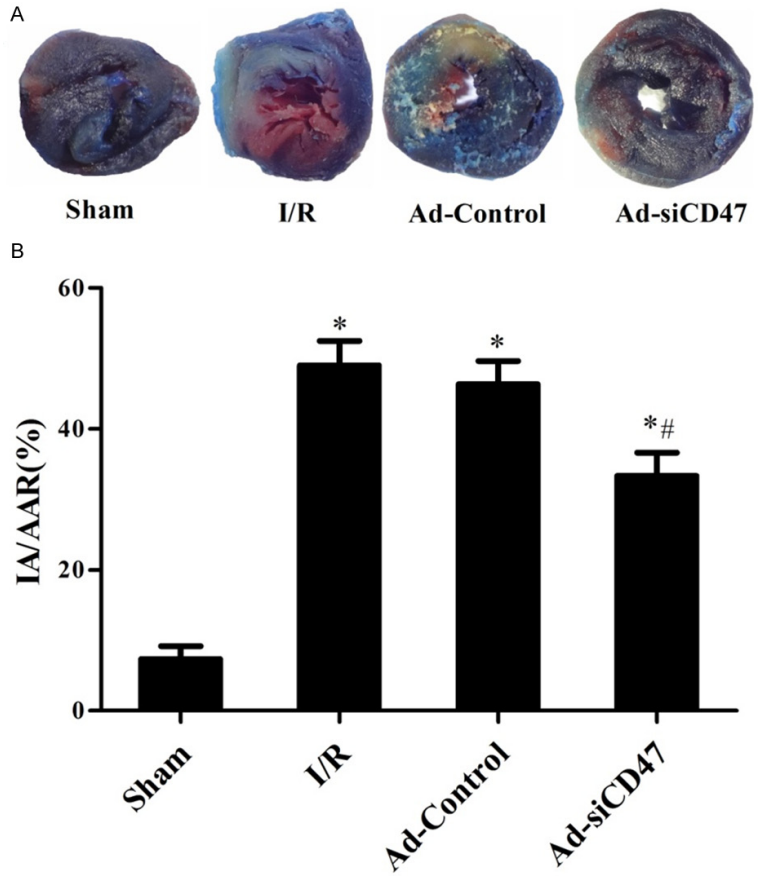

Figure 3. The down-regulation of CD47 decreased the infarct size. A. Representative pictures of Evans and TTC staining. B. IA/AAR was used to evaluate the infarct area. *indicates in comparison with the sham group, *P*<0.05; #indicates in comparison with the I/R group or the Ad-Control group *P*<0.05.

CD47-targeting RNA interference delivery alleviated LDH and CK

LDH and CK are two key markers for myocardial injury. The CK and LDH levels increased significantly in the I/R group. However, after CD47-targeting RNA interference delivery, the CK and LDH levels in the Ad-siCD47 group decreased significantly (P<0.05, **Figure 2**).

Anti-CD47 decreased the infarct area

To quantitatively calculate the myocardial injuries, the IA/AAR was determined as previously demonstrated. The IA/AAR was approximately 49.1± 7.4% in the I/R group. Notably, the CD47 blockade exerted a cardioprotective effect during MIRI. However, no effect was observed in the I/R or Ad-Control groups (P<0.05, **Figure 3**).

Light microscopy evaluation

In the I/R group, the myocardial fibers were disorganized and ruptured, with evident edema. The targeting of CD47 by the siRNA delivery partially reduced the myocardial damage. Furthermore, no effects were observed in the I/R or Ad-Control groups (**Figure 4**).

Anti-CD47 suppressed apoptosis

To determine whether anti-CD47 can attenuate apoptosis during MIRI, TUNEL assays were performed, and the alternations in the Bax and caspase-3 expressions were examined. As shown in **Figure 5**, I/R significantly upregulated the number of the TUNEL-positive cells, as well as the Bax and caspase-3 expressions, compared with the sham group (P<0.05). However, the Anti-CD47 treatment prior to I/R decreased the number of the TUNEL-positive cells and the Bax and caspase-3 expressions compared to the I/R and Ad-Control groups (P<0.05).

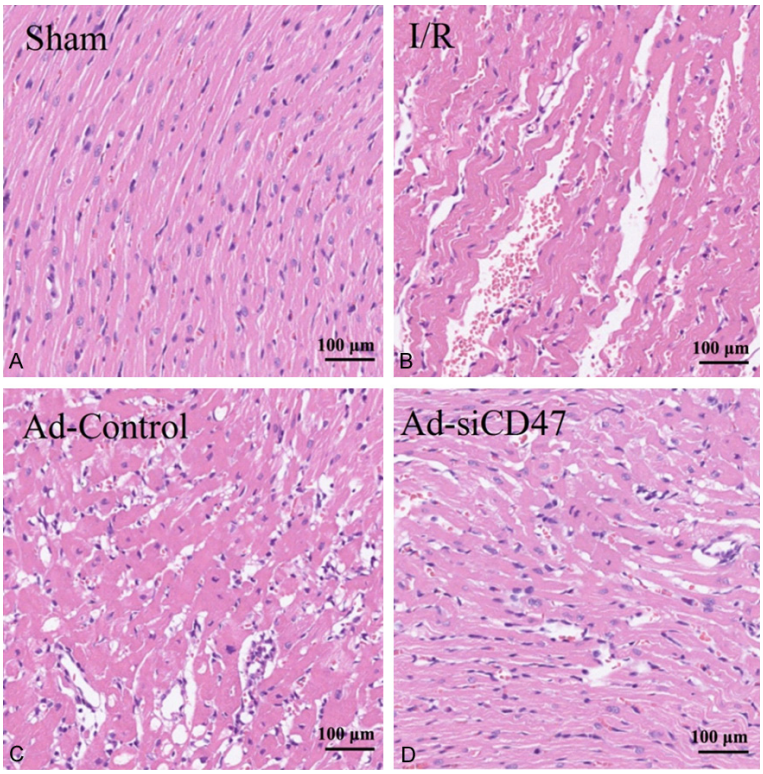

Figure 4. Morphological observation. The myocardial fibers in the sham group are arranged and ordered (A). The myocardial fibers were disorganized, suffered edema, and ruptured in the I/R group (B) and the Ad-Control group (C). Transfection CD47-targeting RNA interference partially reduced the myocardial damage (D). Scale bar, 100 μm.

Determination of the inflammatory mediators

ELISA was used to measure the IL-1β, IL-6, and TNF-α levels. Compared with the sham group, the IL-1β, IL-6, and TNF-α levels were significantly upregulated during MIRI; however, the anti-CD47 treatment attenuated this increase in the expression levels ($P<0.05$; **Figure 6**).

Anti-CD47 inhibited the iNOS activity

In order to explore CD47's possible mechanisms of action during MIRI, the expressions and activity of iNOS and NO were determined. As shown in **Figure 7**, compared with the sham group, I/R significantly upregulated the expression and activity of iNOS ($P<0.05$). However, the anti-CD47 treatment decreased the expression and activity of iNOS compared to the I/R and Ad-Control groups. Moreover, the downregulation of CD47 also reduced the content of NO compared to the I/R group. Therefore, it may be inferred that the I/R injuries were ameliorated by the downregulation of CD47, possibly via the inhibition of the iNOS activity.

Discussion

MIRI involves a number of pathophysiological processes, including apoptosis and inflammation, and it can ultimately lead to cell injury and death. The effective reduction of apoptosis and inflammation is considered a novel therapeutic strategy for MIRI [20]. In recent years, CD47-mediated apoptosis and inflammation have become a research hotspot and have been attracting increased attention [21-24]. However, to the best of our knowledge, no study to date has performed an in-depth analysis of the association between CD47-mediated apoptosis, inflammation and MIRI. In this study, we found that anti-CD47 provided significant protection during ischemic myocardial reperfusion, which was characterized by the upregulation of cell survival and decreased LDH/CK levels, a reduced infarct area, and decreased inflammatory factor levels. In addition, it was observed that the downregulation of CD47 significantly inhibited the expression and activity of iNOS. Our results indicate that the targeting of CD47 in the myocardium following reperfusion may provide a novel therapeutic approach to MIRI.

CD47 has 5 transmembrane domains and is involved in the innate immune response [16]. CD47 can regulate oxidative stress, autophagy, apoptosis, and inflammation following activation by TSP1 [7-9]. Recently published studies have highlighted the potential of CD47 blocking strategies in I/R injury [12-15]. Wang *et al.* [16] and Li *et al.* [17] also indicated that CD47 blockades exert a protective effect against MIRI by attenuating the oxidative stress and rescuing the autophagic clearance. However, to the best of our knowledge, it has not been determined yet whether CD47 blockades reduce apoptosis and inflammation in the

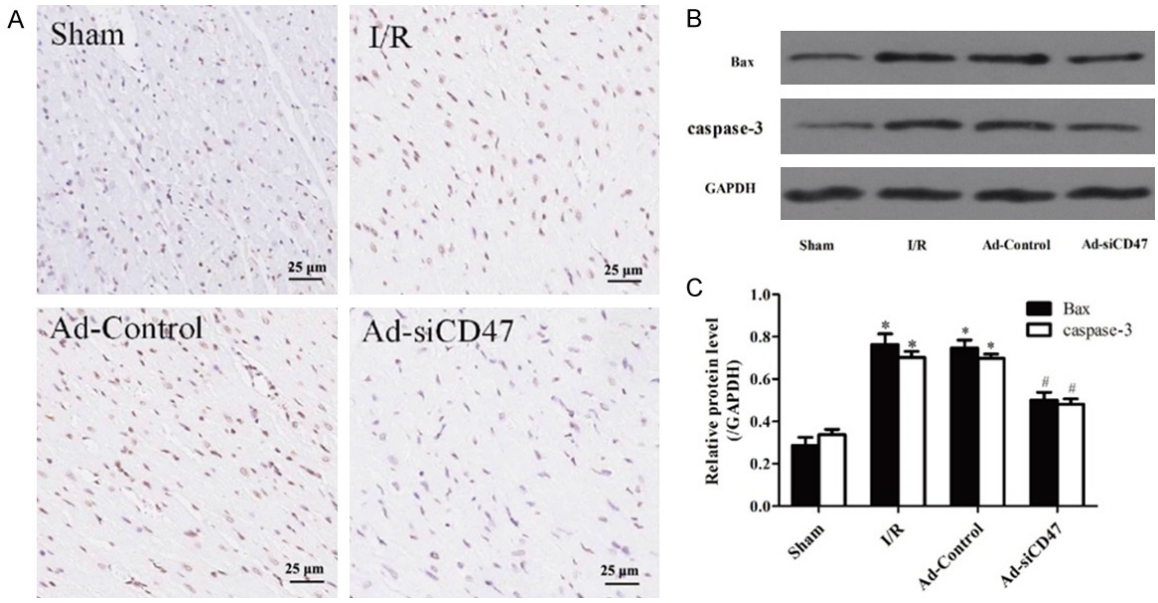

Figure 5. The down-regulation of CD47 reduced the apoptosis. We performed TUNEL assays and measured the alternations in the Bax and caspase-3 levels. The TUNEL-positive cells, Bax, and caspase-3 levels increased sharply but the down-regulation of CD47 was able to reduce (A) the TUNEL-positive cells and (B, C) the Bax and caspase-3 expressions. Scale bar, 25 μm. *indicates a comparison with the sham group, $P<0.05$; #indicates a comparison with the I/R group or the Ad-Control group $P<0.05$.

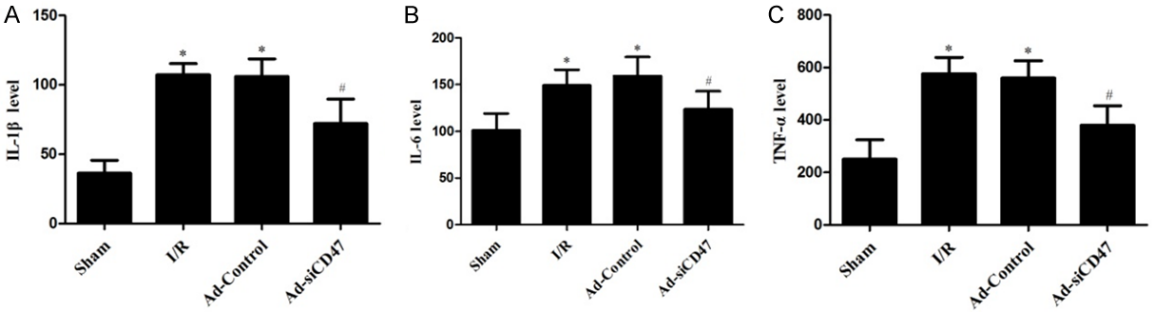

Figure 6. The down-regulation of CD47 reduced the inflammatory response. The ELISA method was used to measure the IL-1β, IL-6, and TNF-α levels in the harvested hearts. The inflammatory factors increased sharply but the down-regulation of CD47 reduced the (A) IL-1β, (B) IL-6, and (C) TNF-α levels. *indicates in comparison with the sham group, $P<0.05$; #indicates in comparison with the I/R group or the Ad-Control group $P<0.05$.

development of MIRI, so the potential mechanisms remain elusive. iNOS is a key molecule that regulates apoptosis and inflammation in various cell types. Nakazawa *et al.* [18] indicated that Sirt1 increased the acetylation (Ac) and activation of p65 NF-κB and p53 via the iNOS signaling pathway, which in turn induced and/or enhanced the inflammatory response and apoptosis, all of which were reversed or ameliorated by the iNOS deficiency. Xiao *et al.* [14, 15] confirmed that iNOS expression was significantly reduced following the treatment with CD47 monoclonal antibodies during liver I/R injury. In terms of the possible involvement of CD47 in the iNOS-related apoptosis and inflammation, it was thus hypothesized that CD47 may also play a pro-apoptotic and pro-inflammatory role in organ I/R insult. Notably, consistent with the aforementioned studies, the present study found that CD47 was not only associated with iNOS overexpression, but also with apoptosis and a persistent inflammatory response; the downregulation of CD47 suppressed the iNOS expression and activity and reduced the apoptosis and inflammation during I/R injury.

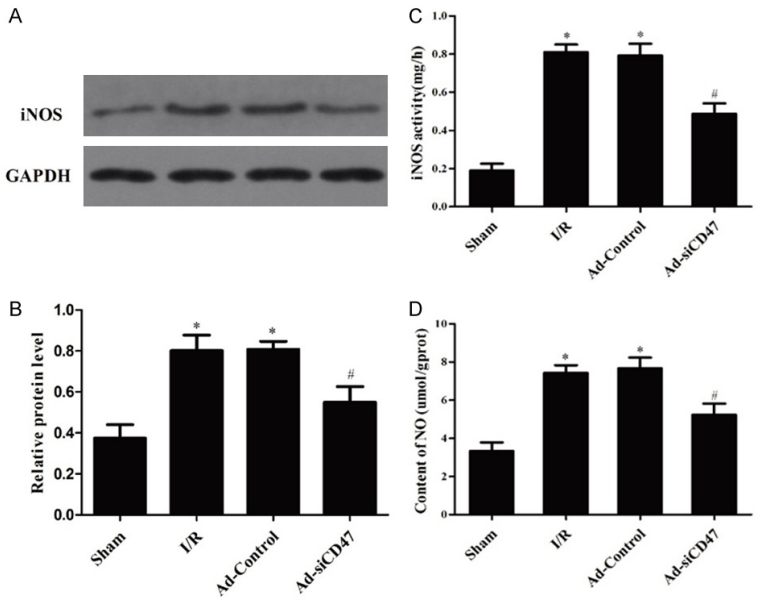

Figure 7. The down-regulation of CD47 inhibited the iNOS activity. In order to explore the underlying mechanism of CD47 in I/R injuries, we determined the expressions and activity of iNOS and NO. The down-regulation of CD47 inhibited the expression and activity of iNOS (A-C). Also, the down-regulation of CD47 was also able to reduce the content of NO compared to the I/R group (D). *indicates in comparison with the sham group, $P<0.05$; #indicates in comparison with the I/R group or Ad-Control group $P<0.05$.

There is increasing evidence to suggest that the consequent elevation of apoptotic cascade events is a crucial mechanism in the pathogenesis of MIRI. In recent years, CD47-mediated apoptosis has become a research hotspot and has been attracting increased attention. In patients with non-small cell lung cancer, Barrera *et al.* [6] indicated that the overexpression of CD47 is strongly related to decreased neutrophil apoptosis/phagocytosis and poor prognoses. In a porcine model of donation, Xu *et al.* [12] and Lin *et al.* [13] demonstrated that a CD47 blockade by treatment with monoclonal antibody reduces I/R injury in renal allografts, partly by decreasing apoptosis. In a rat liver transplantation model, Xiao *et al.* [14, 15] demonstrated that anti-CD47 treatment improved survival and reduced I/R injury, partly by inhibiting apoptosis. Consistently, the data from the present study proved that anti-CD47 plays a crucial protective role in MIRI by decreasing apoptosis, as evidenced by the decreased Bax and caspase-3 levels.

CD47 also controls a number of immunomodulatory genes (including IL-1β, IL-6, and TNF-α) and can activate immune cells to play a pivotal role in inflammation [25]. As a principal mediator of the immune response, TNF-α is involved in inflammatory reactions and cell necrosis. In addition, IL-1β and IL-6 trigger neutrophil recruitment during MIRI [26]. In the present study, the downregulation of CD47 led to significantly lower concentrations of the pro-inflammatory cytokines, such as IL-1β, IL-6, and TNF-α. Therefore, the reduction of these pro-inflammatory cytokines by targeting CD47 appears to reduce heart damage. In turn, the TNF inflammatory pathway can independently contribute to the CD47 expressions that are dependent on the NF-κB signaling pathway and other signaling pathways [21]. Therefore, on the one hand, the downregulation of CD47 reduces the IL-1β, IL-6, and TNF-α levels directly. On the other hand, anti-CD47 also reduces the positive regulation between CD47 and the inflammatory factors.

NO, a bioactive gas produced by the constitutive NOS isoforms, is involved in a number of biological functions which promote tissue perfusion and reduce apoptosis and inflammation. Hence, NO has been used as a cytoprotective element against ischemic damage [16]. Under normal physiological conditions, the heart only expresses eNOS, and small quantities of NO are produced to reduce myocardial injury [16]. Of note, the nitrate circulating levels do not undergo significant changes with low levels of NO. However, abundant levels of iNOS augment NO production under ischemic conditions. The tissue is then damaged by the high levels of NO and peroxynitrite caused by the augmented NO levels [27]. In addition, apoptosis and inflammation are also induced with the enhanced levels of NO during I/R [28, 29]. Furthermore, CD47 has been shown to be a key receptor for TSP-1 influence on the NO signaling pathway and the inhibition of CD47/iNOS/NO can attenuate I/R tissue injuries following ischemic insults [12-15, 18, 19]. In this study, we found

that a CD47 blockade significantly reduced the content of NO compared with the I/R group, and it may be an important mechanism through which the CD47 blockade plays a protective role in MIRI. Of note, the imbalance of the NO amount is also an important mediator of ischemia.

In conclusion, the present study demonstrated that the downregulation of CD47 may alleviate MIRI by reducing apoptosis and inflammation through the regulation of the iNOS/NO pathway. Due to the complexity of the pathophysiological process of MIRI, further studies are required to explore additional signaling pathways which have a close association with CD47 and MIRI. However, the findings of the present study indicate that CD47 may hold promise as a therapeutic target for MIRI.

Disclosure of conflict of interest

None.

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