Maresin 1 alleviates myocardial ischemia-reperfusion injury in rats by suppressing inflammation

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ABSTRACT

Myocardial ischemia-reperfusion injury (MIRI) induces severe inflammatory damage to cardiac tissue, leading to structural impairment and functional decline. Maresin 1 (MaR1) is an anti-inflammatory lipid mediator derived from macrophages that has shown protective effects in various inflammatory conditions. This study investigated the anti-inflammatory properties and underlying mechanisms of MaR1 in the context of MIRI, both *in vivo* and *in vitro*. A rat model of MIRI was established, and MaR1 was administered subcutaneously once daily for one week prior to model induction. Cardiac function was monitored intraoperatively, and serum and myocardial tissue samples were collected postoperatively for analysis. Structural alterations, myocardial injury biomarkers, and inflammatory cytokines were evaluated. *In vitro* experiments using H9c2 rat cardiomyocytes assessed the effects of MaR1 on cell viability and proliferation. MaR1 treatment significantly improved cardiac function impaired by MIRI, preserved myocardial architecture, and reduced serum and tissue levels of creatine kinase, lactate dehydrogenase, cardiac troponin I, and pro-inflammatory cytokines (IL-1β, IL-6, IL-8, MCP1, and TNF-α). In contrast, MaR1 enhanced the expression of the anti-inflammatory cytokine IL-10. In cultured cardiomyocytes, MaR1 promoted viability and proliferation. Collectively, these findings demonstrate that MaR1 confers protection against MIRI by attenuating inflammation, preserving myocardial structure, improving cardiac function, and enhancing cardiomyocyte survival, underscoring its potential as a therapeutic agent for ischemic cardiac injury.

Key words: Maresin 1; myocardial ischemia-reperfusion injury; inflammation.

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Contributions: all authors made a substantive intellectual contribution, read and approved the final version of the manuscript and agreed to be accountable for all aspects of the work.

Conflict of interest: the authors declare no competing interests and all authors confirm accuracy.

Ethical approval: this study was approved by the Animal Experiment Ethics Committee of the Fourth Affiliated Hospital, Guangzhou Medical University (approval no. S2024-592).

Availability of data and materials: the datasets used and/or analyzed during the current study are available upon reasonable request from the corresponding author.

Funding: this work was supported by the Guangzhou Basic and Applied Basic Research Foundation (202201010796).

Introduction

Acute myocardial infarction is a major cause of death from cardiovascular diseases.[1] In recent decades, with the development of interventional technology, timely opening of the infarct-related arteries and restoring the blood supply of the ischemic myocardium has become the key to saving the dying myocardium. Therefore, the prognosis and quality of life of the patients are improved to a great extent.[2] However, reperfusion of cardiac muscle after ischemia for a period of time will lead to myocardial ischemia-reperfusion injury (MIRI), resulting in increased damage to myocardial structure and function.[3] Severe MIRI can induce myocardial stunning, decreased heart function and malignant arrhythmia.[4] There are many evidences showing that MIRI was accompanied by acute inflammatory response, which involved a variety of cytokines.[5] The inflammatory response caused by MIRI continuously releases pro-inflammatory cytokines such as tumor necrosis factor-α (TNF-α), interleukin (IL)-6, reactive oxygen species (ROS) and neutrophils.[6] Studies have shown that MIRI leads to an increase in the expression of TNF-α and IL-6, and antagonizing TNF-α and IL-6 can promote the recovery of cardiac function after ischemia-reperfusion.[7]

Lipid mediators derived from polyunsaturated fatty acids have recently attracted great attention as regulators of inflammation in MIRI. Resolvins, protectins, and lipoxins have been reported to exert cardioprotective effects by attenuating inflammatory cell infiltration, reducing oxidative stress, and improving cardiac function in experimental models of MIRI.[8] These findings highlight the therapeutic potential of pro-resolving lipid mediators in limiting ischemia-reperfusion damage.

Maresin is a class of pro-resolution mediators derived from docosahexaenoic acid (DHA) synthesized by macrophages and can promote the resolution of inflammation.[9] Under inflammatory stimulation, DHA is catalyzed by 14-lipoxygenase to synthesize intermediate metabolites 14S-hydro(peroxy)-4Z, 7Z, 10Z, 12E, 16Z, 19Z-DHA (14S-HpDHA), and 14S-HPDHA further condenses under the action of 13/14-cyclooxygenase to form Maresin.[10] Mass spectrometry was used to analyze the anti-inflammatories in mice, and the results confirmed that the Maresin synthesis pathway marker 14S-HpDHA reached the peak during the inflammation resolution period, indicating that Maresin can rebuild the homeostasis.[10] Maresin 1 (MaR1) is the first chemical isomer of Maresin.[11] An *in vitro* experiments have confirmed that MaR1 can inhibit the infiltration of neutrophils, promote the chemotaxis and phenotypic transformation of macrophages, enhance their phagocytic function, stimulate tissue regeneration, control pain, reduce inflammation and promote the reduction of inflammation from multiple links.[12] However, there is lack of relevant research about anti-inflammatory effect of MaR1 on MIRI.

Therefore, we constructed a rat MIRI model to investigate the effect of MaR1 on rat myocardial inflammation and further explore the mechanism of MaR1 *in vitro*.

Materials and Methods

Animals

All animal experiments have complied with ARRIVE guidelines. A total of 60 rats were kept in our hospital Experimental Animal Center. The rats were randomly divided into sham group, MIRI group and MIRI+MaR1 (4 ng/g) group.[13] Forty rats were used to make MIRI models, of which 2 died during or after modeling (1 in MIRI group, 1 in MIRI+MaR1 group). Rats in the MIRI+MaR1 group were injected with MaR1 subcutaneously daily one week before modeling. Rats in the sham group were only exposed to thorax without coronary artery ligation.

This study was approved by the Animal Experiment Ethics Committee of the Fourth Affiliated Hospital, Guangzhou Medical University (approval no. S2024-592).

Establishment of MIRI model

After being fasted for 12 h, the rats were anesthetized by intraperitoneal injection of sodium pentobarbital (50 mg/kg). The anesthetized rat was fixed on the experimental table. The extremities of the rat were inserted with ECG electrodes and connected to the ECG machine. The fur on the neck and chest of the rat was removed and disinfected. After the rat's neck skin was cut, we used a small animal ventilator (CWE SAR-830; CWEW Inc., Orange, CA, USA) to connect the rat's trachea to maintain breathing (70 breaths/min). The right common carotid artery of the rat was separated and clamped, and then a polyethylene catheter soaked in 1% heparin solution was inserted into the left ventricle from the common carotid artery. The catheter was connected to a pressure transducer to detect the relevant indicators of rat heart function. We cut the rib cage in the fifth intercostal space on the left side of the rat's sternum and found the heart. The left coronary artery between the left atrial appendage and the pulmonary artery was ligated. When the myocardium at the distal end of the ligation turned purple and the ECG showed ST-segment elevation, it meant the operation was successful. After 30 min of ligation, the ligature was loosened for 2 h. When the color of the myocardium and the electrocardiogram returned to normal, it meant blood flow was restored.

Measurement of myocardial injury markers

After reperfusion, blood in the rat heart was collected to detect the concentration of creatine kinase (CK), lactate dehydrogenase (LDH) and cardiac troponin I (cTnI). The concentration of CK, LDH and cTnI is positively correlated with the degree of myocardial injury. After the serum was separated, the CK kit (Thermo Fisher Scientific, Waltham, MA, USA), LDH kit (Thermo Fisher Scientific) and cTnI kit (Thermo Fisher Scientific) were used to detect the concentration of relevant indicators in the serum according to the manufacturer's instructions. The same method was also used to detect the concentration of markers in cardiomyocytes.

Measurement of heart hemodynamic parameters

During the operation, the rat's cardiac hemodynamic parameters were monitored through a pressure transducer and a supersonic cardiogram system (Visual Sonics, Toronto, ON, Canada). Left ventricular diastolic pressure (LVDP), left ventricular end diastolic pressure (LVEDP), the maximum (dP/dt_{max}) and minimum (dP/dt_{min}) rates of pressure rise in the left ventricle were analyzed.

Histological staining

The rat heart tissue was collected and fixed in 4% paraformaldehyde for 24 h. After dehydration, the hearts were embedded in paraffin to make paraffin blocks. The paraffin blocks were cut into 5 μm slices. After the paraffin slices were baked in a 37°C oven for 3 days, the slices were placed in xylene and gradient alcohol in sequence. Hematoxylin and eosin (Beyotime, Shanghai, China) were used to stain the nucleus and cytoplasm, respectively. The stained sections were examined using an Olympus BX53 light microscope (Olympus, Tokyo, Japan) equipped with a 200× objective lens, and the changes in rat myocardial structure were observed.

Immunohistochemistry

After deparaffinization and hydration, the slices were heated to 95°C for 20 min in citrate buffer. After natural cooling, the slices were incubated with 3% H_2O_2 for 30 min to inactivate endogenous peroxidase. Ten percent goat serum was used to block non-specific antigens for 1 h at room temperature. The sections were then incubated overnight at 4°C with primary antibodies against IL-1β (ab239517, dilution 1:200; Abcam, Cambridge, MA, USA) and TNF-α (ab270264, dilution 1:200; Abcam). After washing with PBS, the slices were incubated with HRP-conjugated secondary antibody (dilution 1:500; GeneTech, Shanghai, China) for 1 h at room temperature. The immunoreactivity was visualized using DAB chromogenic reagent (GeneTech), and nuclei were counterstained with hematoxylin. Negative controls were performed by omitting the primary antibody under identical conditions. For each experimental group, three independent biological replicates were analyzed. Immunopositivity was estimated by calculating the percentage of positively stained cells relative to the total number of cells in five randomly selected high-power fields (40×). Stained slides were examined using an Olympus BX53 microscope (Olympus) equipped with 20× and 40× objectives, and representative images were captured.

RT-qPCR

Total RNA was extracted from myocardial tissue with TRIzol (Invitrogen, Carlsbad, CA, USA). After dissolving RNA with RNase-free water, we used a spectrophotometer to detect the concentration of total RNA. According to the detected RNA concentration, we used reverse transcriptase (Vazyme, Nanjing, Jiangsu, China) to configure a reverse transcription system according to the manufacturer's instructions to reverse mRNA into cDNA. SYBR Green Master Mix (Vazyme) was used to amplify the corresponding fragments of cDNA according to different primers. The primer sequences were shown in Table 1. The relative expression of RNA was calculated using the method of $2^{-\Delta\Delta CT}$. GAPDH was used as a reference.

Cell culture and hypoxia-reoxygenation (H/R) model

The rat cardiomyocyte cell line, H9c2 cells, was used in this study. DMEM medium (Gibco, Rockville, MD, USA), fetal bovine serum (Gibco) and double antibodies (Gibco) were configured as the complete medium of 10% fetal bovine serum according to the ratio of 89:10:1. H9c2 cells were cultured in a sterile incubator at 37°C and 5% CO_2. After culturing H9c2 cells with complete medium to reach 90% degree of fusion, the complete medium was replaced with phosphate buffered saline (PBS) and the air in the incubator was replaced with 95% N_2. The cells were starved for 4 h under these conditions. Then we used complete medium again to culture the cells in a sterile incubator at 37°C and 5% CO_2.

CCK8 assay

H9c2 cells were seeded into 96-well plates. After the degree of cell fusion reached 30%, we used different concentrations of MaR1 to stimulate H9c2 cells for 24, 48 or 72 h. The 50 nM concentration of MaR1 was selected based on preliminary experiments and previous reports showing significant biological activity of MaR1 *in vitro* within this concentration range, which is widely used in cellular studies to model its anti-inflammatory effects. Then 10 μL of CCK8 reagent (Dojindo Molecular Technologies, Kumamoto, Japan) was added to each well of the 96-well plates. The cells were again placed in the incubator for 2 h. Then the 96-well plates were placed in a microplate reader under dark conditions to measure the absorbance of each well.

EdU cell proliferation assay

H9c2 cells were seeded onto sterile glass slides in 24-well plates and cultured until approximately 30% confluence. The cells were then stimulated with MaR1 for 24 h. After treatment, cells were fixed with 4% paraformaldehyde for 20 min at room temperature and permeabilized with 0.5% Triton X-100 for 10 min. An EdU detection solution (Sigma-Aldrich, St. Louis, MO, USA) was applied according to the manufacturer's protocol to label cells in the DNA synthesis phase. Nuclei were counterstained with 4′,6-diamidino-2-phenylindole (DAPI; Sigma-Aldrich) for 5 min to visualize total cell numbers. Negative controls without EdU labeling were included to confirm specificity. For each group, three independent biological replicates were performed, and five random fields per slide were analyzed. The proliferation index was calculated as the percentage of EdU-positive nuclei relative to the total number of DAPI-stained nuclei. The stained cells were visualized and imaged using an Olympus BX53 fluorescence microscope (Olympus) equipped with a 20× objective, and representative micrographs were captured.

Statistical analysis

Statistical Product and Service Solutions (SPSS) 21.0 (IBM, Armonk, NY, USA) was used for the data analysis of this study. All experimental results were expressed as mean ± standard deviation. Student's t-test was used for paired analysis, and the analysis of variance was used to compare differences between groups. A p-value <0.05 indicated that the difference was statistically significant. All experiments were repeated three times.

Results

Effect of MaR1 on myocardial function during MIRI

In order to investigate the effect of MaR1 on the heart function of MIRI rats, we examined the systolic and diastolic functions of

Table 1. RT-PCR primers.

Name	Sense sequences (5'-3')	Anti-sense sequences (5'-3')
IL-1β	CCCTTGACTTGGGCTGT	CGAGATGCTGCTGTGAGA
IL-6	CACCAGGAACGAAAGTCAA	CAACAACATCAGTCCCAAGA
IL-8	GAGCAACCCATACCCATCGA	TGGTCCCACCATATCTTCTTAATCT
IL-10	AGGGTTACTTGGGTTGCC	GGGTCTTCAGCTTCTCTCC
MCP1	CCCTGCTGCCCTTTCTA	CCAATCTGGGGTCACACT
TNF-α	CAGCCAGGAGGGAGAAC	GTATGAGAGGGACGGAACC
GAPDH	GTTGTGGCTCTGACATGCT	CCCAGGATGCCCTTTAGT

the rat heart during I/R (Figure 1 A-D). The cardiac function of rats in the sham group was normal throughout. After the left coronary artery of the rat was clipped (-30 min - 0 min), the rat's LVDP, dP/dt_{max} and dP/dt_{min} all decreased with the extension of ischemic time. However, LVEDP gradually increased after myocardial ischemia. This indicated that the heart function of MIRI rats was reduced during ischemia, but MaR1 did not significantly improve the heart function of rats during ischemia. After the left coronary artery of the rat was recanalized (0 min - 120 min), the LVDP, dP/dt_{max} and dP/dt_{min} of MIRI rats did not change significantly, but the related indexes of rats in the MIRI+MaR1 group increased significantly. In addition, MaR1 also reduced LVEDP. These data indicated that MaR1 attenuated the disorder of heart function during reperfusion.

Effect of MaR1 on myocardial structure during MIRI

In order to clarify the protective effect of MaR1 on the myocardial structure of MIRI rats, we compared the structural changes of the myocardial tissue of the three groups of rats. The myocardial tissues of rats in the sham group were arranged neatly and the structure was intact. The number of cardiomyocytes of rats in the MIRI group was significantly reduced and there were many inflammatory cell infiltrations between the cardiomyocytes. In the damaged myocardial tissue, the morphology of myocardial cells was deformed and arranged disorderly. In the myocardial tissue of rats treated with MaR1, the range of myocardial injury was significantly reduced. In addition, the morphology of cardiomyocytes and the infiltration of inflammatory cells were also significantly improved (Figure 2A). CK, LDH and cTnI were important indicators of myocardial injury. In the serum of rats in the MIRI group, the expression of CK (Figure 2B), LDH (Figure 2C) and cTnI (Figure 2D) increased significantly, indicating that the myocardium of the rats in the MIRI group was significantly damaged. After treatment with MaR1, the concentration of CK, LDH and cTnI decreased. These data indicated that MaR1 improved the rat myocardial structure after MIRI.

Effect of MaR1 on myocardial inflammation caused by MIRI

In order to evaluate the anti-inflammatory effect of MaR1 on MIRI, we examined the changes of inflammatory factors in rat myocardial tissue. Immunohistochemistry staining detected the expression of inflammatory factors IL-1β and TNF-α in rat myocardium (Figure 3A). The positive cells for inflammatory factors IL-1β and TNF-α in the injured myocardial tissue of MIRI rats increased significantly, while the treatment of MaR1 reduced their expression. In addition, the expression of IL-1β, IL-6, IL-8, MCP1 and TNF-α mRNA in the myocardium of MIRI rats increased, and MaR1 also reduced the mRNA expression of these inflammatory factors. IL-10 is an important anti-inflammatory factor and MaR1 increased the expression of IL-10 mRNA in the myocardium (Figure 3 B-G). Therefore, MaR1 was found to play an anti-inflammatory effect during MIRI.

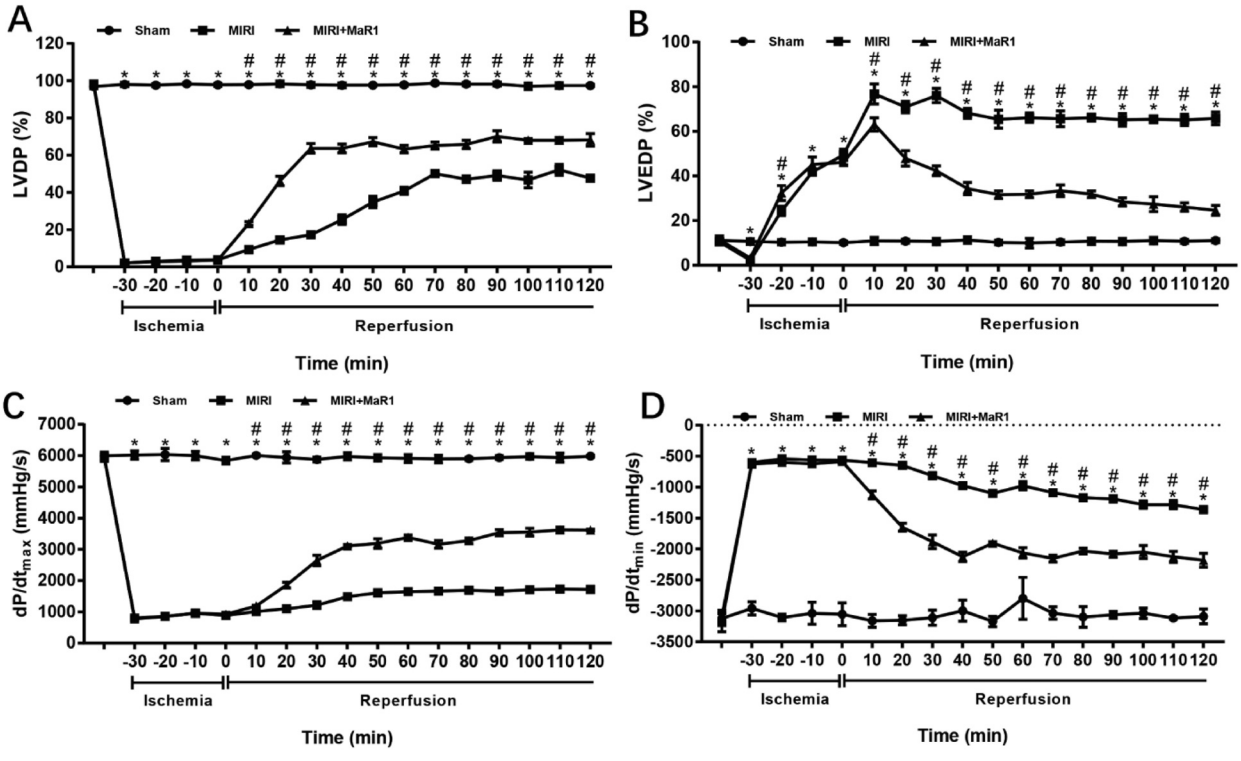

Figure 1. Effect of MaR1 on myocardial function during ischemia-reperfusion injury (MIRI) in rats. Hemodynamic parameters were continuously monitored during ischemia and reperfusion: **A**) Left ventricular developed pressure (LVDP); **B**) left ventricular end-diastolic pressure (LVEDP); **C**) maximum rate of pressure rise (dP/dtmax); **D**) minimum rate of pressure decline (dP/dtmin). Sham-operated rats showed stable function, while MIRI significantly impaired cardiac function. Pretreatment with MaR1 (4 ng/g, s.c.) improved LVDP, dP/dtmax, and dP/dtmin, and reduced LVEDP compared with untreated MIRI rats. Values are expressed as mean ±SD. *p<0.05 *vs* Sham group; #p<0.05 *vs* MIRI group.

Effect of MaR1 on the activity of myocardial cells (H9c2) after H/R

In order to clarify the effect of MaR1 on cardiomyocytes, we cultured H9c2 cell line and induced H9c2 cell damage through H/R. CCK8 assay examined the effects of different concentrations of MaR1 (10, 20, 30, 50, 70 and 100 nM) on the activity of H9c2 cells at different times (24, 48 and 72 h). The results of CCK8 assay showed that when 50 nM of MaR1 stimulated H9c2 cells for 2 days, the cell activity was the highest, so we used 50 nM of MaR1 to stimulate H9c2 cells for 2 days in subsequent experiments (Figure 4A). We examined the expression of CK (Figure 4B) and LDH (Figure 4C) in H9c2 cells. H9c2 cells in the H/R group expressed more CK and LDH compared to the control group, while MaR1 reduced the concentration of CK and LDH. EdU assay examined the proliferation ability of H9c2 cells and found that MaR1 attenuated the inhibition of H/R on the proliferation ability of H9c2 cells (Figure 4 D,E).

Discussion

Ischemia-reperfusion injury plays an important role in the pathogenesis of many organ diseases.[15] Following ischemia, the rapid restoration of blood supply not only fails to recover organ function but also exacerbates tissue damage, ultimately leading to dysfunction.[16] MIRI is a complex pathological process that involves oxidative stress, apoptosis, autophagy, calcium overload, and inflammatory responses. In this study, we focused on the anti-inflammatory effects of the lipid mediator MaR1 on MIRI. Our findings demonstrated that MaR1 exerted significant cardioprotective effects *in vivo* and *in vitro*, suggesting its potential as a therapeutic agent for the prevention and treatment of MIRI.

Inflammation is a central contributor to MIRI. During reperfusion, vascular endothelial cells release large amounts of inflammatory factors, including IL-6 and TNF-α.[17] Elevated TNF-α induces contractile dysfunction, fibrosis, and cardiomyocyte death, while IL-1β and IL-6 amplify the cytokine cascade and promote leukocyte infiltration.[18] Elevated serum levels of IL-6 and TNF-α in patients with heart failure further highlight the clinical relevance of these cytokines.[19,20] Therefore, reducing excessive inflammation is crucial for mitigating MIRI. Our results showed that MaR1 significantly decreased the expression of IL-1β, IL-6, IL-8, MCP1, and TNF-α, while upregulating IL-10, an anti-inflammatory cytokine that plays an essential role in restoring immune homeostasis. Previous studies also demonstrated that IL-10 alleviates lipid metabolic responses and protects against myocardial injury in MIRI models.[21] The ability of MaR1 to enhance IL-10 expression suggests a potential mechanism for sustained myocardial protection by shifting the balance toward an anti-inflammatory and pro-resolving state. Our study is consistent with previous reports of other anti-inflammatory interventions in MIRI. For example, curcumin has been shown to attenuate MIRI through antioxidant and anti-inflammatory pathways, while acetylcholine receptor agonists reduce infarct size and improve cardiac function.[16] Similarly, heat shock protein 70 and photobiomodulation therapies have been proposed as novel cardioprotective strategies.[16] Compared with these interventions, MaR1 belongs to a unique class of pro-resolving lipid mediators that not only suppress pro-inflammatory signaling but also actively promote inflammation resolution and tissue repair. This dual action may provide MaR1 with a therapeutic advantage over conventional anti-inflammatory approaches that

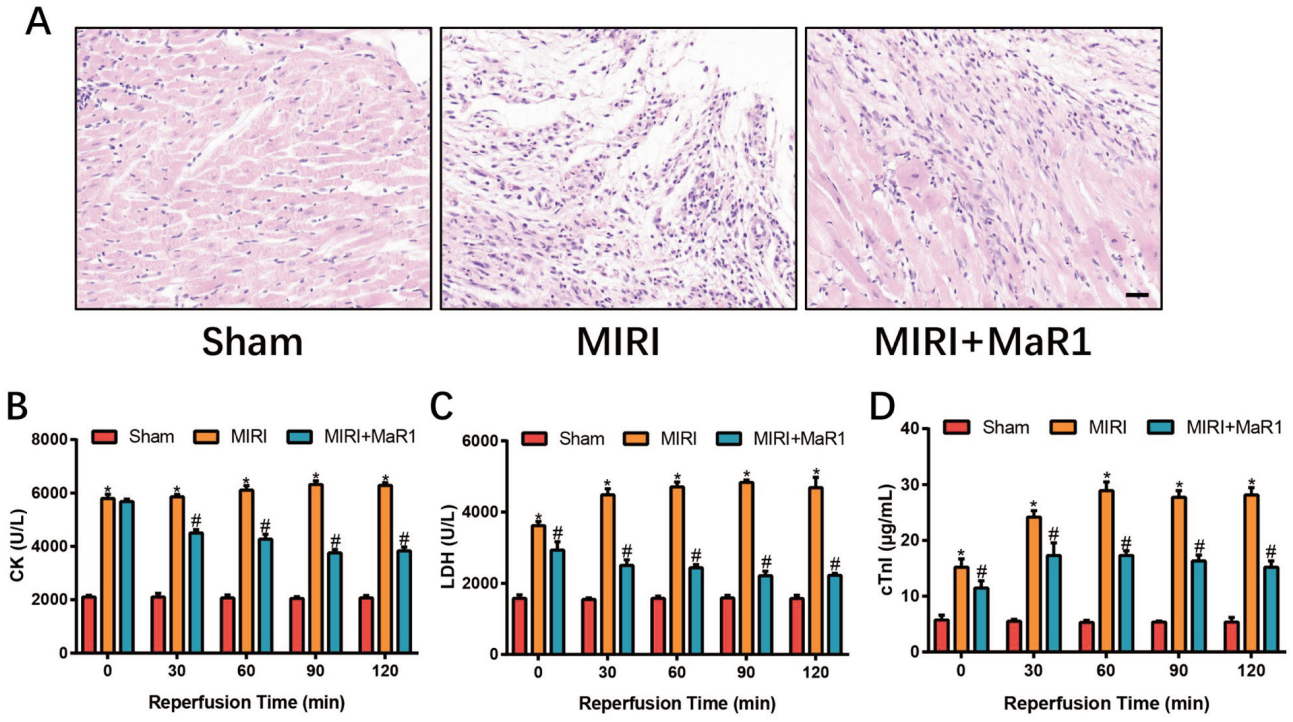

Figure 2. Protective effects of MaR1 on myocardial structure and serum injury markers after MIRI. **A**) Representative hematoxylin-eosin (H&E) staining of left ventricular myocardium; Sham rats showed intact myocardial architecture, while MIRI rats displayed myocyte disarray, necrosis, and inflammatory infiltration; MaR1 treatment markedly preserved myocardial structure and reduced infiltration; scale bar: 50 μm. **B-D**) Serum levels of creatine kinase (CK), lactate dehydrogenase (LDH), and cardiac troponin I (cTnI) were significantly elevated after MIRI but reduced following MaR1 pretreatment. Data are presented as mean ±SD. *$p<0.05$ *vs* Sham group; $^{\#}p<0.05$ *vs* MIRI group.

primarily block inflammatory cascades. Inflammation resolution is a highly regulated process that prevents the transition from acute to chronic inflammation. It is characterized by neutrophil apoptosis, clearance of apoptotic cells by macrophages, reduction of pro-inflammatory cytokines, and increased release of anti-inflammatory and pro-resolving mediators.[22,23] In recent years, several families of pro-resolving mediators (including lipoxins, resolvins, and protectins) have been shown to mitigate MIRI by reducing neutrophil infiltration, limiting oxidative stress, and improving cardiac recovery.[24-26] MaR1, a macrophage-derived DHA metabolite, is among the most extensively studied members of this group. Evidence from peritonitis, neuropathic pain, and acute lung injury models demonstrates that MaR1 potently inhibits neutrophil adhesion, enhances macrophage phagocytosis, and promotes tissue regeneration.[27-29] Our findings extend these observations to the cardiac setting, highlighting MaR1 as a promising therapeutic candidate for MIRI. Despite these promising results, several limitations of the present study must be acknowledged. First, although we evaluated

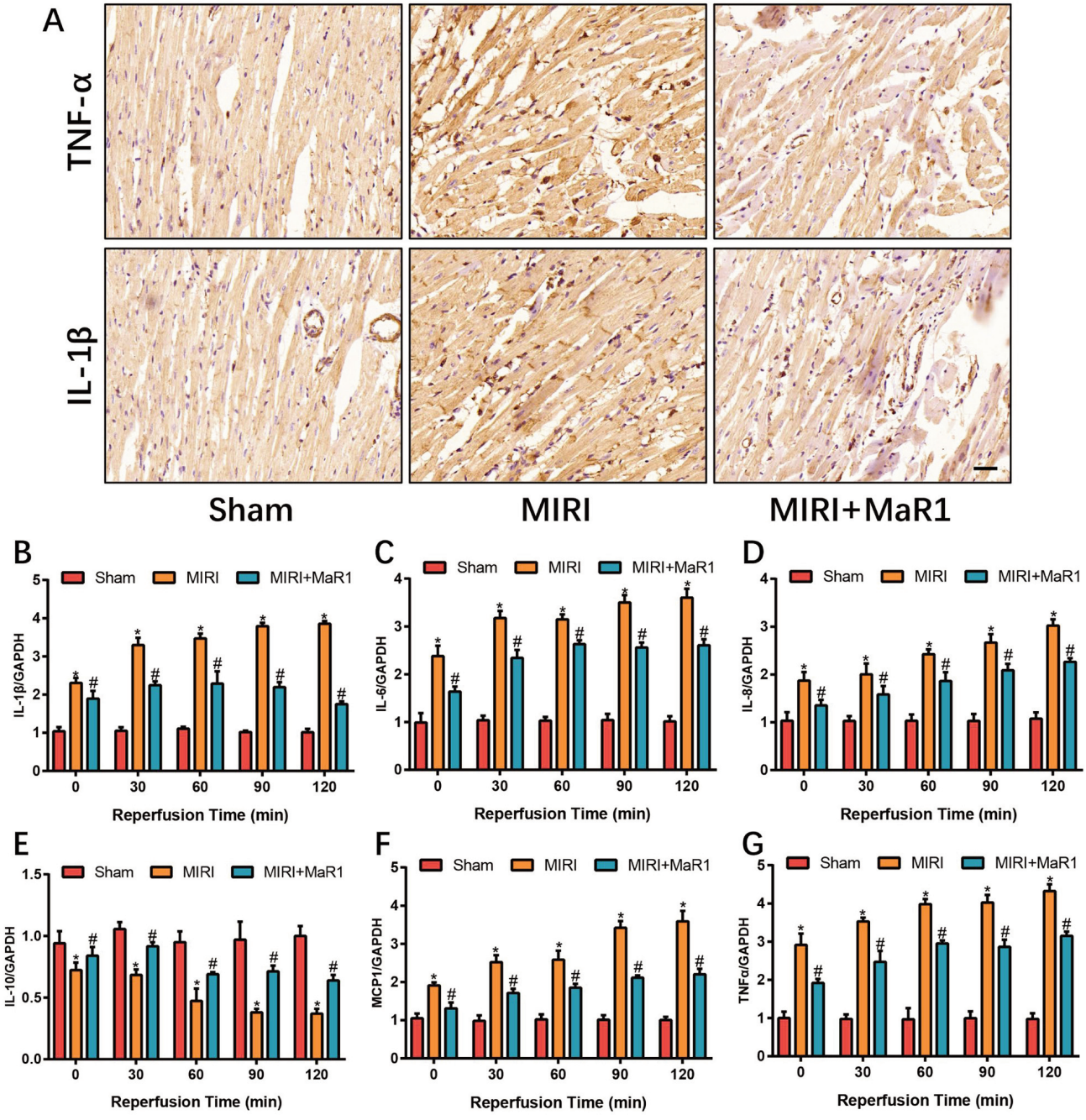

Figure 3. Anti-inflammatory effects of MaR1 in rat myocardium after MIRI. A) Representative immunohistochemical staining for IL-1β and TNF-α in myocardial tissue; brown DAB signal indicates positive immunoreactivity; MIRI increased IL-1β and TNF-α expression, while MaR1 pretreatment reduced their levels; scale bar: 50 μm. **B-G**) Relative mRNA expression of IL-1β, IL-6, IL-8, IL-10, MCP1, and TNF-α determined by quantitative RT-PCR; MaR1 significantly reduced pro-inflammatory cytokine expression and enhanced IL-10 expression compared with MIRI. Data are expressed as mean ±SD. *$p<0.05$ vs Sham group; $^{\#}p<0.05$ *vs* MIRI group.

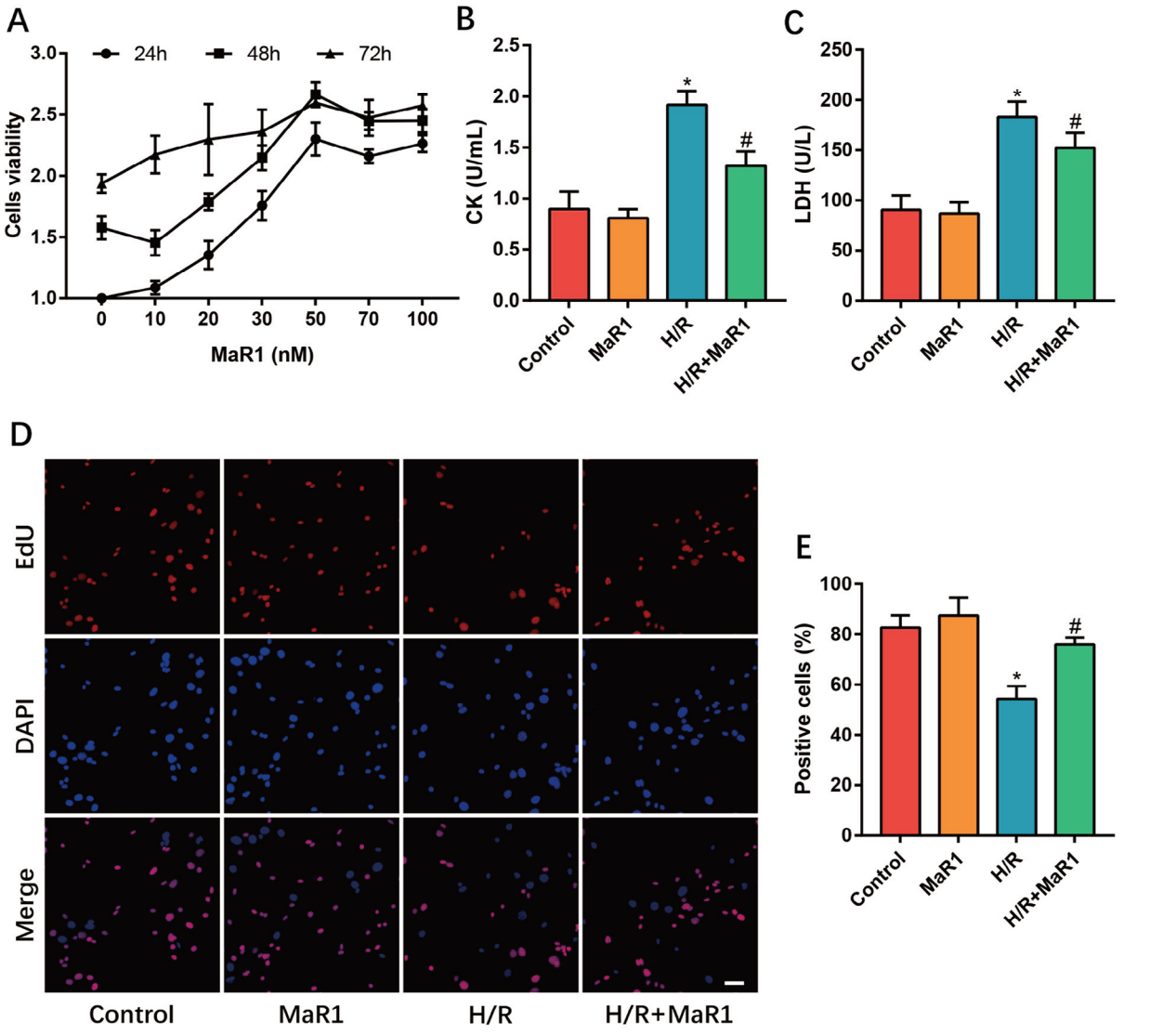

Figure 4. Effects of MaR1 on the viability and proliferation of H9c2 cardiomyocytes exposed to hypoxia/reoxygenation (H/R). **A**) Cell viability assessed by CCK-8 assay after MaR1 treatment at different concentrations and time points. **B,C**) Intracellular CK and LDH levels measured by ELISA; MaR1 reduced CK and LDH release compared with untreated H/R cells. **D,E**) EdU incorporation assay showing proliferating nuclei (red) and DAPI counterstain (blue) in H9c2 cells; quantification of EdU-positive nuclei demonstrated that MaR1 restored proliferation suppressed by H/R; scale bar: 50 μm. Data are expressed as mean ±SD. *$p<0.05$ *vs* Control group; #$p<0.05$ *vs* H/R group.

the effects of a single MaR1 dose, we did not establish an *in vivo* dose-response relationship, which limits our ability to determine the optimal therapeutic concentration. Second, the current experiments focused on short-term outcomes, and no long-term follow-up was performed to evaluate whether MaR1 confers sustained protection against post-ischemic remodeling or heart failure. Third, while we plan to construct MaR1 knockout models to further clarify the endogenous role of MaR1, additional validation in large-animal models and, ultimately, clinical studies will be essential before translation into therapeutic practice.[30]

In conclusion, our findings demonstrate that exogenous MaR1 supplementation alleviates MIRI by improving cardiac function, preserving myocardial structure, reducing inflammatory cytokine expression, and enhancing cardiomyocyte viability. The upregulation of IL-10 may contribute to long-term cardioprotection by promoting an anti-inflammatory and pro-resolving environment. Collectively, these results provide novel insight into the therapeutic potential of MaR1 as a pro-resolving mediator in ischemia-reperfusion injury.

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Received: 22 July 2025. Accepted: 9 September 2025.

European Journal of Histochemistry 2025; 69:4254
doi:10.4081/ejh.2025.4254