ORIGINAL RESEARCH

Mesenchymal Stem Cells Promote the Resolution of Cardiac Inflammation After Ischemia Reperfusion Via Enhancing Efferocytosis of Neutrophils

Zeyu Zhang, MD;* Hongzhen Tian, MD;* Chen Yang, PhD; Jixuan Liu, PhD; Huawei Zhang, MD; Jinda Wang, MD; Shunying Hu, PhD; Zhijun Sun, MD; Kunlun He, PhD; Guanghui Chen, PhD

***Background*—**Neutrophils play a major role in inflammation after myocardial ischemia-reperfusion (I/R) injury. The effects of mesenchymal stem cells (MSCs) on neutrophils in I/R are complex and not fully understood. This study was designed to investigate the effects and mechanism of MSCs on alleviating myocardial I/R injury in rats.

***Methods and Results*—**MSCs induced M2 macrophages polarization in vitro and enhanced macrophage efferocytosis of apoptotic neutrophils, measured by fluorescence-activated cell sorting analysis and immunofluorescence staining. Rats myocardial I/R were induced by transient ligation of left anterior descending coronary. Adipose-derived MSCs or vehicle were infused at initiation (immediate after reperfusion) or peak of inflammation (24 hours after I/R). Hematoxylin and eosin, 2,3,5-triphenyltetrazolium chloride/Evans Blue staining and immunofluorescence staining were applied within 72 hours after cell infusion. Cardiac function was assessed by echocardiography and left cardiac catheterization analysis at 28 days post-operation. MSCs infused immediately and 24 hours later both markedly ameliorated myocardial I/R injury, and immediate infusion had more significant outcome. These improvements were associated with neutrophils infiltration, measured by fluorescence-activated cell sorting analysis and immunofluorescence staining. When infused immediately, MSCs did not significantly change neutrophil number at 24 hours but CD11b expression was significantly higher. When infused at 24 hours, MSCs markedly decreased neutrophil number by enhanced M2 macrophage infiltration and macrophage efferocytosis of neutrophils within 72 hours.

***Conclusions*—**Efferocytosis is pivotal to relieve neutrophil-mediated I/R injury and initial the immune response for healing. MSCs infusion improves cardiac function in rats after myocardial I/R via the possible mechanism of enhancing M2 macrophages-induced efferocytosis of apoptotic neutrophils. **(*J Am Heart Assoc*. 2020;9:e014397. DOI: 10.1161/JAHA.119.014397.)**

Key Words: efferocytosis • inflammation • mesenchymal stem cells • myocardial ischemia • reperfusion injury

Acute myocardial infarction (MI) is a major cause of death and disability worldwide. Tough timely reperfusion therapy is one of the most successful therapies in clinics, the process of reperfusion could lead to additional myocardial damage known as ischemia/reperfusion (I/R) injury.[1,2] Inflammation plays a critical role in this process and the intensity and duration of this inflammatory reaction are intimately associated with myocardial injury and scar formation.[3,4] Since clinical applications of corticosteroid or immunosuppressant have disappointing results,[5,6] optimal method requires not only preventing excessive inflammation but also contributing to adequate stimulation of repair. The strategy of systemic administration of mesenchymal stem cells (MSCs) is an option.

MSCs have been widely used in the treatment of diseases of cardiovascular system and immune system, which shows a broad application prospect.[7,8] MSCs, characterized by paracrine, reparative and immunomodulatory properties,

From the Department of Cardiology, Chinese PLA General Hospital, Beijing, China (Z.Z., H.T., C.Y., J.L., H.Z., J.W., S.H., Z.S., G.C.); Beijing Key Laboratory of Chronic Heart Failure Precision Medicine, Chinese PLA General Hospital, Beijing, China (Z.Z., J.L., K.H.); Department of Cardiology, 969 Hospital of Joint Logistic Support Force of PLA, Huhehaote, China (H.T.).

Accompanying Figures S1 through S4 are available at https://www.ahajournals.org/doi/suppl/10.1161/JAHA.119.014397

*Dr Zeyu Zhang and Dr Hongzhen Tian contributed equally to this work and are co-first authors.

Correspondence to: Guanghui Chen, PhD, Department of Cardiology, Chinese PLA General Hospital, Beijing 100853, China. E-mail: 13910669498@163.com and Kunlun He, PhD, Beijing Key Laboratory of Chronic Heart Failure Precision Medicine, Chinese PLA General Hospital, Beijing 100853, China. E-mail:kunlunhe@plagh.org

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Clinical Perspective

What Is New?

- This is the first study to show that mesenchymal stem cells (MSCs) accelerate M2 macrophage infiltration and thus enhance macrophage efferocytosis of apoptotic neutrophils both in vitro and in vivo.
- In vivo studies showed neutrophil phenotype might have changed with more beneficial activities after infusion of MSCs.
- Our study revealed that MSCs could accelerate the resolution of inflammation by enhancing M2 macrophages-induced efferocytosis of apoptotic neutrophils without changing neutrophil recruitment.

What Are the Clinical Implications?

- Infusions of MSCs intravenously ameliorate myocardial ischemia-reperfusion injury, reduce infarct size, and improve cardiac function. The underlying mechanism partly lies in MSCs mediate neutrophil infiltration by facilitating their removal without affecting their recruitment.
- Our study showed that MSCs could be used as soon as possible after reperfusion therapy, with effectiveness in restricting excessive inflammation and safety in not depriving "the good side" of the inflammation.

ameliorate infarct healing and repair, and also modulate inflammation after myocardial damage.[9] A major contributor to I/R injury is the infiltrating neutrophils, which are also pivotal modulators by regulating reparative processes. However, the effects of MSCs on neutrophils in I/R are complex and not fully understood. In this study, we infused MSCs at initiation or peak of inflammation after myocardial I/R injury in rats which are determined by neutrophil numbers, thus examined the effects and mechanisms of MSCs on cardiac repair and function. Our study provides a novel possible mechanism for the therapeutic potential of MSCs to modulate the post I/R inflammatory response, accelerate repair, and finally prevent heart failure.

Methods

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

Animals

Sprague-Dawley (SD) rats were purchased from the Animal Experiment Center of the Chinese PLA General Hospital (Beijing, China). All animal experimental procedures were approved by the Animal Ethics Committee of the Chinese PLA General Hospital and Military Medical College. The animals were raised under a 12:12-hour light:dark cycle and were given free access to food and water. The room temperature was maintained at 22°C to 25°C, and the bedding was changed twice a week. Ten rats were born within 6 to 7 days for MSCs isolation, 10 were 250 to 300 g for macrophages and neutrophils isolation, and 200 were 220 to 250 g for sham or I/R procedure.

MSCs Isolation and Culture

MSCs were obtained from adipose tissues of SD rats born within 6 to 7 days as previously described.[10] In brief, subcutaneous adipose tissues from the inguinal regions were minced with fine scissors and digested with 1% collagenase (Sigma, #1148089) at 37°C for 40 minutes. After collagenase activity was neutralized, digested adipose tissues were filtered with a 0.074 mm-diameter filter and the suspension was centrifuged at 300*g* for 10 minutes. The cell pellets were resuspended and cultivated for 24 hours in DMEM supplemented with 10% fetal bovine serum (FBS). Non-adherent cells were removed from the cultures, and fresh culture medium was added to the dishes. When reaching 80% confluence, adherent cells were trypsinized, harvested, and expanded. Cells from passages 3-5 were used for further testing or transplantation. Forty-eight hours supernatant was also collected. The purity of MSCs was >98%, assessed by FACS analysis using anti-CD29, anti-CD31, anti-CD34, anti-CD45, and anti-CD90 (Figure S1A).

Macrophages and Neutrophils Isolation and Culture

Macrophages were obtained by peritoneal lavage. Under sterile condition, SD rats (250–300 g) were intraperitoneally injected into 20-mL RPMI medium and peritoneal exudate cells were then harvested. After centrifuged and suspended, total cell numbers were determined by Trypan blue staining. Cells were plated in 24-well culture plates at 2×10^5 cells/well in RPMI medium supplemented with 10% FBS. After 2 hours of incubation at 37°C and 5% CO_2, non-adherent cells were washed away. The purity of macrophage was >90%, assessed by fluorescence-activated cell sorting (FACS) analysis using anti-CD68 (AbD Serotec, #MCA341) and anti-granulocytes (BD Biosciences, #550002) (Figure S1B).

Neutrophils were obtained by density centrifugation over a Ficoll gradient (Ficoll-Paque PREMIUM 1,084; GE Healthcare). After rats were anesthetized with 3% pentobarbital sodium (30 mg/kg) intraperitoneally, blood was taken by puncture of the left heart ventricle. Neutrophils were harvested according to the manufacturer's instructions. To assess the purity, positively selected neutrophils were made into cells smear. After being stained in Wright staining, 200 cells were performed by cell differential counts. Neutrophils were 91.3±1.2% (n=4). The

purity of neutrophils was also >90%, assessed by FACS analysis using anti-granulocytes and anti-CD11b (Figure S1C).

Fluorescent Labeling of Neutrophils and Neutrophil Apoptosis Assay

Positively selected neutrophils were resuspended at 1×10^6 cell/mL in RPMI medium supplemented with 10% FBS, labeled with 2 μmol/L 5-(and 6)-carboxyfluorescein diacetate succinimidyl ester (CFSE, Ebioscience, #65-0850-84), a fluorescent marker, according to the manufacturer's instruction and then were cultured in 37°C and 5% CO_2 for 20 hours in RPMI medium supplemented with 10% FBS. Apoptosis was measured by FACS analysis with Annexin V Apoptosis Detection Kit (BD Biosciences, #559763).

Co-Culture of Macrophages and Aged Neutrophils

Positively selected macrophages were plated in 24-well culture plates at 2×10^5 cells/well in RPMI medium supplemented with 10% FBS and were randomly divided into (1) MSC group, (2) MSC supernatant group, (3) control group. 2×10^4 MSCs, MSC supernatant, or RPMI were added to the upper chambers of Transwell inserts in 24-well tissue culture plates (0.4 μm pore, Corning, USA) in each group. After 48 hours of incubation, 1×10^6 aged neutrophils were added to the lower chamber. After co-culture for 2 hours, neutrophils that had not been phagocytized by the macrophages were washed away. The remaining cells were used afterwards.

Macrophage Efferocytosis of Neutrophils In Vitro

For immunofluorescence staining, the remaining cells (see above) were fixed with 4% paraformaldehyde for 20 minutes at room temperature. After incubation with 5% normal goat serum for 30 minutes, cells were stained using anti-CD163 (Santa Cruz Biotechnology, #sc-58965) overnight at 4°C, followed by incubation with Cy3-conjugated secondary antibody. The chamber slides were photographed using a fluorescence microscope (IX71, Olympus, Japan). Phagocytosis index was calculated and M2 macrophage phagocytosis activity was assessed.

For FACS analysis, the remaining cells (see above) were trypsinized, harvested, and labeled with anti-CD163 (AbD Serotec, #MCA342) and anti-CD68 (AbD Serotec, #MCA341) for 25 minutes. The CD68+CD163+CFSE+ macrophage population was determined by FACS analysis.

Induction of Myocardial I/R Models

Adult male SD rats (220–250 g) were anesthetized with 3% pentobarbital sodium (30 mg/kg) intraperitoneally under sterile conditions. Each animal was helped to breath with a ventilator (30 mL/kg, rr 55/min) after tracheotomy. Heart was exposed by left thoracotomy that the left anterior descending coronary could be visualized. A 6-0 silk suture was passed underneath the left anterior descending coronary 2 to 3 mm from tip of the left auricle. Then the left anterior descending coronary was ligated together with ployethylene-10 (PE-10) tubing and a slipknot was made. Complete occlusion of the artery was confirmed by myocardial blanching and ECG with ST-segment elevation. After 45 minutes of ligation, the ligature was released. Reperfusion was confirmed by ventricular arrhythmia and ECG with ST-segment resolution. The chest was closed and penicillin was used intraperitoneally. Sham-operated animals underwent the same procedure without coronary artery ligation.

Treatment and Groups

First, rats were randomly subject to sham or I/R procedure, and tail vein blood were collected at different time points (6, 12, 18, 24, 30, and 48 hours after operation). Number of neutrophils in blood was determined by Auto Hematology Analyzer (BC-2800Vet, Mindray). Neutrophils infiltrated in hearts were determined by FACS analysis.

More SD rats were subject to I/R procedure and were randomly divided into (1) MI/R group; (2) MSC 0-hour group; (3) MSC 24-hour group; (4) PMN 24-hour group. In MI/R group, rats were intravenously infused with 200-μL saline with a 28G needle through tail vein immediately after I/R. In the latter 2 groups, rats were intravenously infused with 2×10^6 MSCs in 200-μL saline immediately or 24 hours later. In the last group, rats were intravenously infused with 2×10^6 neutrophils in 200-μL saline 24 hours later. Successful infusions were monitored by lack of extravasation at the injection site. The survival rates after I/R procedure in each group were 63.8%, 75%, 69.8%, and 66.7%.

FACS Analysis of Neutrophils and Macrophages Infiltrated Into Hearts

To achieve single cell suspension, rats were anesthetized at each time point (24, 48, and 72 hours after I/R or sham operation). Hearts were harvested, perfused with cold PBS to remove peripheral cells and minced with fine scissors. After digested with 1% collagenase at 37°C for 40 minutes, heart tissues were filtered with a 0.074 mm-diameter filter. The suspensions were centrifuged at 300*g* for 5 minutes and red blood cells were lysed with ACK lysis buffer (BD Biosciences). After being centrifuged and suspended, total cell numbers were determined by Trypan blue staining. The resulting single-cell suspensions were washed with PBS containing 0.1% bovine serum albumin, filtered with a 300-mesh filter and

analyzed by FACS analysis. After washing with PBS containing 0.1% bovine serum albumin, cells were stained with a mixture of antibodies at 4°C for 20 minutes. Results were expressed as cell number per heart. Cells were analyzed using FACS Calibur (BD Biosciences) and FlowJo 7.6 software.

The harvested cells were labeled with the following surface markers: anti-CD45 (BD Biosciences, #559135), anti-CD45 (Ebioscience, #MA5-17425), anti-granulocytes (BD Biosciences, #550002), anti-granulocytes (Ebioscience, #11-0570-82), anti-CD11b (BD Biosciences, #554982), anti-CD68 (AbD Serotec, #MCA341), anti-CD163 (AbD Serotec, #MCA342).

Histopathological Analysis

To evaluate cardiac repair, rats were anesthetized and hearts were harvested at 24, 48, and 72 hours after reperfusion. Hearts were fixed with 4% paraformaldehyde overnight and then embedded in paraffin. Blocks were serially cut 5 μm apart transversely. Hematoxylin and eosin staining was performed to determine the morphological effects and immune cells infiltration.

To evaluate infarct size, rats were anesthetized at 48 hours after reperfusion. Hearts were harvested and perfused with cold PBS to remove peripheral cells. The ligature around the coronary artery was retied. One milliliter of 2% Evans Blue dye (Sigma-Aldrich, #E2129) was injected into the aorta. Subsequently, hearts were frozen at −20°C for 15 minutes and sliced into 2 to 3 mm transverse cross sections and then stained with 1% 2,3,5-triphenyltetrazolium chloride (TTC; Sigma, #T8877) solution in PBS for 15 minutes at 37°C. After fixation for 4 to 6 hours in 10% formalin, both sides of each slice were photographed. The viable myocardium stained brick red, and the infarct tissues appeared pale white. Infarct area, area at risk (AAR) and the total area of left ventricular (LV) myocardium were measured manually in the digital images using Image-Pro Plus 6.0 software. Infarct size, expressed as a percentage of AAR, was calculated.

Cardiac Function Analysis

To evaluate cardiac function, echocardiography was performed at 28 days after reperfusion. The measurements were obtained using a Vevo 2100 ultrasound system (VisualSonics, Canada) equipped with a 30 MHz MicroScan transducer. Graphic acquisition and analysis were performed by a technician who was masked to treatment groups. Fractional shortening and LV ejection fraction were calculated.

To measure internal pressure of LV, a 1.4 French micro manometer-tipped catheter was inserted into the right carotid artery and then advanced into the LV. LV end-diastolic pressure was measured, and maximal (LV +dp/dtmax.) and minimal (LV −dp/dtmin.) first derivative of LV pressure rise and fall were calculated.

Macrophage Efferocytosis of Neutrophils In Vivo

For immunofluorescence staining, hearts were fixed with 4% paraformaldehyde overnight and then embedded in paraffin. Blocks were serially cut 5 μm apart transversely. After blocking non-specific binding with 5% normal donkey serum for 30 minutes at room temperature, the slides were incubated with primary antibodies for anti-MPO (Abcam, #ab9535) and anti-CD163 (Santa Cruz Biotechnology, #sc-58965) overnight at 4°C. After washing in PBS 3 times, the slides were incubated with secondary antibodies conjugated with FITC or Cy3 for 1 hour at room temperature in a darkened humidified chamber. Negative controls were incubated without primary antibodies. Then the slides were incubated with DAPI for 5 minutes and washed in PBS 5 times. Images were captured using a fluorescence microscope (Eclipse Ti-SR, Nikon, Japan).

For FACS analysis, cells isolated from hearts were first labeled with anti-CD163 (AbD Serotec, #MCA342) for 25 minutes, and permeabilized with IntraSure Kit (BD Biosciences). Subsequently, permeabilized cells were labeled with anti-Granulocytes (Ebioscience, #11-0570-82) for 20 minutes. The CD163+Gr+ macrophage population was determined by FACS analysis.

Statistical Analysis

The statistical analysis was performed using GraphPad Prism 5 software, and all values are expressed as the mean±SE unless otherwise stated. The data were compared between groups with an unpaired Student *t* test or ANOVA with Tukey post hoc test as appropriate. $P<0.05$ was considered to indicate statistical significance.

Results

MSCs Induce M2 Macrophages Polarization In Vitro, Thus Enhance Macrophage Efferocytosis of Apoptotic Neutrophils

We examined the ability of MSCs to modulate M2 macrophages polarization by using transwell chambers in vitro. Positively selected macrophages were plated into culture plates. MSCs, MSC supernatant, or RPMI were added to the upper chambers and co-cultured for 48 hours. Representative immunofluorescence staining images showed most macrophages expressed CD163 in MSCs and MSC

supernatant groups (Figure 1A). FACS analysis suggested that ratio of CD163+CD68+ within CD68+ cells in control group was 14.7±3.4%, the value was significantly increased in the MSC group (78.4±4%, $P<0.001$) and in MSC supernatant group (83±3.4%, $P<0.001$), and the value in the latter 2 groups showed no significant differences ($P>0.05$) (Figure 1B).

Then we tested if M2 macrophages could be more efficient to phagocytize early apoptotic neutrophils. After 20 hours incubation, most neutrophils became early apoptotic. The composition of neutrophils was: 16.31±9.1% viable (Annexin V−, PI−), 66.4±7.2% apoptotic (Annexin V+, PI−), 16.16±2.3% necrotic (Annexin V+, PI+) (Figure 2A). After 20 hours incubation, CFSE labeled apoptotic neutrophils were added into the culture plates and co-cultured for 2 hours. Representative immunofluorescence staining images showed most macrophages phagocytized neutrophils in MSC and MSC supernatant groups. FACS analysis suggested that ratio of CD163+CFSE+ cells in control group was 3.8±1.3%, the value was significantly increased in the MSC group (33.8±3.1%, $P<0.01$) and in MSC supernatant group (38±3.7%, $P<0.01$), and the value in the latter 2 groups showed no significant differences ($P>0.05$) (Figure 2B).

These results interestingly demonstrated that MSCs could turn macrophages into M2 types, thus enhance macrophage efferocytosis of apoptotic neutrophils.

Neutrophil Number Increases Following Myocardial I/R Injury

When left anterior descending coronary was ligated, cardiac tissue visually blanched, corresponding with ST-segment of ECG elevation and ventricular arrhythmia accordingly happened within 10 minutes, suggesting complete occlusion of the artery. When the ligature was released 45 minutes later, ST-segment resolution started and ventricular arrhythmia usually happened, showing successful reperfusion. Formation of Q wave and inversion of T wave were observed 1 day after operation. These results suggested successful induction of myocardial I/R models (Figure S2).

Tail vein blood was collected at different time points (6, 12, 18, 24, 30, and 48 hours after operation). Neutrophil number in blood of sham group reached climax at 12 hours, while number of neutrophils after myocardial I/R injury rose progressively until 24 hours and started to decrease but still remained high at 48 hours (Figure 3A). Cardiac cells at different points after I/R were determined by FACS analysis. Related immune cells were

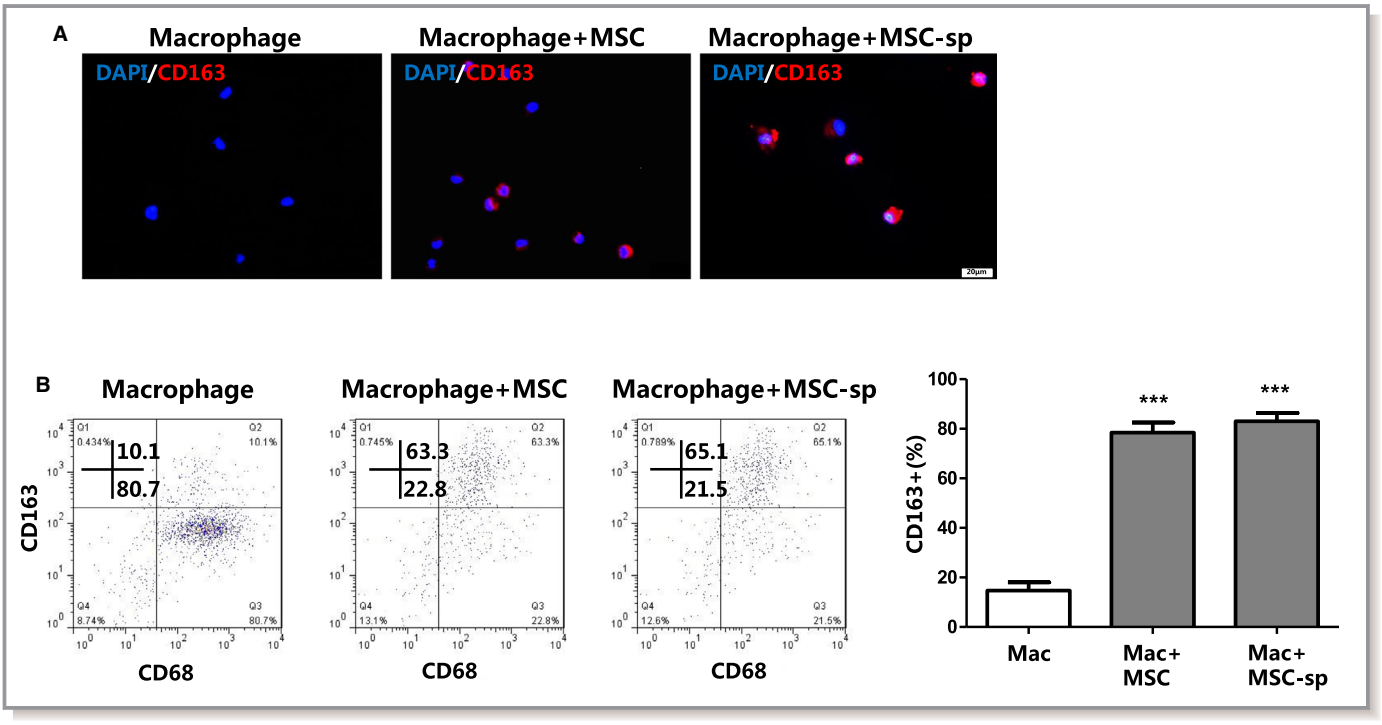

Figure 1. Mesenchymal stem cells (MSCs) turned macrophages into M2 types in vitro. **A**, Representative immunofluorescence staining images (original magnification: ×400). When co-culture with MSCs or MSCs' supernatant, CD163 expression (red) of macrophages was increased. (n=4). **B**, Fluorescence-activated cell sorting analysis showed that MSCs and MSC supernatant both induced M2 macrophages polarization (CD163). (n=4). MSC indicates mesenchymal stem cells. (Data are presented as mean±SD, ***$P<0.001$ vs macrophages, ANOVA with Tukey post hoc test).

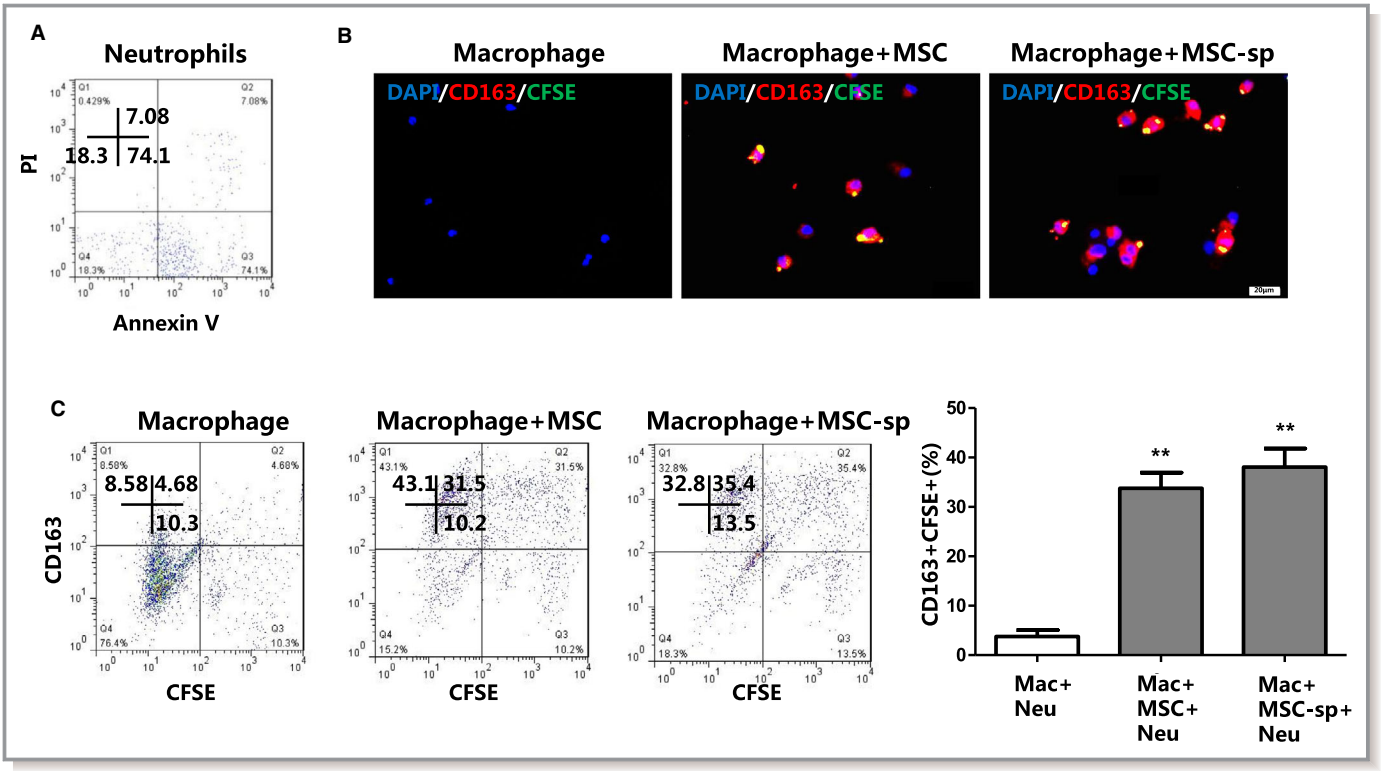

Figure 2. Mesenchymal stem cells enhanced macrophage efferocytosis of apoptotic neutrophils in vitro. **A**, Neutrophil apoptosis was measured with annexin V/PI. (n=4). **B**, Representative immunofluorescence staining images (original magnification: ×400). When co-culture with mesenchymal stem cells or mesenchymal stem cell supernatant, macrophage efferocytosis of early apoptotic neutrophils (green) was increased. (n=4). **C**, Fluorescence-activated cell sorting analysis showed that mesenchymal stem cells and mesenchymal stem cell supernatant both enhanced macrophage efferocytosis of apoptotic neutrophils (CFSE+), which was related to CD163 expression (CD163+CFSE+). (n=4). MSC indicates mesenchymal stem cells; CFSE indicates 5-(and 6)-carboxyfluorescein diacetate succinimidyl ester. (Data are presented as mean±SD, $^{**}P<0.01$ vs macrophages+neutrophils, ANOVA with Tukey post hoc test).

analyzed by following strategy: Neutrophils were recognized by CD45+Gr+, macrophages were CD45+Gr-CD68+, and M2 macrophages were CD45+Gr-CD68+CD163+. Number of neutrophils (CD45+Gr+) in cardiac tissue became maximum 24 hours after myocardial I/R injury ($P<0.01$ versus sham) and remained high at 48 hours (Figure 3B). Immunofluorescence staining and hematoxylin and eosin staining also indicated that neutrophils had infiltrated into heart 24 hours after I/R, compared with sham group (Figure 3C and 3D). These results suggested that neutrophil infiltration is a hallmark of the inflammatory response following myocardial I/R, which is most marked 24 hours after I/R. Accordingly, we regarded immediate after reperfusion as initiation of inflammation and 24 hours after reperfusion as peak of inflammation, at then MSCs were infused to elucidate the impact of MSCs on myocardial I/R injury.

MSCs Ameliorate Myocardial I/R Injury and Improve Cardiac Function

The extents of injury were assayed using TTC/Evans Blue staining. Forty-five minutes ischemia followed by 48 hours reperfusion in MI/R treated group resulted in 33.6±4.7% infarction within the AAR (Infarct/AAR). Infarct/AAR was significantly reduced in MSC 0-hour group (14±4.4%, $P<0.001$) and MSC 24-hour group (25±2%, $P<0.05$), and the value in MSC 0-hour group was less than that in the MSC 24-hour group (14±4.4% versus 25±2%, $P<0.01$) (Figure 4A).

Echocardiography was performed at 28 days after reperfusion. Following I/R, fractional shortening, and left ventricular ejection fraction were significantly decreased in MI/R group ($P<0.001$, versus sham). Fractional shortening and left ventricular ejection fraction in MSC 0-hour group and MSC 24-hour group were significantly increased ($P<0.001$, and $P<0.01$, versus MI/R) (Figure 4B).

Internal pressure of LV was also measured at 28 days after reperfusion. Following I/R, +dp/dtmax and −dp/dtmin were significantly decreased in MI/R group ($P<0.001$, versus sham), +dp/dtmax and −dp/dtmin in MSC 0-hour group and MSC 24-hour group were significantly increased, compared with MI/R group ($P<0.001$, versus MI/R) (Figure 4C).

These findings confirmed that MSCs could ameliorate myocardial I/R injury, reduce infarct size, and improve cardiac

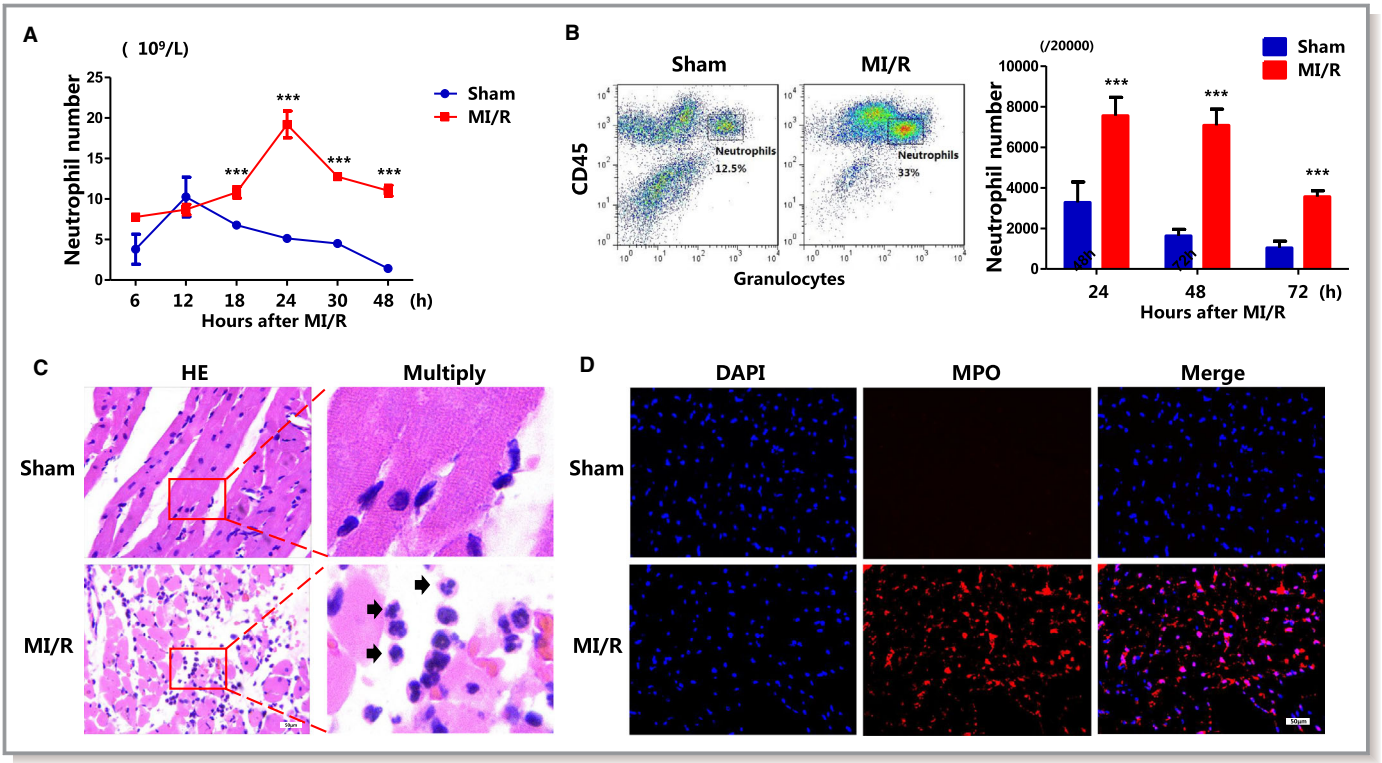

Figure 3. Neutrophil number increases following myocardial ischemia reperfusion injury. **A**, Neutrophil number in tail vein blood of sham group reached climax at 12 hours, while number of neutrophils after myocardial ischemia reperfusion injury rose progressively until 24 hours and it remained high at 48 hours. (n=4). **B**, Fluorescence-activated cell sorting analysis of hearts showed that neutrophil number in cardiac tissue became maximum 24 hours after myocardial ischemia reperfusion injury and remained high at 48 hours, which was significantly different from that of sham group. (n=6). **C** and **D**, Hematoxylin and eosin and immunofluorescence staining indicated that neutrophils had infiltrated into heart 24 hours after ischemia reperfusion, compared with sham group. (n=4). HE indicates hematoxylin and eosin; MI/R indicates myocardial ischemia reperfusion. (Data are presented as mean±SD, ***P<0.001 vs sham, unpaired Student t-test).

function, and immediate infusion might have better outcome than infusion at 24 hours.

MSCs Mediate Neutrophils Infiltration In Vivo, by Facilitating Their Removal Without Affecting Their Recruitment

As determined by FACS analysis of CD45+/Gr+ neutrophils, when MSCs were given at the initiation of inflammation (immediate after reperfusion), neutrophil numbers at peak (24 hours after reperfusion) didn't change significantly, compared with MI/R group (7870±1182 versus 7121±316, P>0.05) (Figure 5A and 5B). Following I/R, the Geom.mean and mean fluorescence intensity of CD11b at neutrophil surface in MI/R group were 370±16.8 and 407±127 at 24 hours, while the values in MSC 0-hour group were greater at 24 hours (523.7±17.9 and 630.7±19.6, P<0.01) (Figure 5C).

When MSCs were given at the peak of inflammation (24 hours after reperfusion), neutrophil numbers were significantly decreased at 48 hours (3811±943.8 versus 6731±470, P<0.01) and 72 hours (1698±616.7 versus 3433±177.2, P<0.01) (Figure 6), demonstrating the potent pro-resolving ability of MSCs. However, the Geom.mean and mean fluorescence intensity of CD11b at neutrophil surface didn't change significantly at 48 hours, compared with MI/R group (656.8±68.5 versus 643.5±87.2, and 716.8±110.6 versus 691±114.6, P>0.05) (Figure S3A).

MSCs Accelerate M2 Macrophage Infiltration and Enhance Macrophage Efferocytosis of Neutrophils In Vivo

Morphological changes using hematoxylin and eosin staining showed that neutrophils had infiltrated into hearts 24 hours after I/R and immune cells were still prevalent at 48 hours. Phagocytotic macrophages infiltrated into hearts 72 hours after I/R, whereas when MSCs were given at the peak of inflammation (24 hours after reperfusion), phagocytotic macrophages had infiltrated into hearts at 48 hours and fibroblasts came into play at 72 hours (Figure 7A), suggesting that MSCs accelerated cardiac healing.

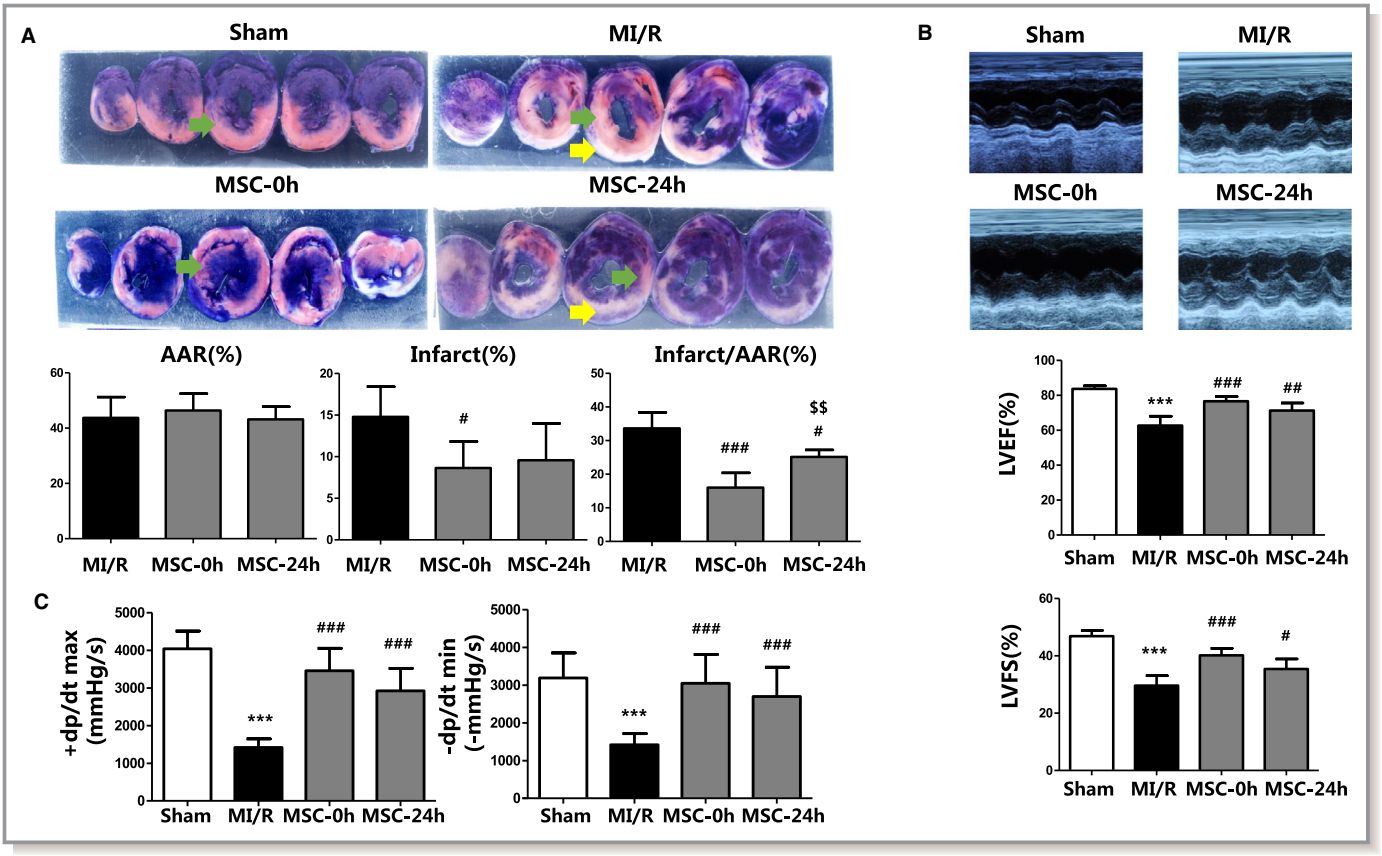

Figure 4. Administration of mesenchymal stem cells ameliorate myocardial I/R injury and improve cardiac function. **A**, Quantification of AAR, infarct size and infarct size/AAR through TTC/Evans Blue staining. AAR was similar between groups. Infarct size and the percentage of infarct size/AAR were significantly decreased in mesenchymal stem cell 0-hour and mesenchymal stem cell 24-hour groups. Mesenchymal stem cell 0-hour group was the most significant. Area at risk was stained as red (green arrow), and infarct area was stained as white (yellow arrow). (n=5,6, or 4 in each group). **B**, Left ventricular fractional shortening and ejection fraction. (n=6). **C**, +dp/dtmax and −dp/dtmin first derivative of left ventricular pressure rise and fall. (n=6). AAR indicates area at risk; LVEF, left ventricular ejection fraction; LVFS, left ventricular shortening fraction; MI/R, myocardial ischemia reperfusion; MSC, mesenchymal stem cells; TTC indicates 2,3,5-triphenyltetrazolium chloride. (Data are presented as mean±SD, ***P<0.001 vs sham, $^{\#}P$<0.05 vs MI/R, $^{\#\#}P$<0.01 vs MI/R, $^{\#\#\#}P$<0.001 vs MI/R, $^{\$\$}P$<0.01 vs MSC 0-hour, ANOVA with Tukey post hoc test).

Representative immunofluorescence staining images of heart slides showed neutrophil and M2 macrophage infiltrations in MI/R and MSCs groups at 48 hours after myocardial reperfusion. Neutrophils were detected using red fluorescence with MPO marker, and M2 macrophages were detected using green fluorescence with CD163 marker. Nuclei were counterstained with DAPI (blue). When MSCs were given at the peak of inflammation (24 hours after reperfusion), M2 macrophage infiltration accelerated at 48 hours, coincidence with enhanced efferocytosis of neutrophils. PMN 24-hour group was used as negative control (Figure 7B, Figure S4).

FACS analysis showed total number of macrophages was decreased after MSCs infusion (6990±267 versus 10 197±1130, P<0.001), and percentage of M2 macrophages was increased at 72 hours (51.6±5.5% versus 30.1±4.4%, P<0.001) (Figure 7B). Percentage of neutrophils that had been phagocytized by M2 macrophages in MI/R group was 13.2±5% at 48 hours, while the efferocytosis was significantly enhanced when MSCs were infused at the peak of inflammation (24h after reperfusion) (20.3±5.3%, P<0.05) (Figure 7C).

These compelling findings suggested that MSCs, rather than neutrophils, could promote M2 macrophages infiltration and thus enhance M2 macrophages-induced efferocytosis of neutrophils, which potentially contributed to the resolution of the inflammation and improved the healing process.

Discussion

In the clinical setting, timely restoration of blood flow is currently thought to be the most effective treatment after MI. However, reperfusion after ischemia causes I/R which triggers a vigorous inflammatory response that ultimately exacerbates tissue injury.[11] General suppression of the immune response, using cortisone, cytostatic, or non-steroidal anti-inflammatory drugs, has shown detrimental

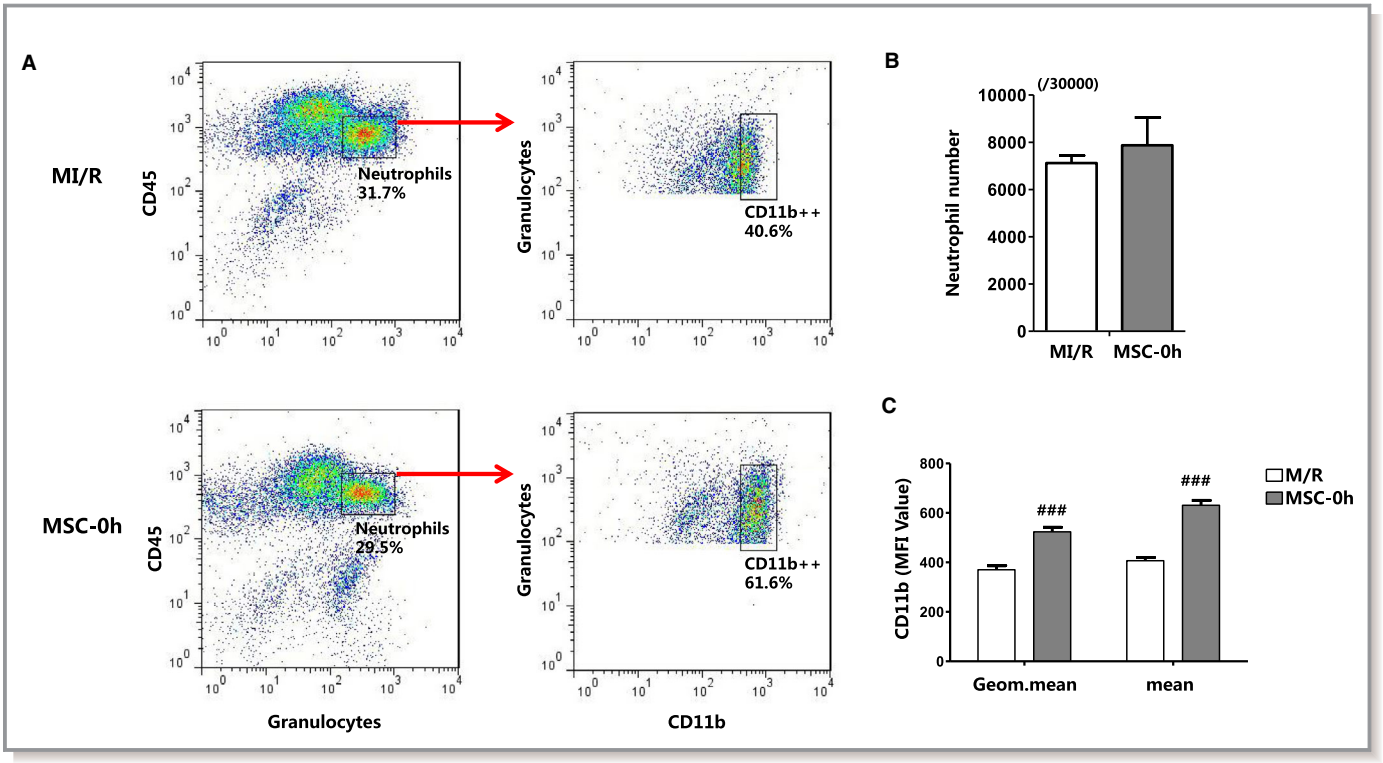

Figure 5. The CD11b expression of neutrophils was higher after mesenchymal stem cells infusion at the initiation of inflammation. **A** and **B**, When mesenchymal stem cells were given at the initiation of inflammation (immediate after reperfusion), neutrophil numbers at peak (24 hours after reperfusion) didn't change significantly. (n=6). **C**, The CD11b expression of neutrophils in mesenchymal stem cell 0-hour group was higher compared with myocardial ischemia reperfusion group. (n=6). MI/R indicates myocardial ischemia reperfusion; MSC, mesenchymal stem cells; MFI indicates median fluorescence Intensity; Geom. indicates geometric. (Data are presented as mean±SD, $^{###}P<0.001$ vs myocardial ischemia reperfusion, unpaired Student *t*-test).

outcomes.[12] Recently, MSC treatment provides a new therapeutic opportunity to improve cardiac outcome after acute injury,[13] but the mechanisms are not fully understood. Systemic immune regulation and resultant tempering of inflammation may be more important than cell transplantation and differentiation in cardioprotective effects of MSC therapy.[14] Intravenously administered MSCs attenuate the progressive deterioration in cardiac function and adverse remodeling through systemically mediated anti-inflammatory activities.[15] In this study, we operated on rats with 45 minutes myocardial ischemia followed by reperfusion. Infusions of MSCs intravenously ameliorate myocardial I/R injury, reduce infarct size, and improve cardiac function. The underlying mechanism partly lies in MSCs mediate neutrophil infiltration by facilitating their removal without affecting their recruitment.

Neutrophil is a double-edged sword. On one hand, neutrophils arrive first to clear debris and release inflammatory mediators,[13] which initiate the wound healing process and the scar formation.[16] On the other hand, they are terminally differentiated which have a short lifespan and easily become apoptosis.[17] Once left within tissues, these apoptotic neutrophils may undergo secondary necrosis, release toxic contents, and subsequently do harm to their neighboring cells.[18] In our study, when MSCs were given at the peak of inflammation (24 hours after reperfusion), neutrophil numbers were significantly decreased afterwards, coincidence with acceleration of interstitial fibrosis, which demonstrates the potent proresolving ability of MSCs. Apoptotic neutrophils are removed by scavenger macrophages through the process of efferocytosis.[19] Zizzo et al[20] discovered that efficient clearance of early apoptotic cells requires CD163+ macrophages. An important characteristic of macrophages is their plasticity, which enables them to switch from one subset to another.[21] CD163+ macrophages belong to M2 subset.[20] MSCs have been certified to reprogram macrophages from a proinflammatory M1 phenotype toward an anti-inflammatory M2 phenotype, coincidence with decreased secretion of inflammatory cytokines such as interleukin-1a, TNF-a, interleukin-12, and interleukin-17, and increased secretion of anti-inflammatory cytokines such as interleukin-10 and VEGFs.[21,22] The mechanisms involved in macrophage polarization by MSCs depend on the secretion of soluble factors, including prostaglandin E2 (PGE2), tumor necrosis factor

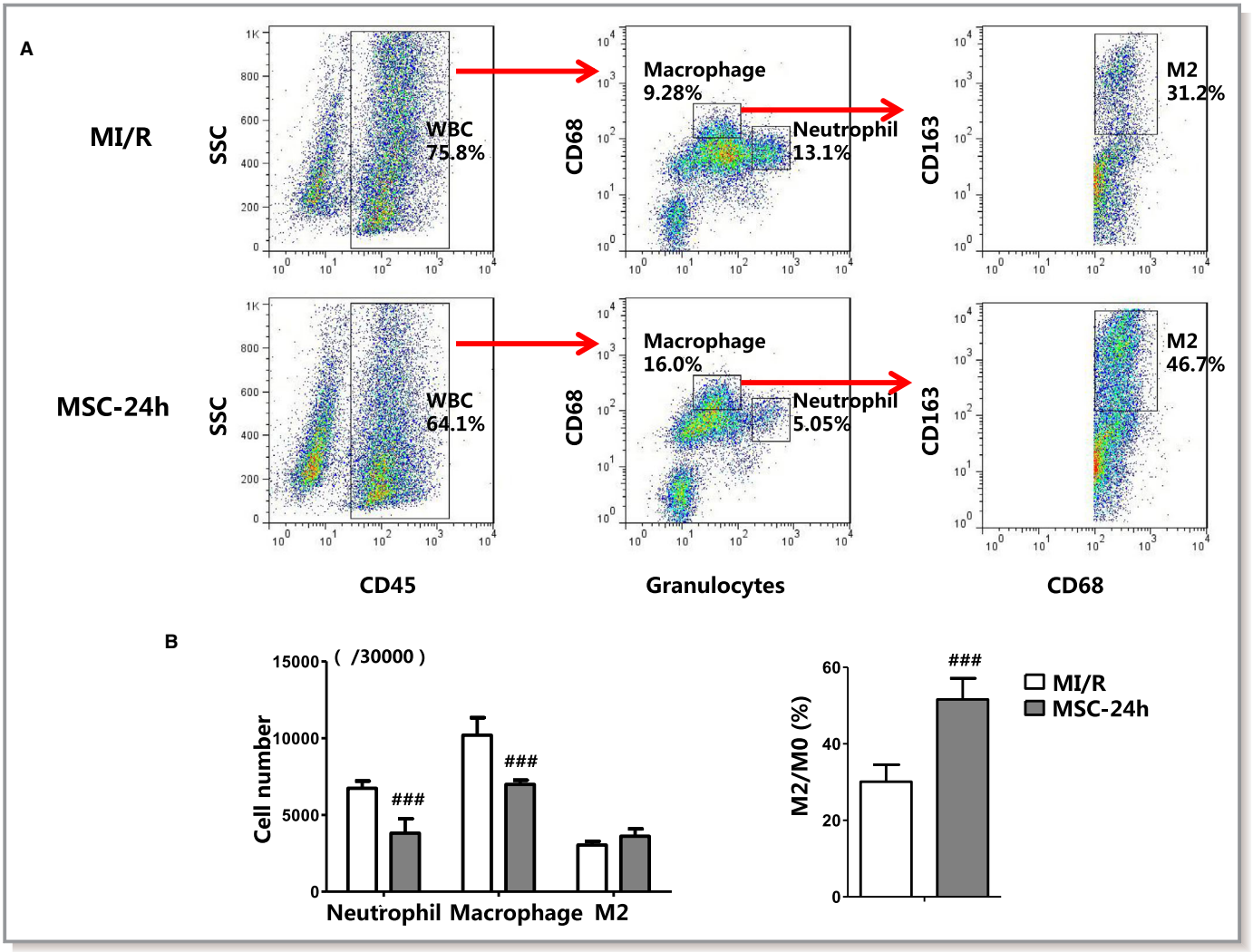

Figure 6. Immune cell numbers changed after MSCs infusion at the peak of inflammation. **A**, Gating strategy of fluorescence-activated cell sorting analysis. Neutrophils were recognized by CD45+Gr+, Macrophages were CD45+Gr-CD68+, and M2 macrophages were CD45+Gr-CD68+CD163+. **B**, When mesenchymal stem cells were given at the peak of inflammation (24 hours after reperfusion), White blood cell absolute numbers and percentage of white blood cells in cells collected from hearts were decreased at 48 and 72 hours after reperfusion, neutrophils' absolute numbers and percentage of neutrophil in white blood cells were significantly decreased at 48 and 72 hours after reperfusion, macrophages' absolute numbers were decreased at 72 hours but the percentage of macrophages in white blood cells were significantly increased, M2 macrophages' absolute numbers were unchanged while percentage of M2 macrophages in all macrophages were significantly increased. (n=6). M2 indicates M2 macrophages; MI/R, myocardial ischemia reperfusion; MSC, mesenchymal stem cells; WBC, white blood cells; SSC indicates side scatter. (Data are presented as mean±SD, $^{###}P<0.001$ vs MI/R, unpaired Student *t*-test).

alpha stimulated gene-6 (TSG-6), interleukin-6 (IL-6), indoleamine 2,3-dioxygenase (IDO), and transforming growth factor-β1 (TGF-β1).[22] Along with the evidences above, we co-cultured macrophages with MSCs or MSCs supernatant in vitro, and most macrophages became M2 polarization coincidence with enhanced ability to phagocytize apoptotic neutrophils. When we infused MSCs, not neutrophils, at peak of inflammation (24 hours after reperfusion), percentage of M2 macrophages increased, and M2 macrophages-induced efferocytosis of neutrophils enhanced. These compelling evidences suggested that MSCs infusion improve cardiac repair after myocardial I/R in rats through enhancing M2 macrophages-induced efferocytosis of apoptotic neutrophils.

Henning et al[23] found that MSC injections quickly and markedly decreased the number of neutrophils which infiltrated into hearts following MI. However, in our study, when given MSCs at initiation of inflammation (immediately after reperfusion), neutrophils' number didn't change significantly at 24 hours after I/R, compared with control groups. It is widely accepted that inflammation after I/R and MI should be considered separately.[24] After MI, the immune response, depending on ischemic effects, is long-lasting and persistent, whereas after reperfusion the response is relatively minor and

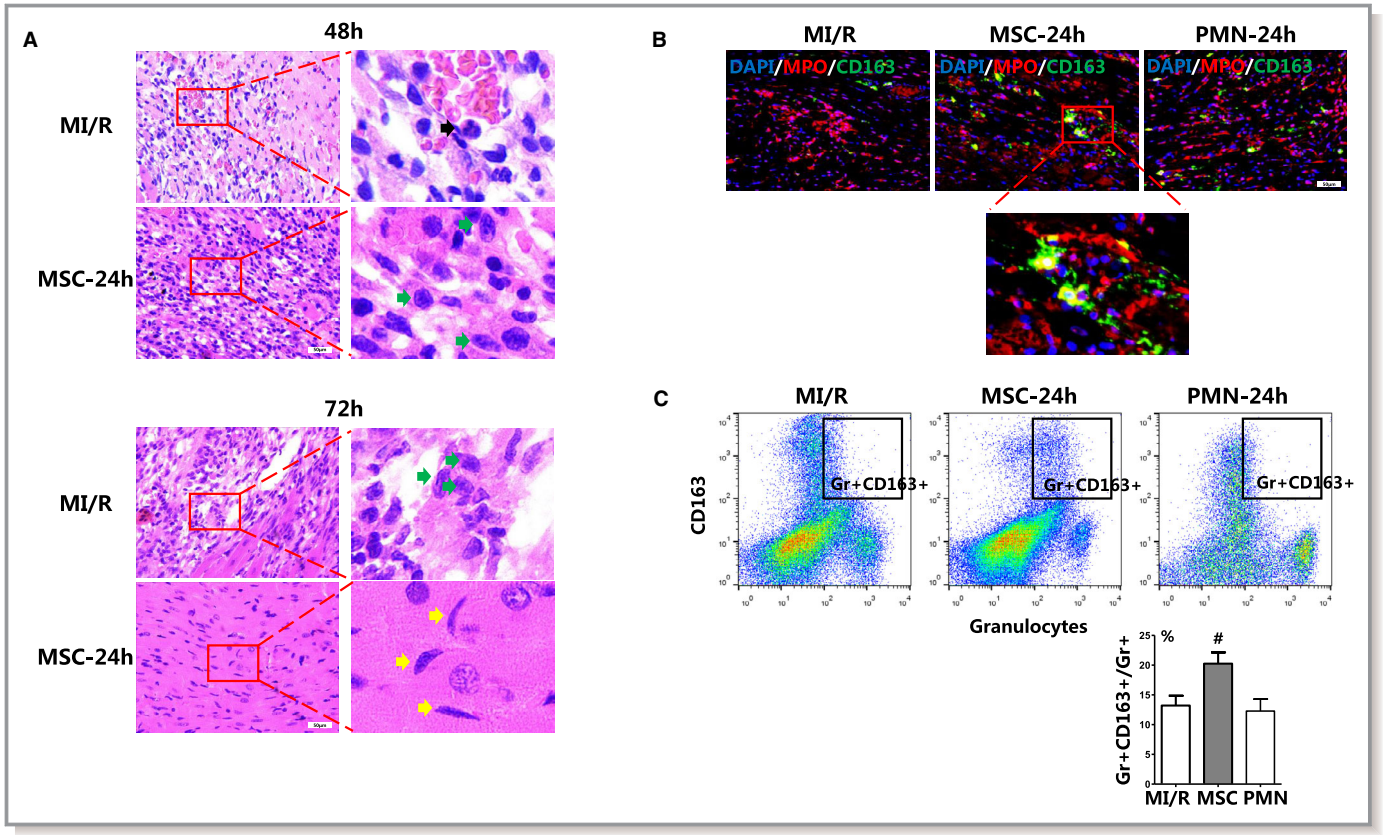

Figure 7. Mesenchymal stem cells (MSCs) accelerate M2 macrophages infiltration and enhance efferocytosis of neutrophils in vivo. **A**, Hematoxylin and eosin staining of heart slides (original magnification: ×400). Neutrophils lasted for >48 hours after ischemia reperfusion (black arrow) and phagocytotic macrophages infiltrated into hearts 72 hours after ischemia reperfusion (green arrow), whereas when MSCs were given at the peak of inflammation (24 hours after reperfusion), phagocytotic macrophages had infiltrated into hearts at 48 hours (green arrow) and fibroblasts came into play at 72 hours (yellow arrow). (n=4). **B**, Representative immunofluorescence staining images of heart slides (original magnification: ×400). When MSCs were given at the peak of inflammation (24 hours after reperfusion), macrophage efferocytosis of neutrophils at 48 hours was enhanced (myeloperoxidase+CD163+). (n=4). **C**, Fluorescence-activated cell sorting analysis showed that when MSCs were given at the peak of inflammation (24 hours after reperfusion), the percentage of M2 macrophages phagocytized neutrophils (Gr+CD163+) was significantly increased at 48 hours. (n=6). MI/R indicates myocardial ischemia reperfusion; MSC, mesenchymal stem cells; PMN, polymorphonuclear cells. (Data are presented as mean±SD, $^{\#}P<0.05$ vs myocardial ischemia reperfusion, ANOVA with Tukey post hoc test).

transient but more complex because of the additional effect of reperfusion injury.[24] With this in mind, we assumed the above mentioned result might depend on neutrophils quickly and largely flocked into heart on account of the rapid blood flow of reperfusion, whereas they gradually infiltrated into heart because of lack of blood flow after MI. McDonald et al[25] discovered that integrin αMβ2 (Mac1, CD11b) was needed in neutrophil adhesion during sterile inflammation. We found that when MSCs were infused at the initiation of inflammation, CD11b expressions of neutrophils infiltrated into heart became enhanced at peak assessed by flow cytometry, which might suggest that neutrophils became somewhat more activated; when MSCs were infused at the peak of inflammation, CD11b expressions of neutrophils at 48 hours didn't significantly change, but seemed higher than those at 24 hours. Importance of neutrophils in sterile environments is highlighted by the fact that simple removal of neutrophils results in negative outcome.[26,27] In line with these, immediate infusion of MSCs after reperfusion had a better outcome in cardiac function than that of 24 hours later in our study, showing that neutrophil activity could be more beneficial after infusion of MSCs.

It is controversial when MSCs should be infused following I/R. Many studies injected MSCs few hours after reperfusion, whereas some of the researches prefer to inject MSCs at the onset of reperfusion.[9] It is indispensable to clarify the golden time for the infusion of MSCs after myocardial I/R. Neutrophils play a major role in myocardial damage after I/R.[28] We observed neutrophil infiltration after I/R and that neutrophil number was dramatically increased compared with sham operation, which is similar to Yan et al's[24] work. Initiation of inflammation is deemed as immediate after injury and resolution of inflammation begins after neutrophils reach climax.[29] Similar with Schwab et al's[30] study, we regarded immediately after reperfusion as initiation of inflammation and 24 hours after reperfusion as peak of inflammation. M2

macrophage-induced efferocytosis of apoptotic neutrophils were strengthened at 48 hours when MSCs were infused at 24 hours, not at beginning (Figure S3B), compared with the saline group. Raffaghello et al[31] showed that MSCs prolong the survival of neutrophils without affecting their phagocytic and chemotactic activities. Under inflammatory conditions both neutrophils and macrophages work together, and MSCs could help them be more harmonious. Results showed that immediate infusion had more benefits than those of infusion at 24 hours, suggesting that early infusion of MSCs might have a better outcome. We hypothesized that MSCs could be used as soon as possible after reperfusion therapy, with effectiveness in restricting excessive inflammation and safety in not depriving "the good side" of the inflammation. More evidences would be needed before clinical application.

A major challenge of the study was the limited availability of monoclonal antibodies to rat cell surface molecules. For example, neutrophils of mice were commonly stained with ly6g and ly6c, but ly6g and ly6c were not applicable for rats. We used "anti-granulocytes", an unofficial antibody used for neutrophils but applicable for rats, which would imply all granulocytes including neutrophils, eosinophils, and basophils. More antibody combinations should be used to identify different cell populations. Amanzada et al[32,33] demonstrated that MPO was only expressed by neutrophils, unless they were phagocytized by macrophages that cells could express both MPO and CD163, which negate the traditional view that the MPO is also expressed by macrophages. Neutrophils have functional heterogeneity and could change their phenotype after cytokine exposure.[34] Compelling evidence has shown that only a selective population of neutrophils was recruited to the site of injury.[35] It cannot not be excluded that neutrophils might have changed their phenotypes after MSCs infusion and as such they do not necessarily represent separate lineages.

The major finding of the present study revealed that some of the favorable effects of MSCs on myocardial ischemic reperfusion are mediated by modulation of neutrophil infiltration. Administration of MSCs intravenously ameliorate myocardial I/R injury, reduce infarct size, and improve cardiac function; MSCs infusion immediately had better outcome compared with 24 hours later. The underlying mechanism partly lies in the fact that MSCs mediate neutrophil infiltration by facilitating their removal without affecting their recruitment. Specifically, MSCs accelerate M2 macrophage infiltration and enhance macrophage efferocytosis of neutrophils both in vitro and in vivo. Taken together, we propose an additional pathway by which MSCs could improve cardiac healing after I/R: MSCs could accelerate the resolution of inflammation by enhancing M2 macrophage-induced efferocytosis of apoptotic neutrophils without changing neutrophil recruitment.

Conclusions

In this study, we interestingly discovered that MSC transplantation improves cardiac repair after myocardial I/R in rats, and immediate transplantation had better outcome. The influx of neutrophils did not significantly change but their removal was accelerated. Furthermore, we discovered for the first time that MSCs could enhance M2 macrophage-induced efferocytosis of apoptotic neutrophils, both in vitro and in vivo. The importance of these findings lies in the fact that targeting neutrophils using MSCs could be a novel approach to ameliorate I/R injury, thus preventing ventricular remodeling and improving cardiac function.

Acknowledgments

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Disclosures

None.

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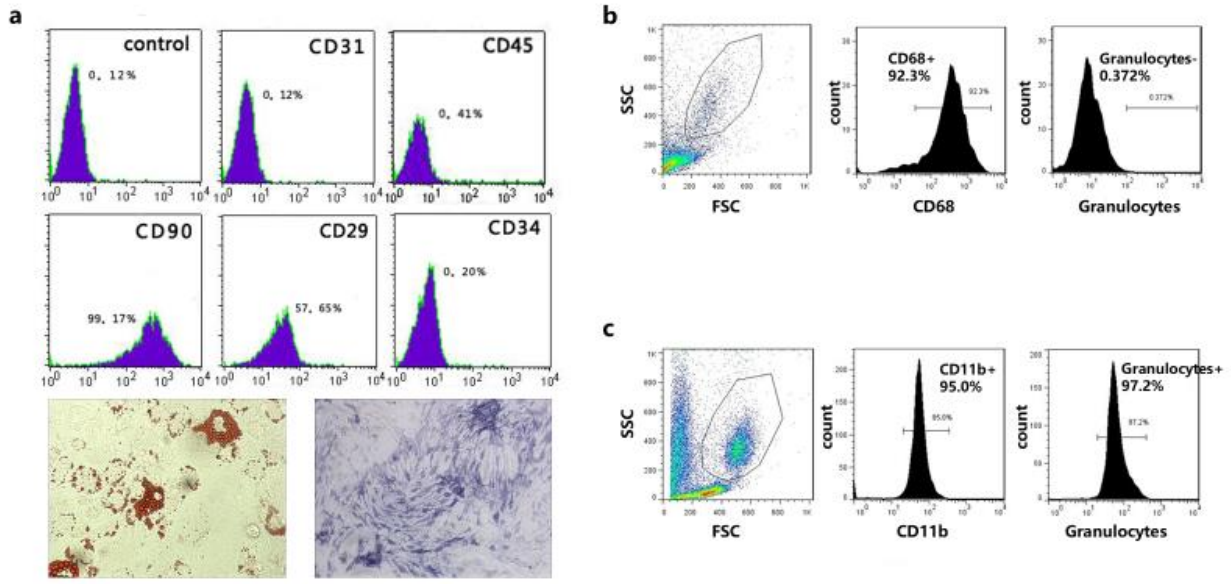
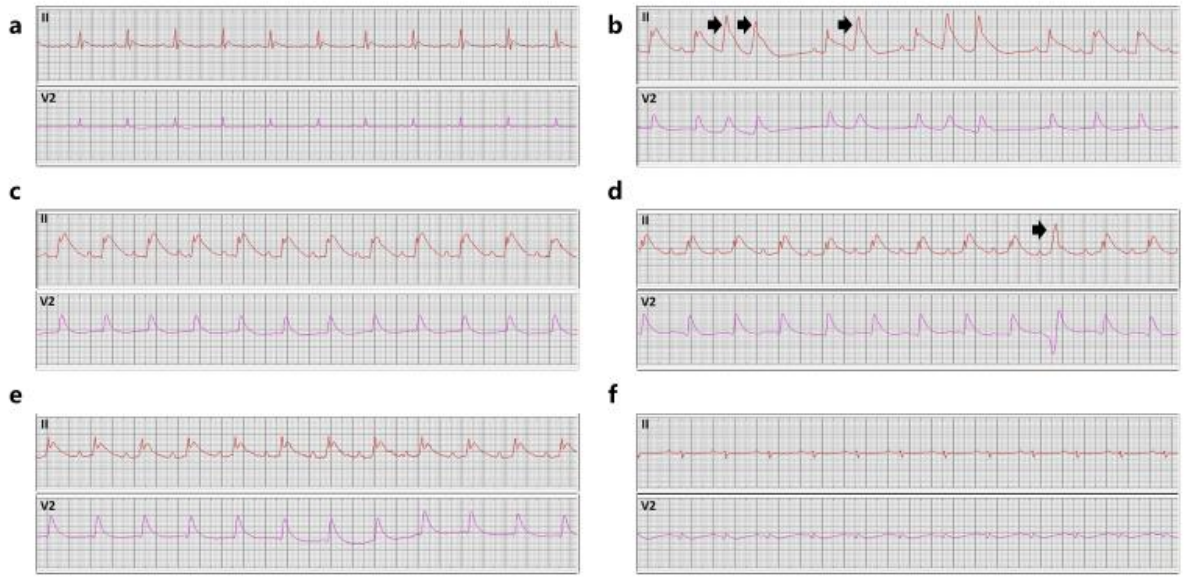
SUPPLEMENTAL MATERIAL

Figure S1. Identification of isolated cells.



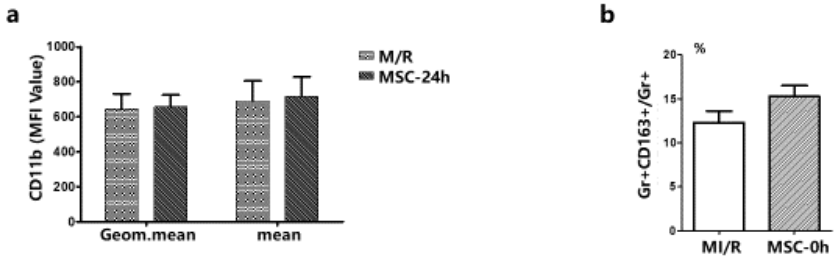
(a) MSCs were identified by FACS analysis as CD31-CD45-CD90+CD29-CD34-, and with adipogenic and myogenic differentiations. (b) Macrophages were identified by FACS analysis as CD68+Granulocyte-. (c) Neutrophils were identified by FACS analysis as CD11b+Granulocyte+.

Figure S2. ECGs during myocardial I/R surgery.



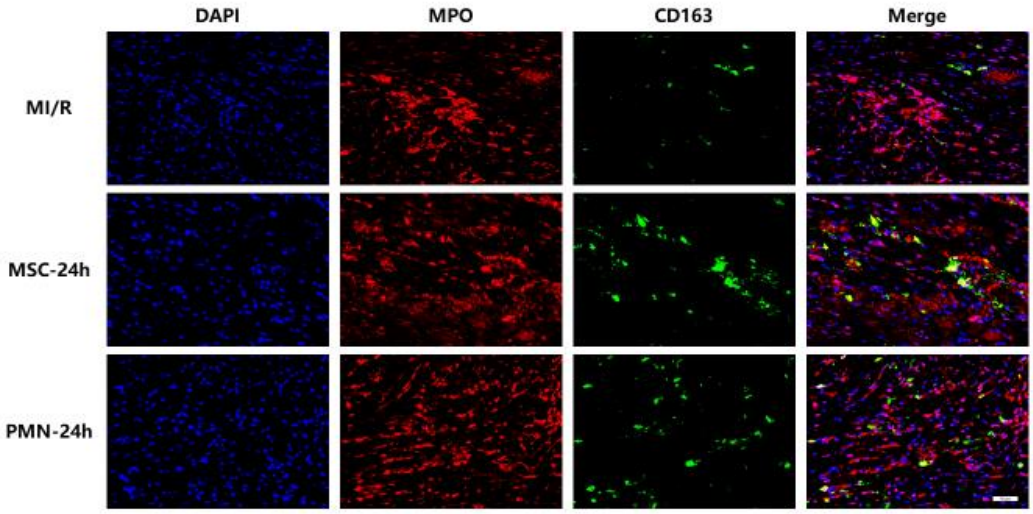
(a) Baseline. (b) Ligation of LAD, ventricular arrhythmia within 10min (Black Arrow). (c)Ligation of LAD, ST segment elevation. (d) Release of ligation, ventricular arrhythmia within 10min (Black Arrow). (e) Release of ligation, ST segment resolution. (f) One day after release, formation of Q wave and inversion of T wave.

Figure S3. FACS analysis.



(a) When MSCs were given at the peak of inflammation (24h after reperfusion), the CD11b expression of neutrophils at 48h didn't change significantly. (n=5). (b) When MSCs were given at the initiation of inflammation (immediate after reperfusion), macrophage efferocytosis of neutrophils at 48h didn't change significantly. (n=5). (Data are presented as mean ± SD, unpaired Student's t-test). MI/R, Myocardial Ischemia Reperfusion; MSC, Mesenchymal Stem Cells; Gr, Granulocytes.

Figure S4. Representative immunofluorescence staining images of heart slides (Original magnification: ×400).



When MSCs were given at the peak of inflammation (24h after reperfusion), macrophage efferocytosis of neutrophils at 48h was enhanced. (blue: DAPI, red: MPO, green: CD163). MI/R, Myocardial Ischemia Reperfusion; MSC, Mesenchymal Stem Cells; PMN, Polymorphonuclear Cells; MPO, Myeloperoxidase.