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IL-6 knockout ameliorates myocardial remodeling after myocardial infarction by regulating activation of M2 macrophages and fibroblast cells

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Abstract. – OBJECTIVE: To investigate the effects of interleukin-6 (IL-6) gene knockout on myocardial remodeling after myocardial infarction (MI) in mice and the potential mechanism, to provide certain references for the prevention and treatment of MI in clinic.

MATERIALS AND METHODS: A total of 40 male C57 mice were divided into two groups, namely Sham group (n=20) and MI group (n=20), using a random number table. Another 20 mice with IL-6 gene knockout were enrolled into the MI + IL-6 KO group. The MI model was established by means of ligating the left anterior descending coronary artery of the mice. 28 d later, the survival status of the three groups of mice was recorded. In addition, the cardiac functions of each group of mice, including two-dimensional echocardiography, ejection fraction (EF%) and fractional shortening (FS%), were measured. The cross-sectional area and pathological change of the myocardial cells in cardiac tissues of each group of mice were detected via hematoxylin and eosin (H&E) staining. Immunohistochemistry was applied to determine the expression of tumor necrosis factor-alpha (TNF-α) in each group of mouse cardiac tissues. Moreover, immunofluorescent staining was utilized to measure the content of M2 macrophages in each group of mouse cardiac tissues.

RESULTS: The 28-d survival rate of the mice with IL-6 gene knockout was remarkably higher than that of the wild-type mice ($p<0.05$). Furthermore, the cardiac functions of the mice in the MI + IL-6 KO group were superior to those in the MI group, with markedly improved FS% and EF% ($p<0.05$). According to the H&E staining results, the cross-sectional areas of the heart and myocardial cells were decreased notably in MI + IL-6 KO group compared with those in the MI group ($p<0.05$). The immunohistochemical staining results showed that IL-6 knockout could lower the MI-induced high expression of TNF-α ($p<0.05$), and Masson's trichrome staining indicated that IL-6 knockout could also repress the degree of cardiac fibrosis. Moreover, it was discovered through immunofluorescent staining that the mice in the MI + IL-6 KO group had markedly elevated content of M2 macrophages in cardiac tissues than those in the MI group ($p<0.05$).

CONCLUSIONS: Inhibiting IL-6 gene expression can prominently ameliorate the MI-induced myocardial remodeling, whose mechanism is possibly associated with the activation of M2 macrophages and reduced collagen production in fibroblast cells.

Key Words:
Myocardial infarction, Myocardial remodeling, IL-6, M2 macrophage activation.

Introduction

Myocardial infarction (MI) is not only a leading cause of death but also a major public health problem around the world[1,2]. Percutaneous coronary intervention in the early stage is recognized as the most efficacious method to treat MI at present[3]. After MI, the left ventricle undergoes a train of repairing and wound healing responses such as inflammation and scar formation, which is collectively referred to as left ventricular remodeling. The degree of left ventricular remodeling after MI directly determines the structure and physiology of the left ventricle as well as the long-term survival rate of the patients[4]. Excessive left ventricular remodeling after MI can develop into heart failure, making the patient's 5-year mortality rate due to heart failure close to 50%[5]. Therefore, further clarifying the mechanism of the occurrence and development of myocardial remodeling after MI is of great significance for the early prevention and precise treatment of the disease.

After MI, massive leukocyte infiltration exists in the MI region, to eliminate the necrotic myocardial tissues. In the first hour after myocardial injury, the levels of pro-inflammatory cytokines

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[including tumor necrosis factor-alpha (TNF-α), interleukin-1 (IL-1), and IL-6] rise rapidly, which serve as early stresses against the myocardial injury[6]. At 3-5 d after MI, the inflammatory responses transform into anti-inflammation and repair responses mainly characterized by accelerated activation and proliferation of fibroblast cells[7]. At 1-7 d after MI, the heart is capable of recruiting reparative M2 macrophages, releasing transforming growth factor-β (TGF-β), IL-10, and other cytokines and inhibiting the inflammatory responses and myocardial fibrosis[8,9]. Inflammations have important significance in the early left ventricular remodeling, but excess inflammatory responses may promote left ventricular dilation and scar formation, thus damaging the normal physiological functions of the left ventricle[10]. Therefore, repressing the inflammations in the heart timely is the key to inhibiting the ventricular remodeling.

IL-6 is a kind of pro-inflammatory cytokine possessing a strong ability to activate the inflammations[11]. In animal models, the serum IL-6 in the mice is increased markedly within 6 h after myocardial ischemia/reperfusion[12]. IL-6 can also promote the synthesis and release of pro-inflammatory mediators in multiple types of cells[13]. In the patients with acute MI, the serum IL-6 level is closely related to the development of heart failure within 24 h after angioplasty[14]. However, the action and mechanism of IL-6 in the myocardial remodeling after MI have not been reported yet.

In this work, the MI model was established in the mice with IL-6 gene knockout, the effects of IL-6 knockout on the cardiac functions, cardiac hypertrophy and myocardial fibrosis after MI in the mice were detected, and the potential molecular mechanism of IL-6 knockout in the myocardial injury was analyzed at the same time.

Materials and Methods

Grouping and Treatment of Laboratory Animals

Our study was approved by the Animal Ethics Committee of Central South University Animal Center. A total of 40 male C57 mice aged 8-10 weeks and weighing (24.61±1.64) g were divided into Sham group (n=20) and MI group (n=20) by means of a random number table. Another 20 mice with IL-6 gene knockout were enrolled into the MI + IL-6 KO group. There were no statistical differences in the age in weeks, weight and other basic information among the three groups of mice. The specific surgical approaches to establish the MI model are as follows: the mice in each group were anesthetized by intraperitoneal injection of 50 mg/kg pentobarbital and, then, the cannula was inserted into the left carotid artery to detect their blood pressure. The ECG limb leads were applied to measure the heart rate. The thoracic cage was opened from the fourth intercostal space, and the pericardium was excised to expose the heart. Next, the left anterior descending coronary artery was ligated at 2 mm above the left atrial appendage using a 6-0 silk suture to induce local myocardial ischemia, and the chest was closed layer by layer after the surgery. The mice in the Sham group were subjected to the same surgical procedures except for the ligation with silk suture. 28 d later, the mice were sacrificed to acquire the myocardial tissues on the left ventricular anterior wall, and the tissues were placed into a refrigerator at -80°C for standby use after the blood was rinsed away with normal saline.

Detection via Echocardiography

To determine the cardiac functions of the mice in each group, a MyLab 30 CV ultrasound system (Esaote S.p.A, Genova, Italy) and a 10-MHz linear ultrasonic transducer were used to obtain the echocardiography. After the hair in the anterior thoracic region was shaved off, the mice were anesthetized and put on a heating plate at 37°C, with the left side facing upward. Parameters including ejection fraction (EF%), fractional shortening (FS%) and heart rate (bpm) were measured.

Hematoxylin and Eosin (H&E) Staining

The hearts in each group were placed in 10% formalin overnight, followed by dehydration and embedding in paraffin blocks. Subsequently, all the myocardial tissues were sliced to 5-μm-thick sections, fixed on glass slides and dried by baking; then, they could be utilized for staining. According to the instructions, the tissues were soaked in xylene, graded ethanol and hematoxylin and mounted in resin. After air drying, the tissues could be observed and photographed under a light microscope. The morphology of myocardial cells, myocardial interstitium and myofilament were observed.

Immunohistochemical Staining

The sliced myocardial tissue sections were baked in the oven at 60°C for 30 min, followed by deparaffinization in xylene (5 min × 3 times) and dehydration with 100%, 95% and 70% ethanol 3 times, respectively. The activity of endog-

enous peroxidase was inhibited by 3% hydrogen peroxide-methanol, and the tissues were sealed in goat serum for 1 h. The anti-TNF-α antibody was diluted at 1:100 with Phosphate-Buffered Saline (PBS; Gibco, Grand Island, NY, USA) and incubated at 4°C overnight, followed by washing with PBS on a shaking table 4 times. After the addition of secondary antibodies, the diaminobenzidine was adopted for color development. After that, 6 samples were randomly selected from each group, and five fields of vision were randomly selected from each sample for photography under the light microscope (200× and 400×).

Detection via Western Blotting

The myocardial tissues of each group of mice were fully ground in lysis buffer, followed by ultrasonication. Then, the lysis buffer was centrifuged, and the supernatant was absorbed and subpackaged into Eppendorf (EP; Eppendorf, Hamburg, Germany) tubes. Later, the protein concentration was determined through bicinchoninic acid (BCA) method (Pierce, Waltham, MA, USA) and ultraviolet spectrophotometric assay, and the volume of all the sample proteins was maintained at equal concentration. Next, the proteins were subpackaged and preserved in the refrigerator at -80°C. Then, the total protein was extracted and subjected to sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). After that, the protein in the gel was transferred onto a polyvinylidene difluoride (PVDF) membrane (Millipore, Billerica, MA, USA), followed by incubation with primary antibody at 4°C overnight, incubation with goat-anti-rabbit secondary antibody in the dark for 1 h, and scanning and quantification of protein bands using an Odyssey membrane scanner. The level of the protein to be detected was corrected *via* glyceraldehyde 3-phosphate dehydrogenase (GAPDH).

Masson's Trichrome Staining

First, the paraffin sections were deparaffinized and dehydrated, followed by chromate treatment or elimination of mercury salt precipitation. Subsequently, the sections were washed with tap water and distilled water sequentially, followed by visualization of cell nuclei using Regaud hematoxylin staining solution or Weigert hematoxylin staining solution for 5-10 min. Next, the sections were washed in water sufficiently and stained with Masson's ponceau acid fuchsin liquid for 5-10 min. After the sections were immersed in 2% glacial acetic acid solution for a while, they were differentiated in 1% phosphomolybdic acid solution for 3-5 min. Finally, the sections were cleared in 95% alcohol, absolute alcohol and xylene and then mounted in neutral balsam.

Statistical Analysis

All the data were analyzed using Statistical Product and Service Solutions (SPSS) 22.0 software (IBM, Armonk, NY, USA), the measurement data were presented as mean ± standard deviation, and *t*-test was performed for comparison of data between the two groups. $p<0.05$ suggested that the difference was statistically significant.

Results

IL-6 Expression in Myocardial Tissues of MI Mice

As shown in Figure 1, the expression levels of IL-6 protein in the myocardial tissues of MI mice in the Sham group, MI group and MI + IL-6 KO group were detected separately. The results indicated that the expression level of IL-6 protein in the myocardial tissues in the MI group was elevated remarkably ($p<0.05$), which was substantially completely inhibited in the MI + IL6 KO group ($p<0.05$), suggesting that the mouse model of IL-6 knockout is induced successfully.

Effects of IL-6 Knockout on 28-d Survival Rate of Each Group of Mice After MI

It was discovered through plotting the Kaplan-Meier survival curves that IL-6 knockout could improve the 28-d survival rate of MI mice prominently ($p<0.05$) and reduce the prevalence rates of cardiac rupture and adverse cardiovascular events caused by myocardial ischemia and arrhythmia in the mice (Figure 2).

Effects of IL-6 Knockout on Cardiac Functions of Each Group of Mice

According to the results of echocardiography (Figure 3), there was no statistically significant difference in the heart rate among the three groups of mice, so the differences in EF% and FS% due to the heart rate among the three groups of mice could be excluded. Compared with those in the Sham group, the chambers of the mouse heart were expanded, the cardiac wall was thinned, and IL-6 knockout could markedly ameliorate the MI-induced abnormal changes in the cardiac structure in MI group. Furthermore, the FS% and EF% in each group were measured, and it was

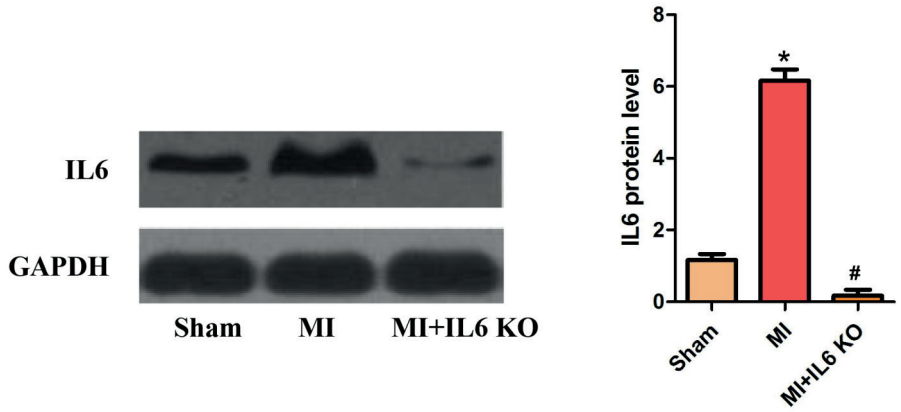

Figure 1. IL-6 expression in myocardial tissues of MI mice. Sham: Sham group, MI: MI group, MI + IL-6 KO: MI + IL-6 gene knockout group, *p<0.05 *vs.* Sham group, # p<0.05 *vs.* MI group, with a statistically significant difference.

revealed that IL-6 knockout remarkably reversed the decreases in FS% and EF% in the MI group (p<0.05), manifesting that the inhibition on IL-6 can prominently improve the cardiac functions of the MI mice.

H&E Staining Results for Heart in Each Group of Mice

H&E staining was performed for the myocardial tissues to assess the microstructural changes of myocardial cells in the cross-section of a mouse heart in each group. The results showed (Figure 4) that there was apparent edema in myocardial cells, disordered arrangement, degradation and different degrees of necrosis of myofilaments, and infiltration of inflammatory cells at the same time in the MI group. After IL-6 knockout in the mice, however, the cardiac tissue edema was relieved evidently, and the myofilament abnormality was improved significantly, indicating that repressing IL-6 can alleviate MI-induced myocardial injury.

Expression Level of TNF-α in Myocardial Tissues of Each Group of Mice

Meanwhile, immunohistochemistry was applied to analyze the expression level of TNF-α protein in the myocardial tissues of each group of mice, and it was manifested (Figure 5) that the expression level was elevated markedly at 28 d after MI, while the mice in the MI + IL-6 KO group had a notably lower expression level of TNF-α protein in the myocardial tissues than the MI group, indicating that IL-6 knockout may affect the release of pro-inflammatory cytokines.

Effects of IL-6 Knockout on Cardiac Fibrosis of Each Group of Mice

To evaluate the effects of IL-6 knockout on the cardiac fibrosis of mice, Masson's trichrome staining was adopted to detect the expression of collagens in the cardiac tissues of each group of mice. According to the results (Figure 6), IL-6 was able to prominently reduce the myocardial interstitial fibrosis after MI, thus suppressing the cardiac remodeling.

Effects of IL-6 Knockout on Infiltration of M2 Macrophages in Myocardial Tissues of Each Group of Mice

Considering that M2 macrophages are crucial anti-inflammatory macrophages, CD206 positive cells in the cardiac tissue in each group were labeled *via* immunofluorescent staining. It was shown that the percentage of CD206 positive cells in the MI group was markedly lower than

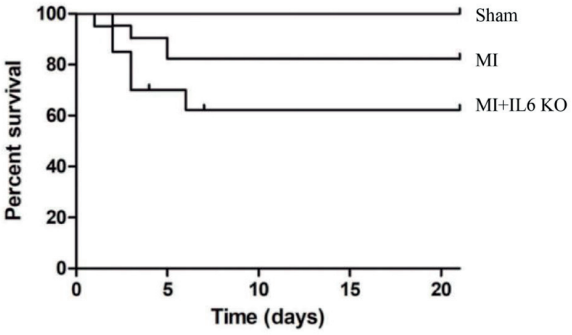

Figure 2. Effects of IL-6 knockout on 28-d survival rate of each group of mice after MI. Sham: Sham group, MI: MI group, MI + IL-6 KO: MI + IL-6 gene knockout group.

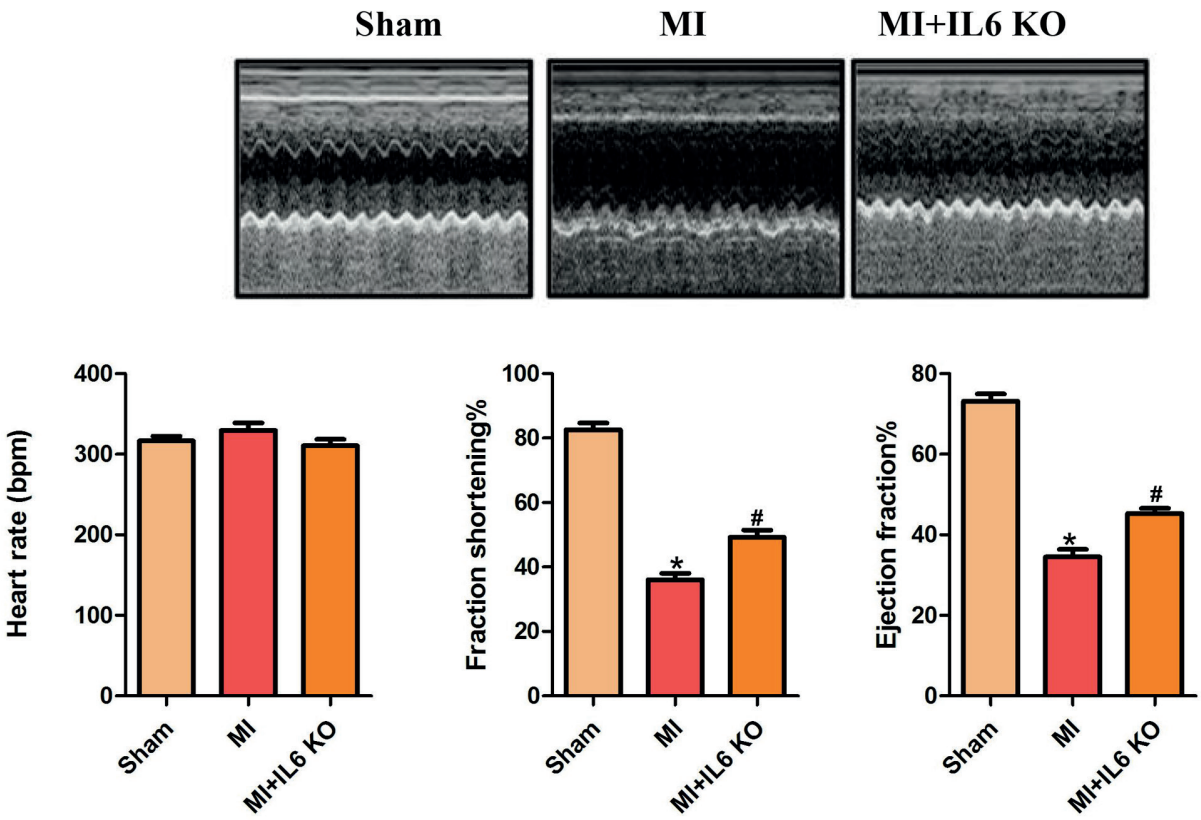

Figure 3. Effects of IL-6 knockout on cardiac functions of each group of mice. Sham: Sham group, MI: MI group, MI + IL-6 KO: MI + IL-6 gene knockout group, *p<0.05 *vs.* Sham group, # p<0.05 *vs.* MI group, with a statistically significant difference.

that in the Sham group, which was increased notably after IL-6 knockout (p<0.05), illustrating that IL-6 knockout is capable of promoting M2 macrophage polarization.

Discussion

This work aims to evaluate the therapeutic applicability of IL-6 in ameliorating wound healing after MI by regulating the functions of macrophages and fibroblast cells. The results showed that IL-6 gene knockout could improve the physiological functions of the heart and M2 macrophage polarization after MI and decrease collagen deposition by repressing fibroblast cell activation simultaneously. IL-6 inhibits the inflammation and enhances the self-healing capability of the heart after MI by directly acting on the macrophages and indirectly acting on the fibroblast cells.

It is known that IL-6, as a common kind of pro-inflammatory cytokine, can induce the generation of pro-inflammatory cytokines. In aged mice, IL-6 deficiency can aggravate left ventricular dysfunction and cardiac inflammations[15]. However, in young mice, IL-6 deficiency can remarkably lower the expression levels of TNF-α and CCL2[16]. Studies[17] have demonstrated that Exendin-4 is able to increase the expression of IL-6, thus suppressing myocardial remodeling after MI. In addition, it is revealed through investigations that the subcutaneous injection of IL-6 antibodies after MI can notably ameliorate the left ventricular dysfunction and inhibit the attenuation of theinfarcted wall at the same time[17]. Consistent with all those research findings, it was discovered in this research that IL-6 knockout can significantly improve the physiological functions of the mice after MI. Moreover, this research and several studies mentioned above support that inhibiting IL-6 can prevent poor ventricular remodeling after MI by controlling the inflammatory responses.

Macrophages have dual effects on the wound healing process after MI, so regulating their specific effects can promote myocardial repair. The

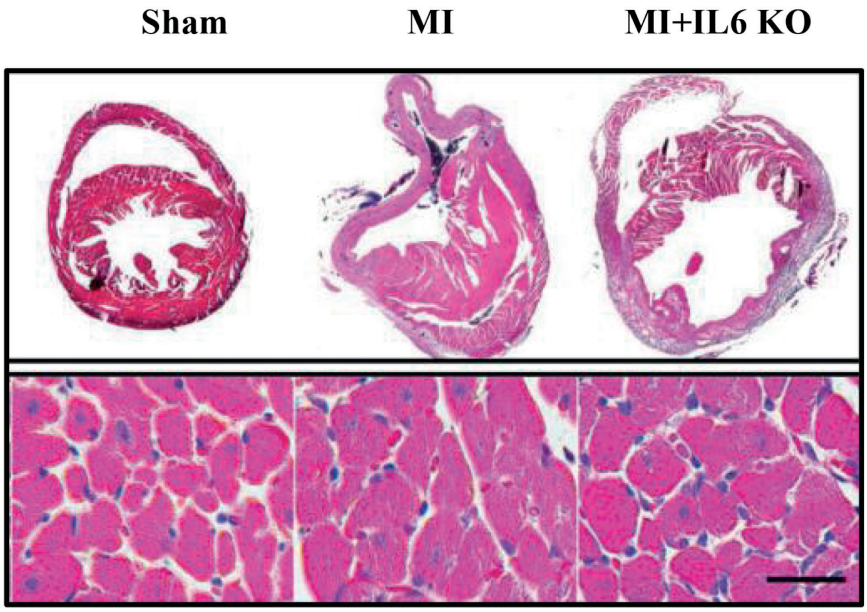

Figure 4. Pathological staining for effects of IL-6 knockout on cardiac tissues of each group of mice (magnification: 100×). Sham: Sham group, MI: MI group, MI + IL-6 KO: MI + IL-6 gene knockout group.

inflammatory responses are activated by the production of pro-inflammatory cytokines (also known as M1 cytokines) within several hours after MI[18]. Classically activated (M1) macrophage plays a dominant role in the early pro-inflammatory stage after MI, while the alternatively activated (M2) macrophage takes the dominant place in the late anti-inflammatory stage. The prognosis of MI can be improved by accelerating the transition from pro-inflammatory M1 macrophage to anti-inflammatory M2 macrophage in time[19]. IL-6 knockout is capable of inducing the polarization of myocardial M2 macrophage, thus creating an infarction environment conducive to tissue repair[20]. It was also discovered in this research that the impacts of IL-6 on myocardial remodeling after MI are not limited on the regulation of inflammations, but including those on myocardial fibrosis. The macrophages also stimulate the activation of fibroblast cells in addition to coordinating the inflammations. Studies have manifested that paracrine factors of the M1 macrophage and M2 macrophage are able to activate or repress the fibroblast cells. The M2 macrophage is particularly correlated with tissue repair and fibroblast cell activation[21]. IL-6 induces the polarization of M2

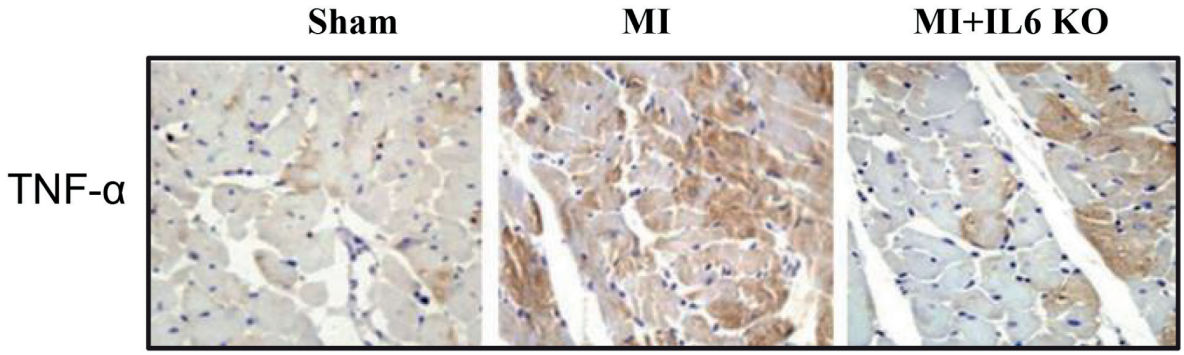

Figure 5. Effects of IL-6 knockout on TNF-α in myocardial tissues of each group of mice (magnification: 100×). Sham: Sham group, MI: MI group, MI + IL-6 KO: MI + IL-6 gene knockout group.

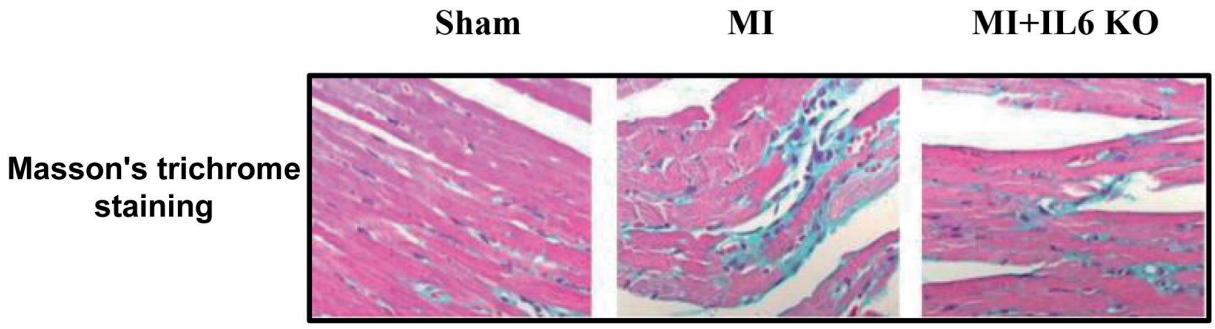

Figure 6. Effects of IL-6 knockout on myocardial fibrosis of each group of mice (magnification: 100×). Sham: Sham group, MI: MI group, MI + IL-6 KO: MI + IL-6 gene knockout group.

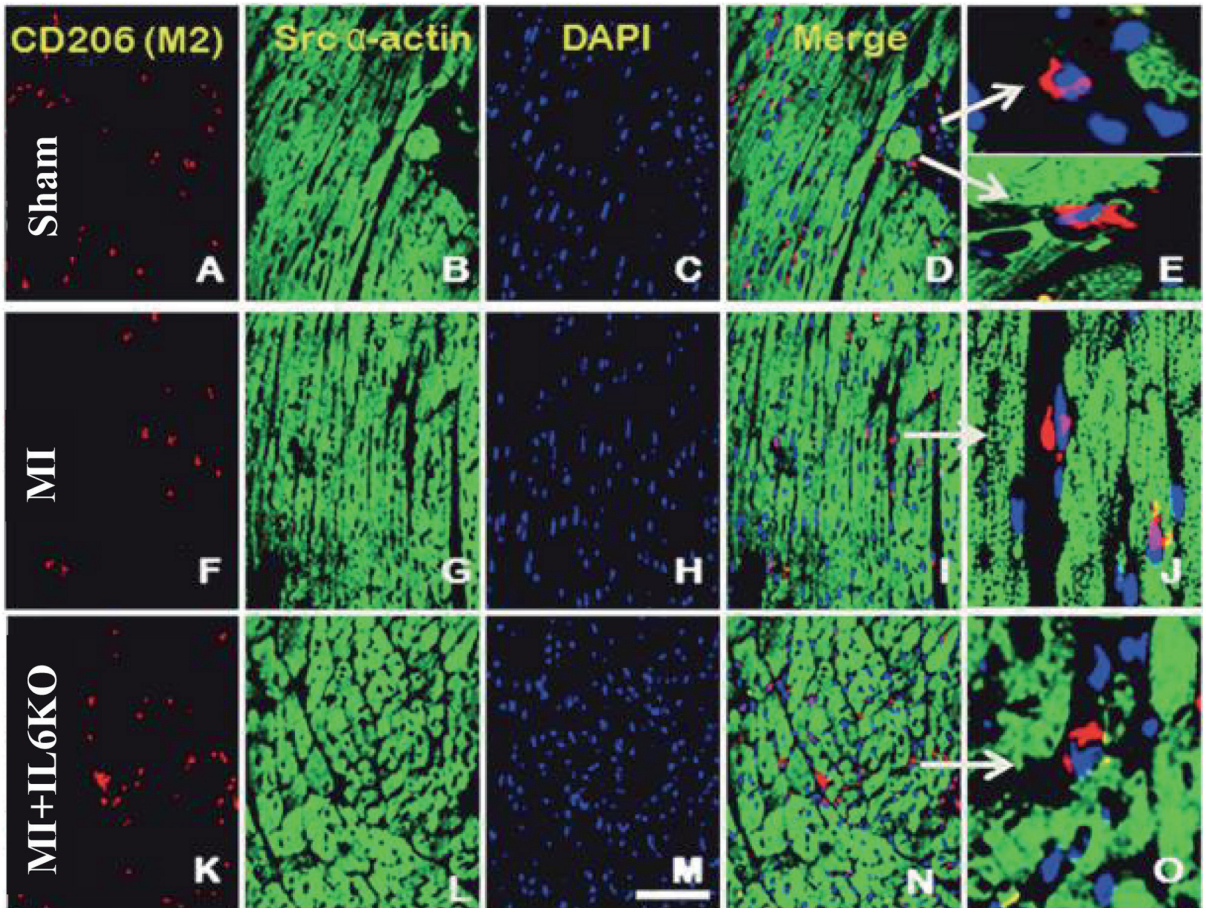

Figure 7. Effects of IL-6 knockout on infiltration of M2 macrophages in myocardial tissues of each group of mice (magnification: 100×). Sham: Sham group, MI: MI group, MI + IL-6 KO: MI + IL-6 gene knockout group.

macrophage which can activate the cardiac fibroblast cells, implying that the macrophages in infarction focus are cross-linked with the fibroblast cells. Although IL-6 does not have direct effects on the cardiac fibroblast cells, it is capable of triggering the M2 macrophage to secrete cytokines that increase the fibroblast cell activation, which plays a vital role in the cross-linking between the two types of cells. The activated fibroblast cells can produce extracellular matrix to recombine the

myocardial contractility, and extracellular matrix accumulation is a prerequisite for scar formation, which can prevent the thinning and rupture of the cardiac wall after MI. However, excessive deposition of extracellular matrix and type I-III collagen is closely associated with the declined compliance of left ventricular walls[22]. Therefore, the myocardial fibrosis after MI is a double-edged sword, and excessive or insufficient fibrosis can immediately affect the myocardial contractility, further influencing the cardiac functions and even leading to heart failure. Nevertheless, there are still some limitations in this research: 1) no cell experiments were designed to verify the results, 2) no animal models of overexpressed IL-6 was used for negative verification, and 3) the signaling pathways were not explored.

Conclusions

It is proven for the first time in this study that IL-6 knockout can improve the cardiac functions of MI mice and inhibit the cardiac remodeling, whose mechanism may be related to the regulation of IL-6 on the M2 macrophage and myocardial fibrosis.

Conflict of interest

The authors declare no conflicts of interest.

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