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VSIG4 regulates macrophages polarization and alleviates inflammation through activating PI3K/AKT and inhibiting TLR4/NF-κB pathway in myocardial ischemia-reperfusion injury rats

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ABSTRACT

Background: Myocardial infarction is the primary cause of high disability and mortality in patients with cardiovascular disease worldwide. The pathological process of myocardial ischemia/reperfusion (I/R) may trigger harmful inflammatory response and ultimately lead to serious cardiac dysfunction. The mechanism of myocardial repair post myocardial infarction has not been fully elucidated. The present study speculated that VSIG4 is related to the regulation of heart injury. *Methods:* The myocardial I/R injury model was established in Sprague-Dawley (SD) rats. Before I/R operation, the viral solution containing AAV-NC or AAV-VSIG4 was intravenously injected into rats. Cardiac function indicators, mRNA expression, the apoptosis ratio of cardiomyocytes, myocardial infarct area, phenotype polarization of macrophage, and the protein expression of apoptosis or macrophage phenotype were measured. *Results:* Myocardial I/R injury

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decreased the expression of VSIG4 and subsequently triggered myocardial apoptosis. The induction of AAV-VSIG4 produced a protective effect on general cardiac function and attenuated the I/R-induced cellular apoptosis in rats. Moreover, VSIG4 signaling might potentially modulate macrophage M1/M2-related inflammatory disorders via activation of PI3K/AKT and inhibition of TLR4/NF-κB expression. *Conclusion:* In summary, the present study provided evidence that VSIG4 had cardiac protective role in myocardial I/R injury. More importantly, enhanced VSIG4 expression inhibited M1 polarization of macrophages by blocking TLR4/NF-κB activation, subsequently suppressing cardiomyocyte apoptosis. This finding provides vital insights into the role of VSIG4 in I/R injury and may provide a new target for I/R therapy.

KEYWORDS

VSIG4, myocardial infarction, ischemia-reperfusion, PI3K/AKT, TLR4/NF-κB

INTRODUCTION

Myocardial infarction is the primary cause of high disability and mortality in patients with cardiovascular disease worldwide [1]. The mechanism of myocardial repair post myocardial infarction is exceptionally complicated. Currently, therapies for myocardial infarction can only alleviate the symptoms, but cannot solve the problem of myocardial cell apoptosis induced by myocardial infarction [2]. The pathological process of myocardial ischemia/reperfusion (I/R) may trigger a harmful inflammatory response and ultimately lead to serious cardiac dysfunction [3, 4]. After myocardial infarction, M1 (pro-inflammatory) macrophages infiltrate the damaged heart tissue, trigger the inflammatory response and release pro-inflammatory cytokines, such as IL-6, IL-1β, TNF-α and iNOS [5, 6]. Conversely, anti-inflammatory cytokines are produced by anti-inflammatory macrophages (Arg1, IL-4 and IL-10), which can support the progression of scar formation and wound healing [6]. Therefore, changing the dynamic polarization of macrophages and converting macrophages from M1 to M2 have great pathological significance for suppressing inflammatory response and repairing cardiac function.

V-set immunoglobulin-domain-containing 4 (VSIG4) is a member of the immunoglobulin superfamilies that functions as an essential membrane protein accessory to the complement receptor [7]. In addition, VSIG4 is highly expressed in macrophages and has powerful physiological functions that affect tumor regulation [8]. Previous studies have proved that overexpression of VSIG4 is associated with the malignant process of high-grade glioma, gastric cancer and lung cancer [9–11]. Moreover, VSIG4 can also modulate the phenotype of glial cells or macrophages [12]. A recent study has shown that VSIG4 inhibited the activation of glial cells by suppressing TLR4 expression and promoting the conversion of microglia to M2 in ischemic stroke mice [13]. VSIG4 modulates the activation of macrophages by activating the PI3K/AKT-STAT3 pathway and reprogramming mitochondrial pyruvate metabolism to alleviate the occurrence of hepatitis [7]. In macrophages, VSIG4 can also inhibit the expression of NLRP3 and IL-1β, which cause autoimmune encephalomyelitis and colitis [12]. In VSIG4 knockout mice, the high-fat diet-induced inflammation was apparently accelerated by activating the nuclear factor-kB (NF-kB) signaling pathway [14]. Furthermore, some studies have indicated that VSIG4 can be expressed in healthy tissues, but its expression will be significantly decreased when

the tissues are dysfunctional [8, 14]. Therefore, the present study speculated that VSIG4 is related to the regulation of heart injury. This study investigated the role of VSIG4 and its influence on PI3K/AKT or TLR4/NF-κB signaling pathways in myocardial I/R injury rats.

METHODS

Animal model

Sprague-Dawley (SD) rats (weighing 220–260 g, 10–12 weeks) were randomly separated into four groups (Sham, I/R, AAV-VSIG4 and AAV-NC groups, with 10 rats in each group) for these experiments. Three weeks before I/R injury, 4 mL viral solution containing AAV-NC or AAV-VSIG4 was intravenously injected into the rats. AAV-VSIG4 was subcloned into a pAAV-CMV bGlobin-MCSeGFP-3Flag vector, which was also used as control vector (AAV-NC). Rats were first anesthetized by intraperitoneal injection of 400 mg kg^{-1} 10% chloral hydrate. Then, ischemia was achieved in all animals by using a 7.0 prolene suture around the left anterior descending coronary artery and ligated for 30 min, after which the knot was relaxed and the heart was allowed reperfusion for 120 min. The rats in the sham group only underwent left thoracotomy. After reperfusion, the rats were sacrificed and hearts were harvested for RNA isolation and apoptosis assay. All animal experiments were approved by the guidelines of the Animal Care and Use Committee of the First Affiliated Hospital of Kangda College of Nanjing Medical University (Approval No.2020-061).

Quantitative real-time PCR (qRT-PCR)

The total RNA was isolated from heart tissues using the TRIzol method (Invitrogen, USA) according to the manufacturer's protocols. The Prime-Script RT reagent kit (TIANGEN, Beijing, China) was used to synthesize cDNA. qRT-PCR was performed on a CFX96 Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA) and the relative expression of target genes was detected by using SYBR Green PCR Master Mix (TaKaRa).

The following primers were used:
VSIG4:
forward: 5′-GGATCCCACCCCACCCTAAAAACA-3′;
reverse: 5′- CTCGAGTCAGCAGGCAGGAATAGA-3′;
iNOS:
forward: 5′- CAGCTGGGCTGTACAAACCTT -3′;
reverse: 5′- CATTGGAAGTGAAGCGTTTCG -3′;
IL-1b:
forward: 5′- CAGGCAACCACTTACCTATTTA -3′;
reverse: 5′- CCATACACACGGACAACAACTAGAT -3′;
Arg1:
forward: 5′- AACACTCCCCTGACAACCA -3′;
reverse: 5′- CATCACCTTGCCAATCCC -3′;
IL-10:
forward: 5′- CGACTGTTGCCTCTCGTACA -3′;
reverse: 5′- AGGAGGTTCACAGCCCTTTT -3′

Western blot

Heart tissue from each treatment group was extracted and homogenized. Protein samples (20 μg protein/sample) were separated by 12% SDS-PAGE and then electro-transferred onto nitrocellulose membranes. The membranes were incubated with primary antibodies against VSIG4 (1:2000, Novus Biologicals, USA), Cleaved Caspase-3 (1:3000, Abcam, Cambridge, UK), PI3K (1:5000, Abcam, Cambridge, UK), p-PI3K (1:5000, Abcam, Cambridge, UK), Bax (1:5000, Abcam, Cambridge, UK), Bcl-2 (1:3000, Invitrogen, USA), p-AKT (1:3000, Abcam, Cambridge, UK), AKT (1:3000, Abcam, Cambridge, UK), TLR4 (1:3000, Invitrogen, USA), p-NF-κB (1:3000, Abcam, Cambridge, UK), NF-κB (1:3000, Abcam, Cambridge, UK), and β-actin (1:20000, Invitrogen, USA) overnight at 4 °C, and subsequently incubated with secondary antibody for 1 h. The specific protein signals were detected by enhanced chemiluminescence reagent (Bio-Rad, USA).

TTC staining

Rats were sacrificed and heart slices were taken at an interval of 2 mm rapidly at identical basal, middle, and apical levels in each group for measurement of myocardial infarct size. The tissue slices were stained by 2% 2,3,5-triphenyltetrazolium chloride (TTC) and infarct volume was analyzed. Tissues were stained for 30 min and then fixed in 4% paraformaldehyde for 24 h. The sections were photographed and the infarct area was measured by the Image-Pro Plus 6.0 software (Media Cybernetics, Silver Springs, MD, USA). The calculated infarct area was expressed as the percentage ratio of infarct area to the total cross-sectional area of the left ventricle in each section for comparison.

Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay

The apoptosis ratio of cardiomyocytes was determined by TUNEL Assay Kits (R&D Systems) according to the manufacturer's instructions. The cardiac tissue from different treatment groups was cut into sections with a thickness of 30 μm and stained. The average number of apoptotic cells/field at 400 × magnification was counted, and the cardiomyocyte apoptosis index was calculated as follows: apoptosis index = the number of TUNEL-positive cells/the total number of cells.

Flow cytometry

The cardiac tissues post I/R injury were collected and rinsed into PBS for digestion, which was stopped by the addition of cold flow cytometry staining buffer. The mixture was filtered and centrifuged. Next, cardiomyocytes were resuspended in staining buffer. To separate macrophages from the cardiac extract, CD45+ (BioLegend), Ly6G− (Abcam), F4/80 + (BioLegend) and LysM-EGFP+ (BioLegend) were used to sort macrophages. The isolated cells were cultured with anti-CD86 (BioLegend) and anti-CD206 (BioLegend) antibodies for subdividing the macrophages into different phenotypes. Cell sorting was analyzed by flow cytometry (Beckman Coulter, Inc., CA, USA).

Statistics

All the experiments were performed in triplicates and analyzed using GraphPad Prism Software 5.0 (GraphPad Software, La Jolla, USA). All data are presented as mean ± SEM. Two-way

ANOVA was used to analyze the difference between groups. A $P < 0.05$ was considered as statistically significant difference.

RESULTS

VSIG4 was aberrantly expressed in myocardial tissue of myocardial ischemia-reperfusion injury rats

To determine the potential role of VSIG4 in I/R injury, the myocardial I/R model was established in rats. The present study compared the expression of VSIG4 in myocardial tissue of I/R injury rats and normal ones by RT–PCR and western blotting analysis. The mRNA level and protein expression level of VSIG4 were significantly decreased in the myocardial I/R injury rats compared to the normal group (Fig. 1). These results suggest that VISG4 may be used as a prognostic factor in I/R injury.

VSIG4 alleviated the ischemia-reperfusion induced myocardial injury

To further investigate the potential biological functions of VSIG4 in the heart, VSIG4 was overexpressed through an adeno-associated virus (AAV) in rats. The animals were injected with AAV-VSIG4 or AAV-NC, and the results from western blotting showed that the animals infected by AAV-VSIG4 had higher VSIG4 expression in rats post I/R injury (Fig. 2A). TTC staining showed that the infarct area was particularly larger in the I/R group than that in the sham group. Conversely, the infarct area was remarkably decreased in the IR+AAV-VSIG4 group compared to the IR+AAV-NC group (Fig. 2B). These data indicated that the enhanced expression of VSIG4 exhibited protective effect on general cardiac function in rats post I/R injury.

VSIG4 suppressed the ischemia-reperfusion induced myocardial apoptosis

To investigate whether VSIG4 was involved in the cardioprotective effect of the ischemia-reperfusion induced myocardial injury, the apoptosis ratio of myocardial tissue was measured by TUNEL staining and western blot analysis. The results showed that the myocardial apoptosis

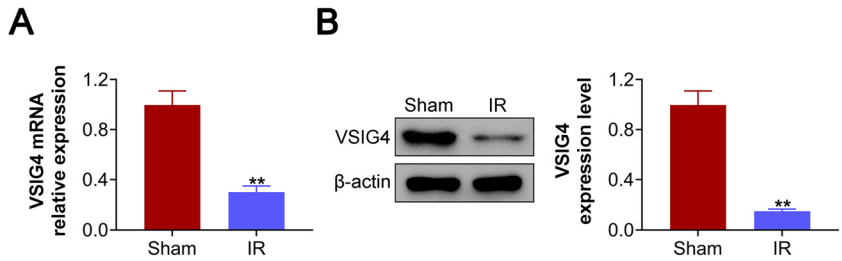

Fig. 1. **The expression of VSIG4 in myocardial ischemia-reperfusion injury rats.** (A) qRT-PCR analysis of VSIG4 mRNA expression level. (B) Western blot analysis of VSIG4 protein expression. ** $P < 0.01$ vs. Sham. Data are expressed as mean ± SEM

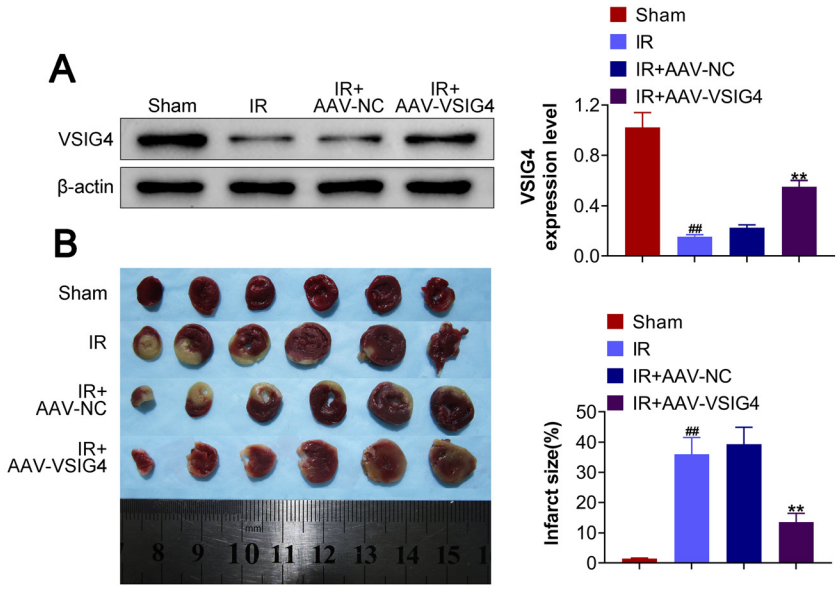

Fig. 2. **Overexpression of VSIG4 reduced myocardial infarct size in myocardial ischemia-reperfusion injury rats**. (A) qRT-PCR analysis of VSIG4 mRNA expression level. (B) Myocardial infarct size assessed by TTC, the infarction zone (white). $^{\#\#}$ $P < 0.01$ vs. Sham. ** $P < 0.01$ vs. IR+AAV-NC. Data are expressed as mean ± SEM

ratio assessed by TUNEL staining was remarkably enhanced in the I/R group, which was significantly reversed by the AAV-VSIG4 treatment (Fig. 3A). Western blot analysis showed similar results. The expression levels of Bax and Cleaved Caspase-3 were significantly increased, but the expression of Bcl-2 was down-regulated in the I/R group. Compared with the IR+AAV-NC group, the abnormal expression of apoptosis-related proteins was remarkably ameliorated in the IR+AAV-VSIG4 treatment group (Fig. 3B). These results clearly suggested that the overexpression of VSIG4 attenuated I/R-induced cellular apoptosis, which may contribute to cardioprotection.

VSIG4 modulated the phenotype polarization of M1/M2 macrophage

To explore the biological function of VSIG4 in macrophage-derived inflammation in rats post myocardial I/R injury, AAV-VSIG4 or AAV-NC was pre-injected into the rats. Flow cytometry results indicated that $CD86^+$ cells were significantly increased in rats post I/R injury. Conversely, VSIG4 overexpression decreased the number of $CD86^+$ cells (Figs. 4 and S1). Compared to the controls and the I/R injury group, the number of $CD206^+$ cells was markedly increased (Figs. 4 and S1). In parallel, the RT-qPCR result indicated that the mRNA levels of pro-inflammatory cytokines including IL-1β and iNOS were distinctly enhanced in the I/R group, and attenuated in AAV-VSIG4 rats (Fig. 4B). Moreover, the decreased expression of anti-inflammatory meditators such as IL-10 and Arg1 in I/R injury rats was obviously reversed by the overexpression of VSIG4 (Fig. 4C). Collectively, these results

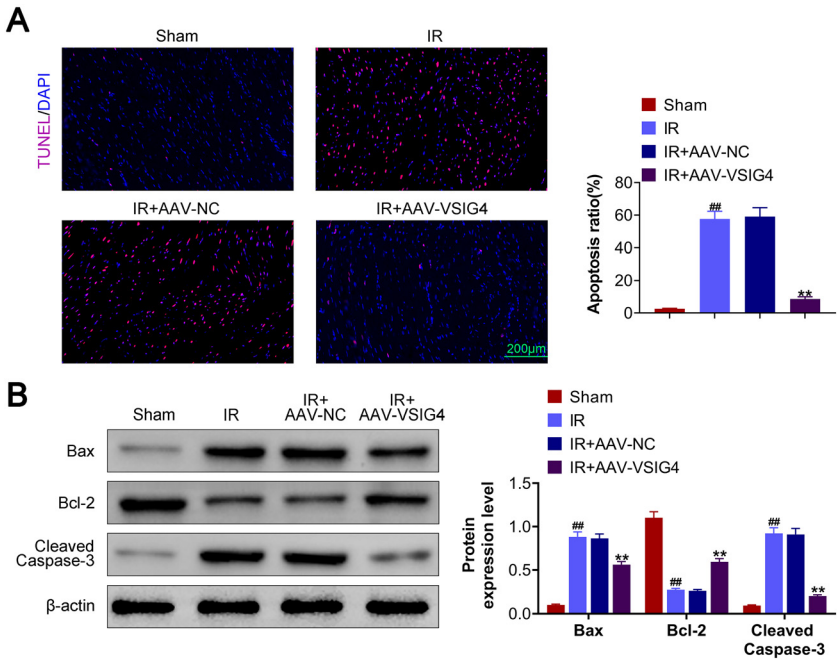

Fig. 3. **VSIG4 regulated the myocardial apoptosis ratio in ischemia-reperfusion injury rats.** (A) Analysis of apoptosis ratio in myocardial tissue by TUNEL staining. (B) Western blot analysis of Bax, Bcl-2, Cleaved Caspase-3 expression. $^{\#\#}$ $P < 0.01$ vs. Sham. ** $P < 0.01$ vs. IR+AAV-NC. Data are expressed as mean ± SEM

suggested that enhancing VSIG4 expression might have the potential for modulating macrophage M1/M2-related inflammatory disorders.

VSIG4 triggered the activation of PI3K/AKT and inhibited the TLR4/NF-κB expression in myocardial ischemia-reperfusion injury rat

To address the underlying mechanism by which VSIG4 expression was up-regulated, PI3K/AKT and TLR4/NF-κB signaling was investigated in I/R injury rats. Western blot analysis showed that the expression levels of p-PI3K and p-AKT in the I/R group were lowest among all the groups, and the AAV-VSIG4 injection obviously reversed the I/R-induced effect (Fig. 5A). Moreover, the expression levels of TLR4, NF-κB, and p-NF-κB were remarkably upregulated in the I/R group compared to other groups. After AAV-VSIG4 treatment, the proteins levels of TLR4, NF-κB, and p-NF-κB were reduced (Fig. 5B). To confirm the mechanism of VSIG4 regulating the TLR4/NF-κB pathway in myocardial ischemia-reperfusion injury rat, the effect of the NF-κB inhibitor PDTC (ammonium pyrrolidine dithiocarbamate) was investigated. The RT-qPCR result indicated that the mRNA levels of IL-1β and iNOS were decreased in both the I/R + PDTC group, and the I/R + AAV-VSIG4 + PDTC groups, but the AAV-VSIG4 plus PDTC treatment showed a more obvious influence than PDTC treatment (Fig. S2 A). Moreover, the anti-inflammatory cytokines including IL-10 and Arg1 reduced by I/R injury were increased

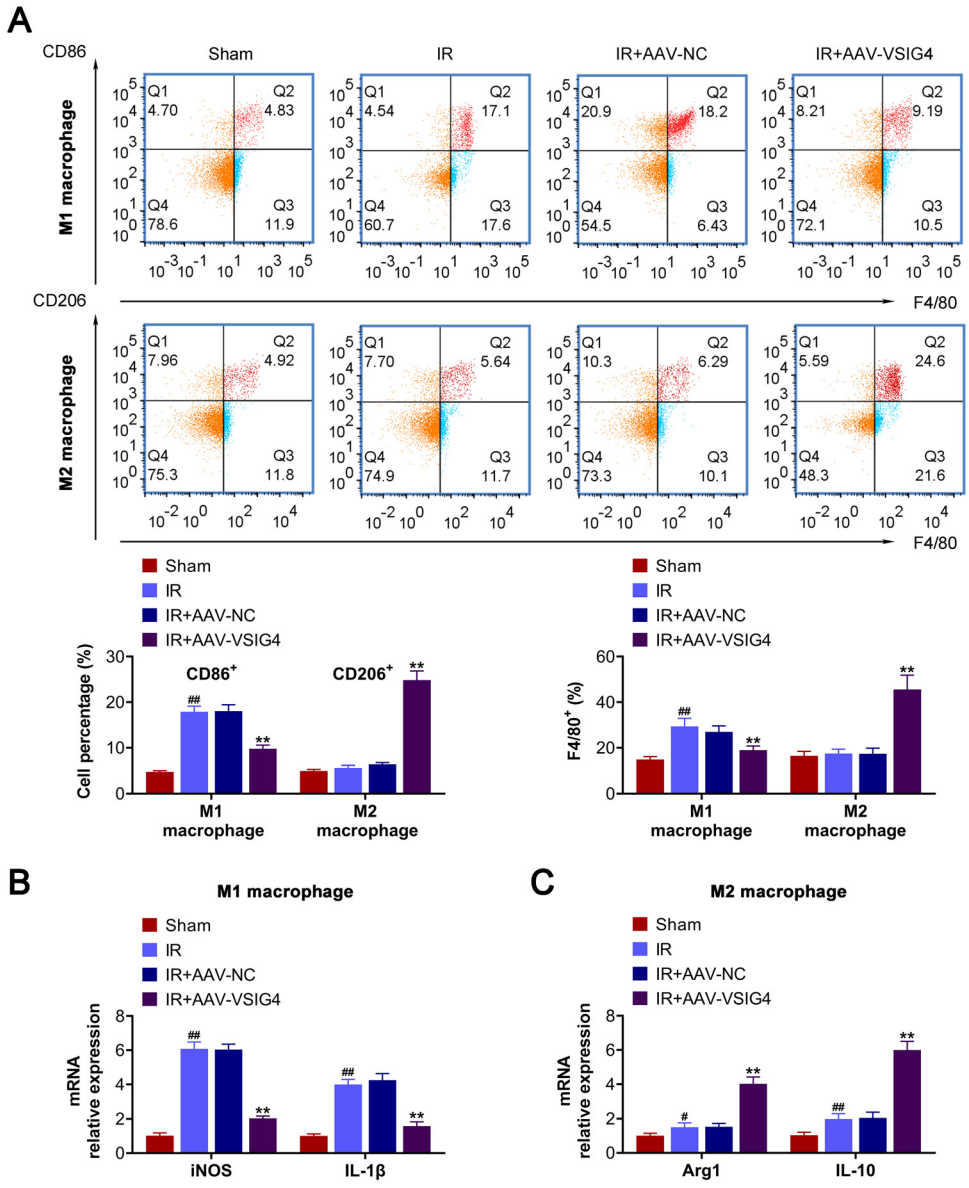

Fig. 4. **Overexpression of VSIG4 modulated the phenotype polarization of M1/M2 macrophages**. (A) Representative flow cytometry plots for CD86+ and CD206+ macrophages in myocardial tissue. (B) qRT-PCR analysis of iNOS and IL-1β mRNA expression levels. (C) qRT-PCR analysis of IL-10 and Arg1 mRNA expression levels. $^{\#}$ $P < 0.01$ vs. Sham. $^{\#\#}$ $P < 0.01$ vs. Sham. ** $P < 0.01$ vs. IR+AAV-NC. Data are expressed as mean ± SEM

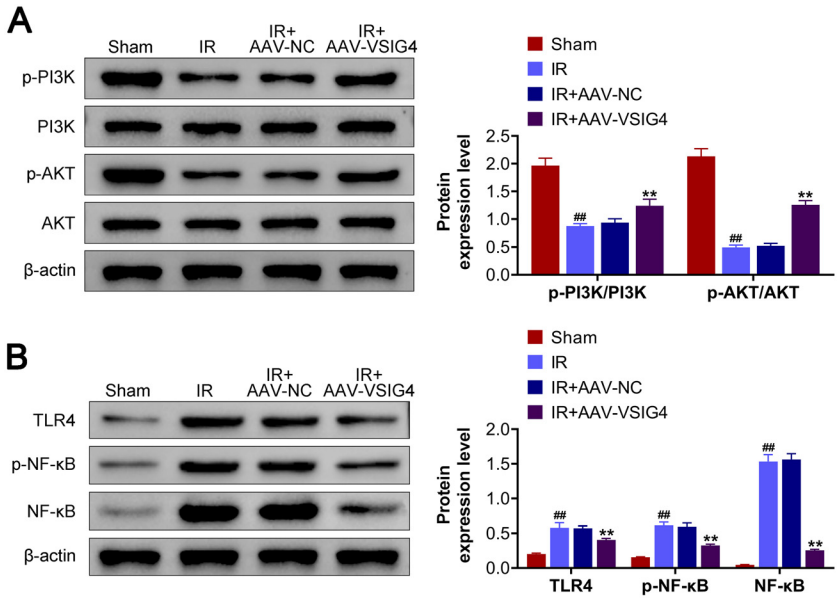

Fig. 5. **The changes of PI3K/AKT and TLR4/NF-κB activation in myocardial ischemia-reperfusion injury rats after VSIG4 transfection**. (A) Western blot analysis of p-PI3K and p-AKT expression. (B) Western blot analysis of TLR4, NF-κB, and p-NF-κB expression. $^{\#\#}$ $P < 0.01$ vs. Sham. ** $P < 0.01$ vs. IR+AAV-NC. Data are expressed as mean ± SEM

by PDTC treatment, but were more enhanced in the I/R + AAV-VSIG4 + PDTC groups (Fig. S2 B). TUNEL staining showed similar results. The I/R injury-induced myocardial apoptosis was reversed by PDTC treatment, but the AAV-VSIG4 + PDTC treatment had a larger inhibitory effect on myocardial apoptosis (Fig. S2 C). Furthermore, the above results indicated that there was an interaction between VSIG4 and PI3K/AKT or TLR4/NF-κB activation in myocardial I/R injury rats.

DISCUSSION

Myocardial I/R injury is the major pathological mechanism leading to ischemic cardiac damage [15]. Effective treatment of ischemic heart infractions is to rapidly restore blood flow reperfusion to the cardiac ischemic area. However, reperfusion may induce serious myocardial I/R injury. The therapy and precaution of ischemia-reperfusion injury are critical in treating ischemic heart disease and have become a hotspot issue. After the I/R injury, collagenous remodeling occurs, and cardiomyocyte death and endothelial leakage lead to the infiltration of immune cells to remove cellular debris [16]. Monocytes and macrophages are regarded as the key effector cells for the healing of cardiac infarction [5, 17]. Metabolic adaptation activates the macrophages, whose plasticity determines their biological functions in immunity and inflammation. Studying the mechanisms of macrophage metabolic regulation is critical for understanding the pathology

of inflammatory disorders in myocardial ischemia-reperfusion injury. VSIG4 is one of the members of the B7 family, and has recently been identified as an immunomodulatory molecule. The constitutive expression of the VSIG4 protein is generally restricted in macrophage-lineage cells [18]. VSIG4 can suppress macrophage activation by remodeling mitochondrial pyruvate oxidation [19]. In this study, a rat myocardial I/R model was established to demonstrate the protective effect of VSIG4 on myocardium reperfusion. The mRNA and protein expression levels of VSIG4 in myocardial I/R injury rats were significantly reduced compared to the control group. Furthermore, the cardioprotective effect of VSIG4 was also observed by TTC staining. Overexpression of VSIG4 can dramatically decrease the heart infarct volume in myocardial I/R injury rats. Apoptosis is a prominent pathophysiological feature that triggers heart failure. A previous study suggested that inhibition of Bax expression and overexpression of Bcl-2 can improve myocardial pro-survival progression [20, 21]. To understand whether VSIG4 is involved in I/R-induced myocardial apoptosis, the expression of apoptosis-related proteins was measured. TUNEL staining proved that VSIG4 protected the rats from I/R-induced myocardial apoptosis. Moreover, overexpression of VSIG4 reduced the cleaved-caspase-3 expression and enhanced the Bcl-2/Bax ratio in myocardial ischemia-reperfusion injury rats.

Inflammation plays a critical role in the recovery of myocardial I/R injury. Numerous reports have indicated that inordinate inflammatory responses could be found in ischemic models [22, 23]. The increase in endothelial cell permeability and abnormal chemokine release were both triggered by inflammatory mediators, which further caused the accumulation of neutrophils and macrophages. Macrophages are the critical mediators of cardiac inflammation and contribute to the initiation and recovery of inflammation [24]. Recent research reported that stem cells could modify anti-inflammatory M2 macrophages after heart damage, thereby improving cardiac rehab [25].

Moreover, macrophage phenotypes could be polarized during the process of the pro-inflammatory cytokine generation and suppress their harmful effects on cardiac tissue. This study indicated that VSIG4 expression could transform macrophages from M1 phenotype to M2 phenotype, triggering an immunomodulatory regulation after I/R injury. This present study found that the pro-inflammatory macrophages ($CD86^{+}$) were significantly suppressed and anti-inflammatory macrophages ($CD206^{+}$) were remarkably increased by AAV-VSIG4 treatment. On the other hand, the expression of M1 biomarkers (IL-1β and iNOS) was reduced, whereas the expression of M2 biomarkers (IL-10 and Arg1) was obviously enhanced by the overexpression of VSIG4. It is reported that TLR4 is partly involved in the regulation of inflammatory responses through facilitating NF-kB activation and the subsequent release of pro-inflammatory cytokines or chemokines [26, 27]. Inhibition of TLR4 has shown the protective effects on tissue homeostasis and ameliorated the pathogenesis of myocardial I/R damage [28, 29]. Interestingly, this study also observed that VSIG4 activated PI3K/AKT signaling and inhibited TLR4/NF-κB signaling in myocardial ischemia-reperfusion injury rats.

CONCLUSION

In conclusion, the present study has given evidence confirming the cardiac protective effect of VSIG4 in myocardial I/R injury. More importantly, enhanced VSIG4 expression inhibited M1 polarization of macrophages by blocking TLR4/NF-κB activation, and subsequently suppressed cardiomyocyte apoptosis. These findings provide new insights into the role of VSIG4 in the

cardioprotective mechanism after I/R injury and may provide new therapeutic target for I/R therapy.

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Competing interests: The authors state that there are no conflicts of interest to disclose.

Ethics approval: Ethical approval was obtained from the Ethics Committee of First Affiliated Hospital of Kangda College of Nanjing Medical University (Approval No.2020-061).

Contribution of authors: Yanan Wang and Jie Ding designed the study, supervised the data collection, Hejian Song analyzed the data, interpreted the data, Yanling Teng and Xiaoling Fang prepared the manuscript for publication and reviewed the draft of the manuscript. All authors have read and approved the manuscript.

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Appendix

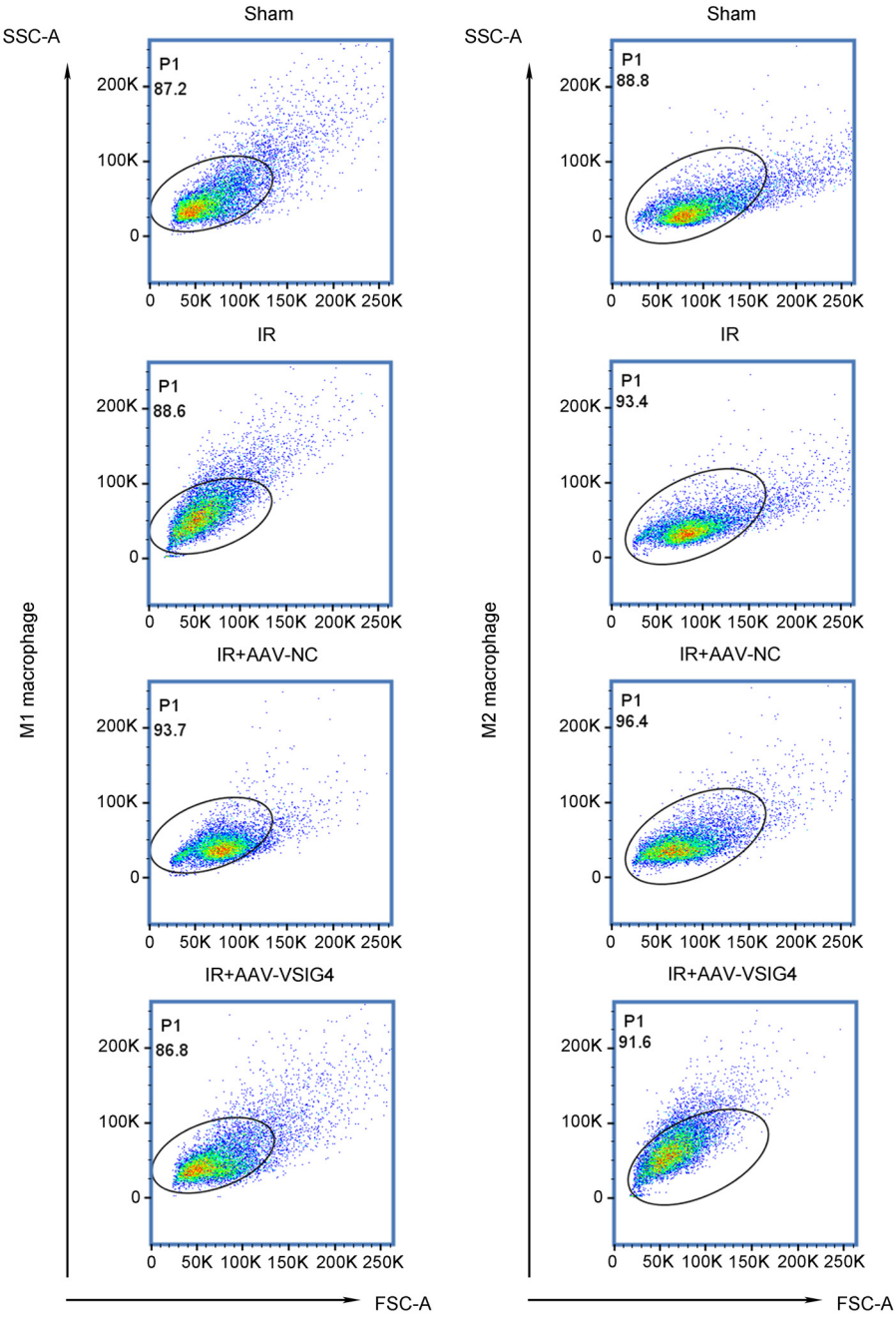

Fig. S1. The gating strategy of macrophages

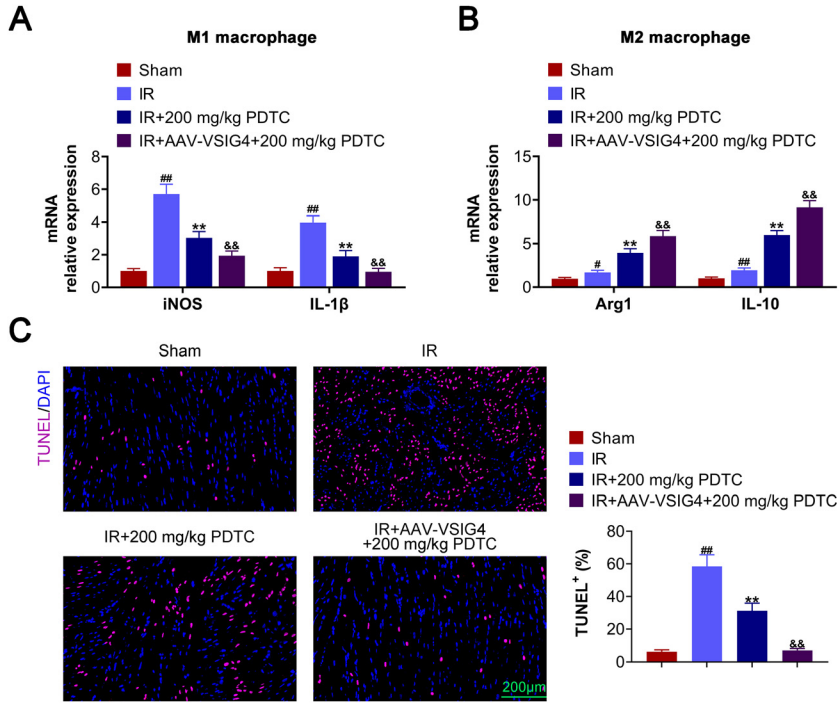

Fig. S2. **Inhibition of NF-κB modulated the myocardial apoptosis ratio and phenotype polarization of M1/M2 macrophage in ischemia-reperfusion injury rats**. (A) qRT-PCR analysis of iNOS and IL-1β mRNA expression levels. (B) qRT-PCR analysis of IL-10 and Arg1 mRNA expression levels. (C) Analysis of apoptosis ratio in myocardial tissue by TUNEL staining. $^{\#}P < 0.05$ vs. Sham. $^{\#\#}P < 0.01$ vs. Sham. $^{**}P < 0.01$ vs. IR. $^{\&\&}P < 0.01$ vs. IR+ 200 mg kg^{-1} PDTC. Data are expressed as mean ± SEM