Original Article
Resveratrol improves cardiac function by promoting M2-like polarization of macrophages in mice with myocardial infarction

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Abstract: Macrophage polarization determines the transition from the inflammation phase to the inflammation resolution phase after myocardial infarction (MI). The aim of the present study was to investigate whether resveratrol (RSV) could inhibit the inflammatory mediators associated with the regulation of macrophage phenotypes and functions in MI mice. We initially discovered that RSV significantly improved cardiac function and suppressed the expression of fibrosis markers, such as collagen-I, collagen-III, and fibronectin, and pro-inflammatory cytokines, including interleukin-1β (IL-1β), interleukin-6 (IL-6), and tumor necrosis factor alpha (TNF-α). RSV inhibited the expression of M1-like macrophage-related biomarkers (e.g., TNF-α and MCP-1) when bone marrow-derived macrophages (BMDMs) were stimulated with lipopolysaccharide (LPS) and interferon-γ (INF-γ). In contrast, it upregulated M2-like macrophage-related biomarkers (e.g., CD163 and Arg-1) when BMDMs were stimulated with interleukin-4 (IL-4) and interleukin-10 (IL-10). In addition, we found that RSV promoted M2-like macrophage polarization under anoxic conditions, which could be related to JAK2-SATA3 phosphorylation. In summary, RSV might promote anti-inflammatory M2-like polarization of macrophages after MI to improve cardiac function via the regulation of JAK2-SATA3 phosphorylation.

Keywords: Resveratrol, myocardial infarction, inflammation, macrophage polarization, JAK2-SATA3 phosphorylation

Introduction

Myocardial infarction (MI) is the primary disease amongst cardiovascular events as a leading cause of morbidity and mortality [1, 2]. The post-infarction inflammatory responses determine the path towards cardiac repair and scar formation in infarcted hearts [3]. Macrophages are not only the primary inflammatory mediators but also the essential modulators for healing cardiac wounds [4]. Macrophages exhibit two phenotypes: the classically activated M1-like and the alternatively activated M2-like [5]. During the early post-infarction stage, M1-like macrophages are the predominant pro-inflammatory immune cells infiltrating into the injured cardiac tissues, producing pro-inflammatory cytokines, including tumor necrosis fac-

tor alpha (TNF-α), interleukin-1β (IL-1β), and interleukin-6 (IL-6). With time, M1-like macrophages transform into anti-inflammatory M2-like macrophages, which express interleukin-10 (IL-10) and arginase-1 (Arg-1) and promote the healing and remodeling of cardiac tissues [6, 7]. Therefore, it is pharmaceutically beneficial to promote the phenotypic switch of macrophages from pro-inflammatory M1-like to anti-inflammatory M2-like for effective cardiac repair [8, 9].

Resveratrol (RSV), a compound extracted from plants, such as Gnetales, is a strong antioxidant and is considered a protective molecule for the cardiovascular system [10]. A previous in vitro study demonstrated that RSV was able to reduce oxidative stress in several cell types,

including endothelial cells, smooth muscle cells, cardiomyocytes, and macrophages [11]. Besides its antioxidative effects, RSV has been reported to exert cardioprotective effects against ischemia-reperfusion injury in animal models. Pretreatment of AMI rats with RSV resulted in an increase in micro-vessel density, and preservation of left ventricle (LV) function and blood flow [12, 13]. In addition, RSV has been shown to increase the protein levels of vascular endothelial growth factor (VEGF) and angiotensin II and their receptors, in order to mediate its angiogenic effect [14]. The angiogenic effect of RSV was achieved through increasing the bio-viability and production of nitrogen monoxide, which, in turn, modulates the VEGF signaling pathway [15]. Furthermore, upregulation of VEGF may directly influence the expression levels of SDF-1α and CXC chemokine receptor type 4 (CXCR-4), which are required for signal transduction in stem cell-associated chemotaxis, migration, and homing [16, 17]. However, the precise mechanisms for RSV-modulated infarct repair and restoration of cardiac function post-MI are not completely understood.

Given the critical role of macrophage polarization in MI, in the present study, we investigated the effect of RSV on the polarization and function of macrophages. We investigated how RSV regulates the expression levels of M1-like and M2-like macrophage-related biomarkers *in vitro* as well as the cardioprotective effects of RSV in a mouse MI model. We further attempted to elucidate the mechanisms underlying RSV-mediated regulation of macrophage polarization and proliferation.

Materials and methods

Chemicals and reagents

Monoclonal antibodies against JAK2, p-JAK2, STAT3, p-STAT3, STAT6, p-STAT6, and glyceraldehydes-3-phosphate dehydrogenase (GAPDH) were obtained from Cell Signaling Technology (Boston, MA, USA). Antibodies against CD206, CD11c, F4/80, and Ki67 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). FITC-labeled CD45, CD11b, F4/80, CD11c, and CD206 antibodies were purchased from BD biosciences (BD Biosciences, San Diego, USA). RSV, macrophage colony-stimulating factor (M-CSF), LPS, IFN-γ, IL-4, and IL-10 were purchased from Sigma Aldrich (St. Louis,

MO, USA), and WP1066 was purchased from MedChemExpress (New Jersey, USA). Other chemicals, unless indicated, were obtained from Sigma Aldrich (St. Louis, MO, USA).

Animals

C57BL/6J mice (aged 1-3 days for isolation of primary neonatal mouse cardiomyocytes, aged 4 weeks for isolation of BMDMs, aged 8 weeks for MI model; mice were all male except those aged 1-3 days) were purchased from the Animal Center of Nanjing Medical University. All animal experiments were performed according to the guidelines for animal care set by the Institute for Laboratory Animal Research of Nanjing Medical University. This study was approved by Animal Ethical and Welfare Committee of Nanjing Medical University (Approval No. IACUC-1812020).

Mouse model of myocardial infarction

Permanent ligation of left anterior descending coronary artery was used to achieve MI as described previously [18]. Briefly, mice were anesthetized by the intraperitoneal injection of pentobarbital sodium (50 mg/kg/body weight) prior to surgery, then placed on an aseptic plate supinely, and subsequently, intubated. Afterwards, the artificial mechanical ventilation was performed (tidal volume: 1.8 mL, inspiration and expiration ration: 2:1, respiratory rate: 130 breaths per min). After exposing the heart, an 8-0 nylon suture was passed approximately 2-3 mm below the tip of the left auricle for permanent ligation of left anterior descending coronary artery. The successful induction of MI was confirmed by observing pale color on the anterior portion of the left ventricle and examining electrocardiogram (ECG) ST-segment elevation. Then, the mice were randomly divided into the Sham group (Sham, n = 36), the MI + normal saline group (MI + NS, n = 45), and the MI + Resveratrol group (MI + RSV, n = 45). Mice in the Sham group received the same procedure except for ligating the LAD coronary artery. RSV was administered once a day at a dose of 20 mg/kg/body weight by gavage in the MI + RSV group, and equivalent volume of saline was administered in the Sham and MI + NS groups. The survival rate was measured by Kaplan-Meier survival curve analysis. All mice were sacrificed by asphyxiation at the 4th week after MI.

Echocardiography

Cardiac function was evaluated on the 3rd, 7th, and 28th days following MI using the Vevo 3100 ultrasound system (Visual Sonic, Canada) equipped with a 30-MHz transducer as previously described [18]. Left ventricular ejection fraction (LVEF) and left ventricular fractional shortening (LVFS) of each mouse were calculated.

Masson's trichrome staining

Hearts were harvested from different groups, washed in phosphate buffer saline (PBS), fixed in 4% paraformaldehyde overnight and embedded in paraffin. Then, heart tissues were cut into sequential sections of 5 μm thickness and stained with Masson's trichrome. The extent of cardiac fibrosis was calculated using Image Pro Plus software (version 6.0) and expressed as the percentage of the full ventricle area.

Immunohistochemical staining

Paraffin sections (5-μm) were dewaxed, rehydrated, and subjected to antigen retrieval in 0.01 M citrate buffer (pH 6.0) using a microwave oven. The sections were subsequently incubated in 0.3% H_2O_2 for 10 min, washed with PBS three times, and blocked with 5% goat serum in PBS-T buffer. The tissue sections were further probed with primary antibodies against F4/80, CD11c, and CD206 overnight and detected by the HRP-labeled secondary antibody. The bound antibodies were visualized by DAB (Servicebio, China) staining, and imaged on ZEISS Axiocam 503 color.

Immunofluorescence staining

The tissue sections were prepared using the same method used for immunohistochemical staining. The tissue sections were further probed with primary antibodies against F4/80 and Ki67 overnight, and subsequently detected by FITC (Servicebio, China) and CY3 (Servicebio, China), respectively. After staining the cell nuclei with DAPI, the immunofluorescent images were imaged on Pannoramic MIDI/250 (3D HISTECH, Hungary).

Primary bone marrow-derived macrophage (BMDM) culture and differentiation

BMDMs were isolated from 4 weeks old male C57BL/6J mice. Briefly, femurs of the mice

were isolated after sacrifice by cervical dislocation; and marrow was obtained by irrigation with culture medium and filtered through a 100 μm Nylon Cell Strainer (FALCON, USA). After suspension and washing, the cells were cultured in 60 mm Petri dishes in RPMI 1640 (GIBCO, USA) containing 10% FBS (GIBCO, USA), 1% penicillin/streptomycin (PS) (GIBCO, USA), and macrophage colony-stimulating factor (M-CSF, 50 ng/ml) at 37°C in a humidified 5% CO_2 atmosphere for 2 days. Cells were then maintained for 8 days with medium replacement after every 2 days. Adherent cells used for subsequent experiments were obtained on the 8th day as M0 macrophages. Then, cells were stimulated with 1 μg/mL LPS and 20 ng/mL of IFN-γ for 24 h to induce M1-like polarization, and 20 ng/mL of IL-4 and IL-10 for 24 h to induce M2-like polarization, respectively [19].

Isolation and culture of primary neonatal mouse cardiomyocytes

All neonatal (1-3 d) C57BL/6J mice were anaesthetized by inhaling isoflurane (2%), sacrificed by cervical dislocation, and then, sterilized by soaking in 75% ethanol. Following the isolation of the hearts, the apex cordis was obtained, immersed in precooled PBS, washed three times with PBS to remove residual blood, and uniformly cut into ~1 mm^3 fragments in 0.1% trypsin. Apex cordis fragments were trypsinized in 0.1% trypsin at 37°C for six times (7 min each time). After each trypsinization, the stop solution was added; and the supernatant was collected using elbow dropper. The collected supernatant was centrifuged at 2000 rpm for 5 min at 4°C, and the supernatant of the centrifuged solution was discarded. The cells were seeded in 100 mm Petri dishes after being resuspended in DMEM (GIBCO, USA) containing 10% FBS and 1% PS together with amphotericin B (0.25 μg/mL). The supernatant was moved to a new culture dish after 2 h. The adherent cells represented cardiomyocytes after 48 h-culture.

Cell culture and treatment

The cell-based experiments were divided into two parts after obtaining BMDMs and primary neonatal mouse cardiomyocytes.

The first was to study whether RSV can affect the polarization and proliferation of BMDMs (grouped into M1, M1 + RSV, M2, and M2 + RSV). RSV (final concentration: 30 μM, dis-

solved in DMSO), or equivalent volume of DMSO were used to stimulate cells for 1 h. Then, some BMDMs were stimulated with 1 µg/mL of LPS and 20 ng/mL of IFN-γ for 24 h to induce polarization of the cells into M1 phenotype. Other cells were stimulated with 20 ng/mL of IL-4 and IL-10 for 24 h to induce M2 phenotype. The cells were finally used for Edu⁺ analysis and evaluation of the mRNA levels of macrophage makers by quantitative real-time polymerase chain reaction (qRT-PCR).

Subsequently, BMDMs were co-cultured with cardiomyocytes *in vitro* to mimic the *in vivo* environment after MI, and to study whether RSV could affect the polarization tendency of BMDMs by affecting the phosphorylation of JAK2-STAT3 pathway (grouped into Control, CM, CM + RSV, and CM + RSV + WP1066). Cardiomyocytes were cultured under hypoxic condition (1% O_2, 5% CO_2, 94% N_2) for 1 h. Then the supernatant (CM) from hypoxic cardiomyocytes was extracted and co-cultured with M0 macrophages. Cells were then pre-incubated with WP1066 (an inhibitor of JAK2 and STAT3, 10 µM) or equivalent volume of DMSO 15 minutes before RSV (30 µM, 6 h) treatment. Finally, RNA and protein were isolated for qRT-PCR and western blot analysis, respectively.

Enzyme-linked immunosorbent assay (ELISA)

The levels of IL-6, TNF-α, IL-1β, and HYP in heart tissue and serum on the 3rd, 7th, 28th day post-MI were determined using ELISA method as per manufacturer's instructions (KeyGEN BioTECH, China).

Western blot analysis

Following the drug treatment, the cellular proteins were extracted and analyzed by western blot analysis as previously described [20]. Briefly, the cellular proteins were isolated, resolved by electrophoresis on 10-12% SDS-PAGE, and transferred onto a polyvinylidene fluoride (PVDF) membrane (Millipore, USA). After 2-hour incubation in Tris buffer with 1% Tween-20 (TBS-T) and 5% BSA, the membranes were incubated with specific primary antibodies overnight, and subsequently incubated with horseradish peroxidase-conjugated secondary antibodies at room temperature for 1 h. The activity of peroxidase retained on the blots was measured with enhanced chemiluminescence

(ECL) detection reagents from GE Healthcare (Uppsala, Sweden) according to the manufacturer's instructions.

Heart tissue FACS staining

Heart was obtained from each group (Sham, MI + RSV, and MI + NS), sliced into smaller pieces (~1 mm³), and digested with Neonatal Heart Dissociation Kit (Miltenyi Biotec, Germany) in C tubes (Miltenyi Biotec, Germany) using GentleMACS™ Octo Dissociator with heaters. The cells were washed through the cell strainer with 10 mL cell culture medium, supplemented with FBS, to obtain single-cell suspension. After re-suspending cell pellet in 1 mL PEB buffer, the cells were incubated for 2 min at room temperature, with the concentration adjusted to 1×10^6 cells per tube or per sample. Then, the cells were re-suspended in 200 µL staining buffer containing 1 µg/mL Fc-Block (BD Biosciences, USA) and incubated on ice or 4°C for 15 min in the dark. Then, antibodies for CD45, CD11b, F4/80, CD11c, and CD206 with Fc-Block were added in cell culture and stained for at least 30 minutes on ice in the dark. The reaction solution was centrifuged at 300 × g for 5 minutes and the supernatant was discarded. Then, the cells were re-suspended in 200 µL IC fixation buffer and incubate for 30 min at RT in the dark. After incubation, 2 mL ice cold PBS was added to each tube and the cells were resuspended gently. The tubes were centrifuged at 300 × g for 5 min and the supernatant was discarded. This step was performed twice to wash the cells. Finally, the cells were resuspended in 250 µL sterile PBS and analyzed on cytometer. M1 macrophages were identified as F4/80⁺/CD11c⁺ and M2 macrophages as F4/80⁺/CD206⁺ cells. The data was then analyzed by Kaluza.

RNA preparation and quantitative real-time polymerase chain reaction

Total RNA was extracted from left ventricular myocardial tissues or BMDMs using Trizol reagent (Invitrogen, USA). Reverse transcription was performed using 5 × qRT SuperMix (Vazyme, China) as per the manufacturer's instructions. qRT-PCR was performed using AceQ qPCR SYBR Green Master Mix (Vazyme, China) on StepOnePlus Real-Time PCR system (Applied Biosystems, USA), according to the manufacturer's protocols. Quantification of

Table 1. Primers used for quantitative real time-PCR analysis

Gene	Forward primer	Reverse primer
Arg1	CTCCGGGACCAGGTGCTAAT	ATGAGAGAGAGGGCGGGTAG
IL-10	GCTGCCTGCTCTTACTGACT	CCTGGGGCATCACTTCTACC
CD163	GAGACACACGGAGCCATCAA	CGTTAGTGACAGCAGAGGCA
IL-6	TCCTTCCTACCCCAATTTCCA	GTCTTGGTCCTTAGCCACTCC
IL-1β	TCCTTACTGCTGAGGGCAAC	GTTGAGAGGGCTAGTGAGAAGG
TNF-α	ATGGCCTCCCTCTCATCAGT	TTTGCTACGACGTGGGCTAC
MCP-1	CCACAACCACCTCAAGCACT	TAAGGCATCACAGTCCGAGTC
iNOS	GGTGAAGGGACTGAGCTGTT	CTCTCCACTGCCCCAGTTTT
IL-1β	CTCCGGGACCAGGTGCTAAT	ATGAGAGAGAGGGCGGGTAG
CD206	GTCAGAACAGACTGCGTGGA	AGGGATCGCCTGTTTTCCAG
CD163	GAGACACACGGAGCCATCAA	CGTTAGTGACAGCAGAGGCA
Arg-1	CTCCGGGACCAGGTGCTAAT	ATGAGAGAGAGGGCGGGTAG
Collagen-I	GCTCCTCTTAGGGGCCACT	ATTGGGGACCCTTAGGCCAT
Collagen-III	CTGTAACATGGAAACTGGGGAAA	CCATAGCTGAACTGAAAACCACC
Fibronectin	TGTGACAACTGCCGTAGACC	GACCAACTGTCACCATTGAGG
GAPDH	GACAGCCGCATCTTCTTGTG	AATCCGTTCACACCGACCTT

gene expression was determined by the $2^{-\Delta\Delta Ct}$ method and normalized to GAPDH as an endogenous control. The sequences of the forward and reverse primers used for RT-PCR are listed in **Table 1**. Gene expression assays were purchased from Invitrogen (Invitrogen, USA).

Edu⁺ analysis

For Edu⁺ analysis, BMDMs were seeded in 6-well plates. RSV (30 μM, DMSO was used as control) was used to treat BMDMs for 1 h before the cells were induced into M1 and M2 phenotype, respectively. Then, the Edu⁺ was analyzed according to BeyoClick™ EdU-48 protocol (Beyotime Biotechnology, China).

Statistical analysis

Data were expressed as mean ± SD from at least three experiments. Statistical significance was determined using Student's t-test between two groups and one/two-way analysis of variance (ANOVA) followed by Tukey's test among multiple groups with Graphpad Prism 7. A value of $P < 0.05$ was considered as statistically significant.

Results

RSV mitigated cardiac functional deterioration following MI in mice

First, we investigated the effects of RSV on cardiac function in mice with MI. In the MI + NS group, only 58% mice survived up to 28 days post-MI, while in the MI + RSV group, nearly 80% mice survived, suggesting the beneficial effects of RSV against MI (**Figure 1A**). Echocardiography revealed that RSV exhibited ameliorated pathological remodeling and functional deterioration after MI (**Figure 1B-D**).

Cardiac fibrosis was ameliorated by RSV following MI in mice

Masson staining revealed that myocardial fibrosis was significantly decreased in the MI + RSV group compared with the MI + NS group (**Figure 2A** and **2B**). RSV could significantly suppress hydroxyproline (HYP, a non-essential amino acid needed to manufacture collagen) levels both in serum and cardiac tissue on the 28th day following MI (**Figure 2C** and **2D**). We also examined the mRNA levels of fibronectin, collagen-I, and collagen-III in heart tissues. In the MI + NS group, the content of fibronectin on the 28th day following MI was approximately three-fold compared with that in sham-operated tissues, in which the levels of fibronectin significantly decreased after RSV treatment (**Figure 2E**). Moreover, the mRNA levels of collagen-I and collagen-III in the MI + NS group were approximately 100 and 30 folds compared with those in sham-operated tissues, respectively (**Figure 2F** and **2G**). The collagen-I and collagen-III levels of the MI + RSV group decreased significantly compared with those of the MI + NS group. These results demonstrated that treatment with RSV significantly ameliorated cardiac fibrosis following MI in mice.

RSV reduced pro-inflammatory cytokines following MI in mice

Inflammation plays an important role in post-MI ventricular remodeling. We further investigated the effect of RSV on inflammation in a mouse model of MI, using ELISA and qRT-PCR methods. After MI, the levels of IL-6, TNF-α, and IL-1β in murine hearts were significantly increased and peaked on the 7th day in each group. In con-
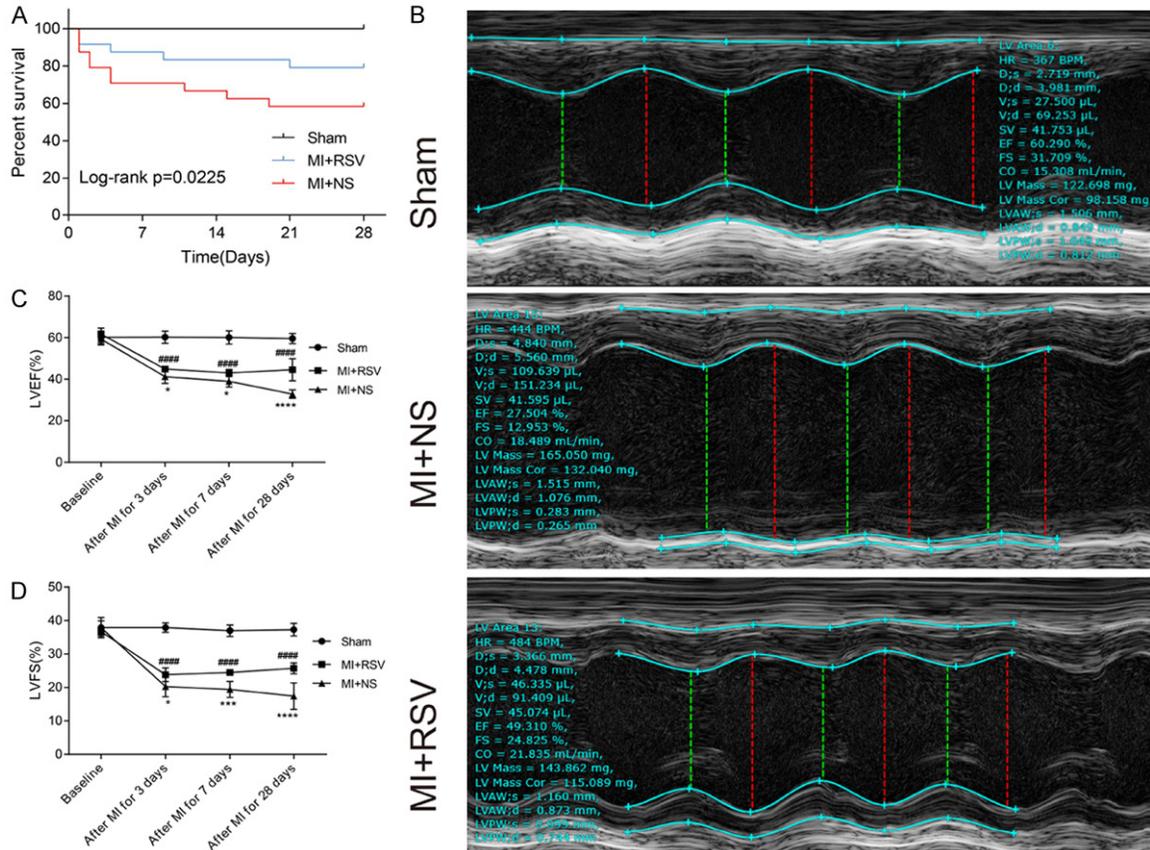
Figure 1. RSV mitigated cardiac functional deterioration following MI in mice. (A) Survival rate of mice at the 4th week following MI. Kaplan-Meier survival curve of the indicated groups (n = 24 per group) showed lower mortality in mice of the MI + RSV group compared with those of both the MI + NS and Sham groups. (B) Representative echocardiographic records obtained from mid-ventricular short-axis views of murine hearts (n = 12). Statistical analysis of the data obtained or derived from original echocardiographs, including LVEF (C) and LVFS (D). *$P < 0.05$, ***$P < 0.001$, ****$P < 0.0001$ *versus* the Sham group; ####$P < 0.0001$ *versus* the MI + NS group.

trast, RSV treatment significantly decreased the serum levels of IL-6, TNF-α, and IL-1β compared with the MI + NS group; though, they were significantly higher compared to the Sham group (**Figure 3A-C**). In addition, we examined the mRNA levels of IL-6, TNF-α, and IL-1β in cardiac tissue on the 3rd and 7th days; the results were similar to those for the serum (**Figure 3D-F**). These results demonstrated that RSV plays a cardioprotective role by suppressing inflammation following MI.

RSV promoted M2-like polarization of macrophages following MI in mice

The classical activation of M1-like macrophages contributes to the poor progression of MI, while the alternative activation of M2-like macrophages promotes the healing and remodeling of cardiac tissues. CD11c and CD206 are,

respectively, M1-like- and M2-like-macrophage surface markers in both mice and humans. We aimed to assess whether RSV could affect the polarization of macrophages. We, thus, first analyzed the effect of RSV on the polarization of macrophages *in vivo* using FACS. Hearts from different groups were harvested on the 3rd and 7th days post MI. Significant upregulation of CD206 expression was observed in cardiac tissues when mice were treated with RSV for 3 days following MI, while the levels of CD11c were significantly decreased (**Figure 4A-C**). Similar conclusions were obtained by immunohistochemical staining: the expression level of CD206 was increased by RSV treatment, while the expression levels of F4/80 and CD11c were reduced (**Figure 4D-G**). These results clearly suggested that RSV promotes macrophage M2-like polarization in MI.
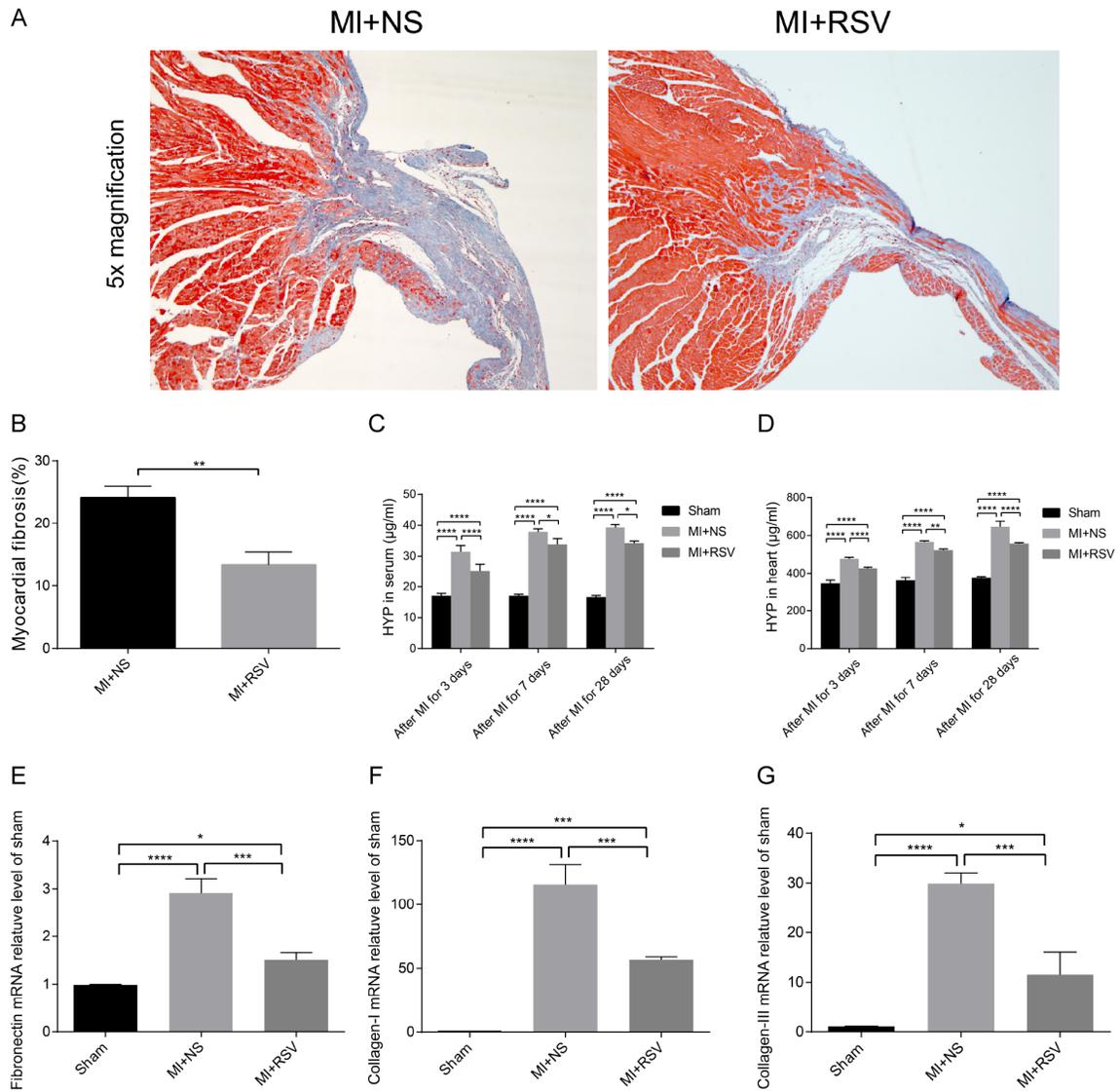
Figure 2. Cardiac fibrosis was ameliorated by RSV following MI in mice. (A) Masson staining of heart tissue following MI. (B) Quantitative analysis of myocardial fibrosis. HYP changes in serum (C) and cardiac tissue (D) following RSV or NS treatment on the 3rd, 7th, and 28th days post MI, determined using ELISA. (E-G) The mRNA levels of fibronectin, collagen I, and collagen III in heart tissue on the 28th day following MI were determined by qRT-PCR, normalized against the mRNA level of GAPDH and expressed relative to the Sham group. The results are representative of three independent experiments. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$.

RSV promoted macrophage proliferation following MI in mice

Next, we determined the expression of Ki67, a cell proliferation marker, by immunofluorescence as an additional independent measure of macrophage proliferation following MI in mice. Hearts were harvested after 3 days of RSV or NS treatment. We found that the number of F4/80-positive cells decreased in the peri-infarct region, while the number of Ki67-positive cells and the percentage of Ki67/

F4/80 double-labeled cells significantly increased (**Figure 5A** and **5B**), suggesting that RSV might promote macrophage proliferation along with M2-like polarization.

RSV promoted both M2-like macrophage polarization and proliferation in vitro

BMDMs treated with LPS and IFN-γ or IL-4 and IL-10 for 24 h showed pronounced induction of M1 or M2 phenotype, respectively. We further explored the effect of RSV on macrophage
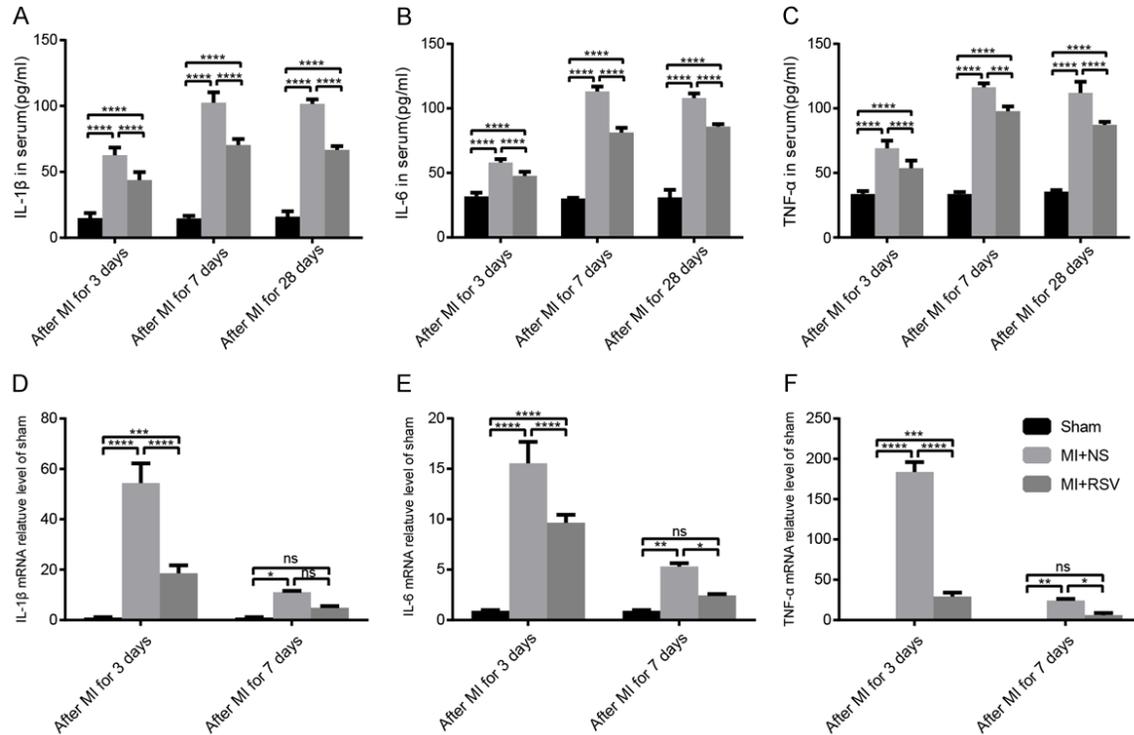
Figure 3. RSV reduced pro-inflammatory cytokines following MI in mice. A-C. Pro-inflammatory cytokines in the serum of the Sham, MI + NS, and MI + RSV groups on the 3rd, 7th, and 28th days post MI, determined using ELISA. D-F. Pro-inflammatory cytokines in heart tissues in different groups after surgery, measured by qRT-PCR. All results are representative of three independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$.

polarization *in vitro*. Following RSV treatment, the mRNA levels of M1-like macrophage related markers, including IL-1β, TNF-α, and IL-6 were significantly decreased in the M1 phenotype groups. In contrast, M2-like macrophage markers, including CD163, IL-10, and Arg-1 were increased in M2-like groups, with significant differences in IL-10 and Arg-1 expression levels (**Figure 6A** and **6B**), which suggested that RSV promoted M2 phenotype polarization. In addition, we observed the BMDM proliferation during polarization. As shown in **Figure 6C**, the Edu-positive cells were significantly increased in RSV-treated M2-like macrophages, which was opposite to the changes induced in M1-like macrophages, suggesting RSV promoted macrophage M2-like polarization along with proliferation.

RSV promoted macrophage M2-like polarization under anoxic condition via JAK2/STAT3 pathway

The JAK2/STAT3 pathway plays an important role in macrophage polarization. A previous study has shown that JAK2/STAT3 could be activated under hypoxic condition [21]. Thus,

we cultured cardiomyocytes under hypoxic condition (1% O_2, 5% CO_2, 94% N_2) for 1 h *in vitro* to mimic the *in vivo* environment after MI. Then, the supernatant from hypoxic cardiomyocytes (CM) was extracted and co-cultured with M0 macrophages, with WP1066 (an inhibitor of JAK2 and STAT3, 10 μM) or equivalent volume of DMSO 15 minutes before RSV (30 μM, 6 h) treatment. As shown in **Figure 7A** and **7B**, the phosphorylation of JAK2, STAT3, and STAT6 was significantly upregulated after RSV treatment under anoxic condition, in which the increased trends were suppressed by WP1066 treatment. Thus, we assumed that RSV might directly act on JAK2/STAT3 pathway during M2-like polarization. To confirm our hypothesis, we examined the M1- and M2-like relevant biomarkers under hypoxic condition. As shown in **Figure 7C**, compared with the control group (M0 without CM), the M1-like related biomarkers in M0 macrophages co-cultured with CM, including MCP-1, iNOS, and IL-1β, increased significantly. These trends were suppressed after RSV treatment. However, when the JAK2-STAT3 was blocked in addition to RSV treatment, the level of MCP-1 significantly increased, and the levels of iNOS and IL-1β displayed similar trends
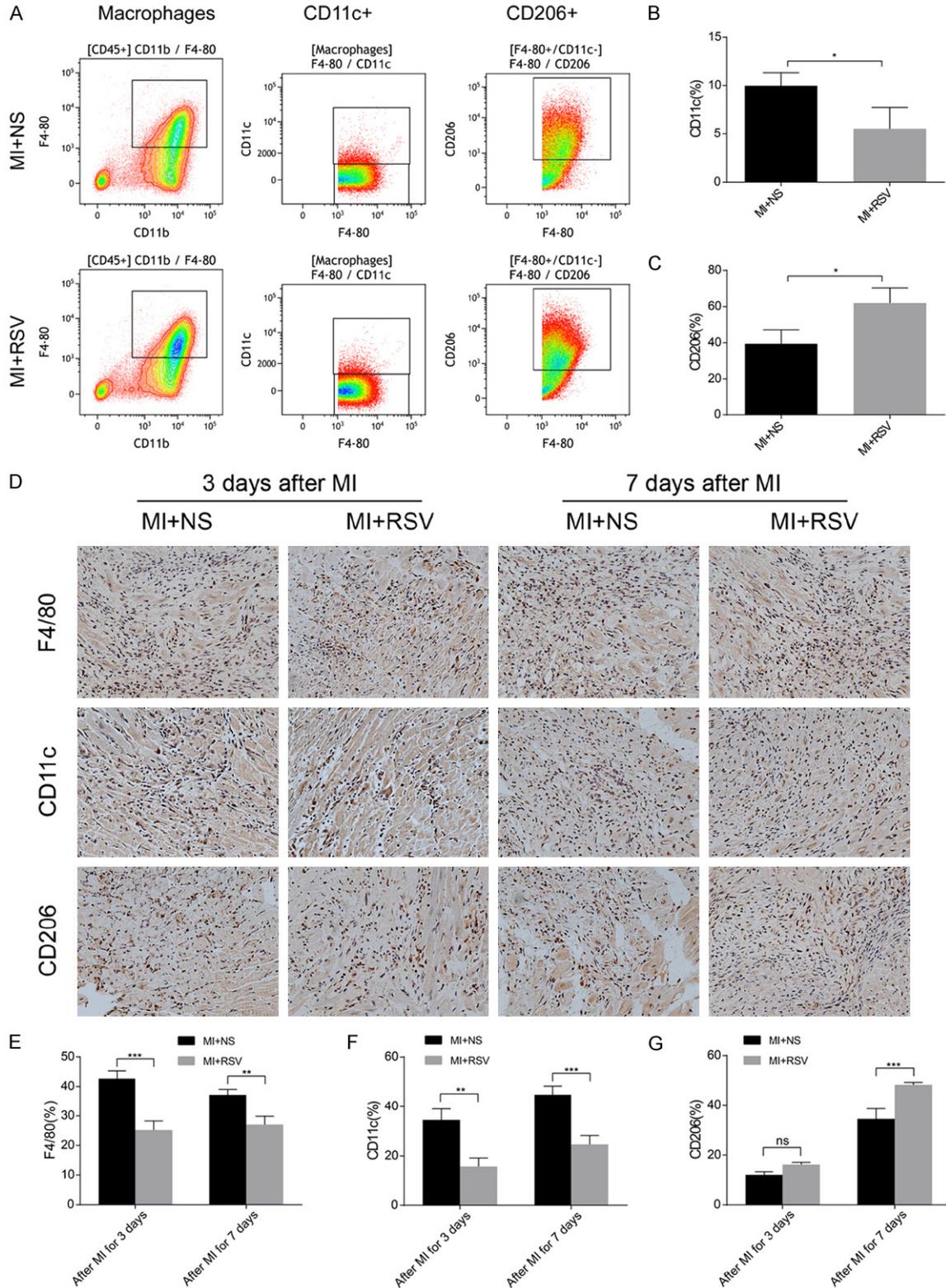
Figure 4. RSV promoted M2-like polarization of macrophages following MI in mice. A-C. Hearts were obtained from the MI + NS and MI + RSV groups on the 3rd day following MI, the subtypes of infiltrating cells in heart tissues were identified by analyzing profiles of cell surface markers by FACS. Macrophages: CD45+/CD11b+/F4-80+, M1-like: F4/80+/CD11c+, M2-like: F4/80+/CD206+, and the data was analyzed by Kaluza. D-G. The expression levels of F4/80+, CD11c+, and CD206+ cells in heart tissues from different groups was analyzed by immunohistochemical staining on the 3rd and 7th days following MI. All results are obtained from triplicate experiments. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.
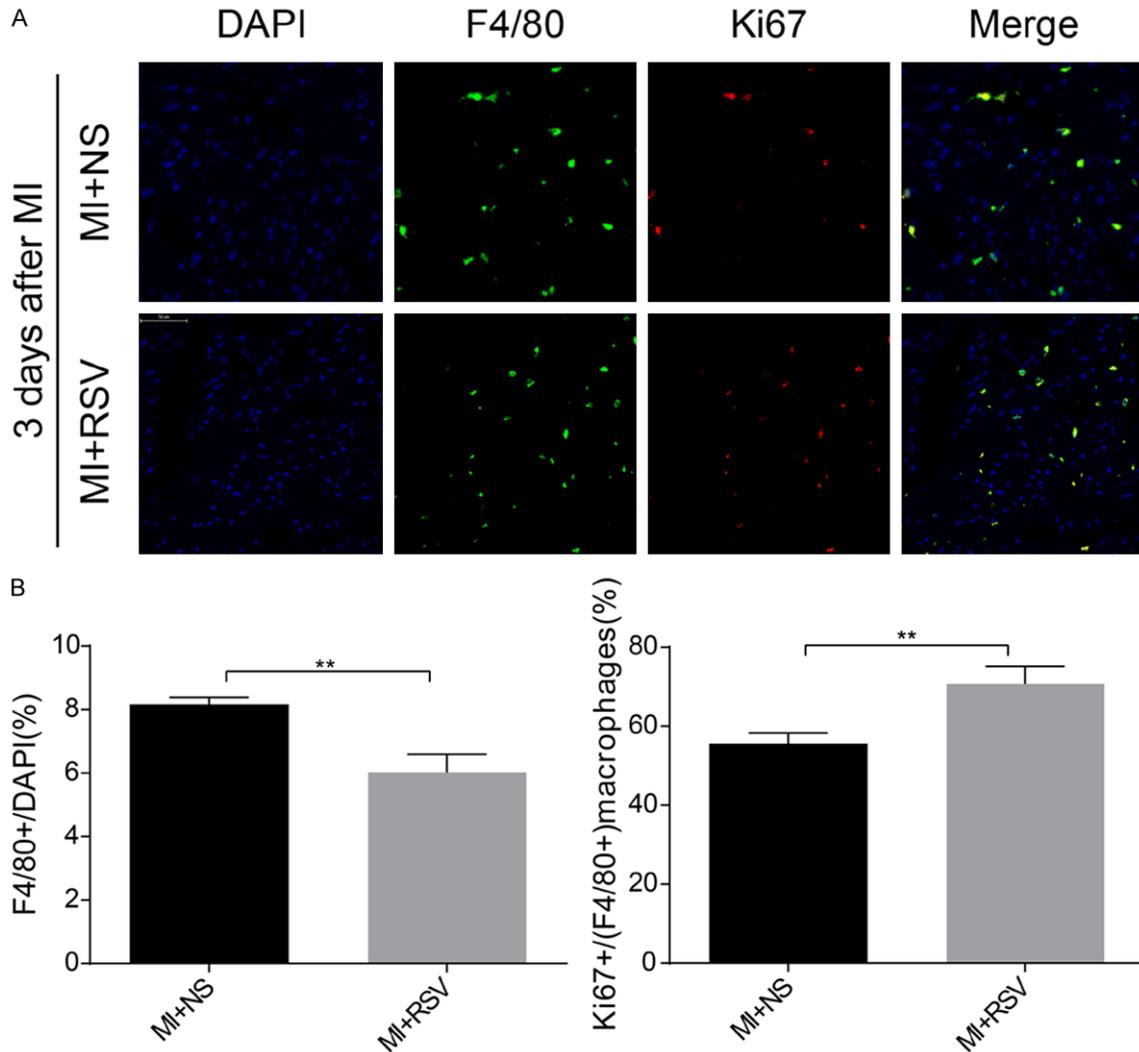
Figure 5. RSV promoted macrophage proliferation following MI in mice. A. The expression levels of F4/80 (green) and Ki67 (red) in hearts harvested after 3 days of RSV or NS treatment were confirmed by immunofluorescence staining. The nuclei position was determined by DAPI (blue). B. Quantitative analysis of Ki67 and F4/80-positive cells. **$P < 0.01$.

(without significant difference). In contrast, the M2-like relevant biomarkers, including Arg1, CD163, and CD206, were significantly increased following RSV treatment under anoxic conditions compared with the control group. These trends were also suppressed after blocking the JAK2-STAT3 pathway (**Figure 7D**). The above data suggested that RSV promoted M2-like polarization by activating the phosphorylation of the JAK2-STAT3 pathway under anoxic conditions.

Discussion

MI, one of the most lethal events globally, might result in heart failure if it does not prove fatal

immediately [9]. Currently, there are no clinically applicable medications for myocardial ischemic injury. In the cardiovascular system, RSV has been reported to play a cardioprotectant role in MI [22, 23]. In this study, we found that RSV treatment significantly improved cardiac function in MI mice while cardiac dysfunction and mortality post-MI were apparent in the Sham group. In addition, RSV ameliorated myocardial injury by promoting the transition of macrophages toward the M2 phenotype, which was involved in the amelioration of inflammation.

Inflammatory reactions triggered by myocardial necrosis post-MI clear the wound of dead cells
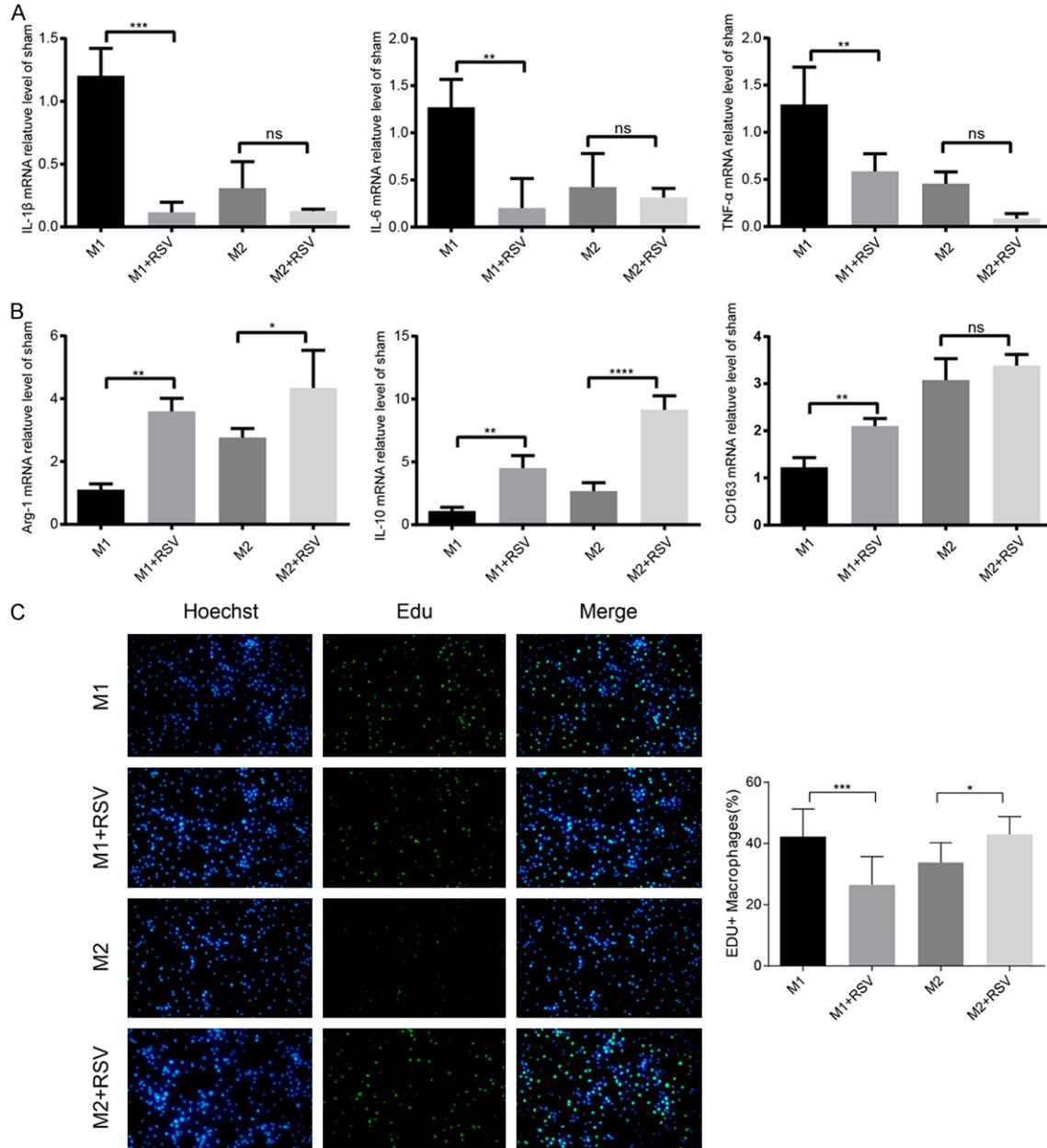
Figure 6. RSV promoted both M2-like macrophage polarization and proliferation *in vitro*. BMDMs were treated with RSV (30 μM) or equivalent volume of DMSO for 1 h before being induced to M1 and M2 phenotype, respectively. A. The relative mRNA expression of M1-like macrophage markers, including IL-1β, IL-6, and TNF-α, were measured using qRT-PCR. B. The relative mRNA expression of M2-like macrophage markers, including Arg-1, IL-10, and CD163, were measured using qRT-PCR. C. Edu[+] expression in BMDMs. Data are obtained from three separate experiments. [*]P < 0.05, [**]P < 0.01, [***]P < 0.001, [****]P < 0.0001.

and matrix debris and are believed to be critical for infarct healing, vascularization, and restoring cardiac function [9, 24]. Macrophages are the principle inflammatory cell types involved in the regulation of the healing process and modulate the inflammatory response to MI at multiple levels [24, 25]. Early during MI, Th1 cyto-

kines and C-C chemokines promote classical activation of macrophages, exacerbating the inflammatory response and cardiac dysfunction [26]. Subsequently, alternatively activated macrophages accumulate in the damaged tissue and initiate the repair process since M2-like macrophages antagonize pro-inflammatory
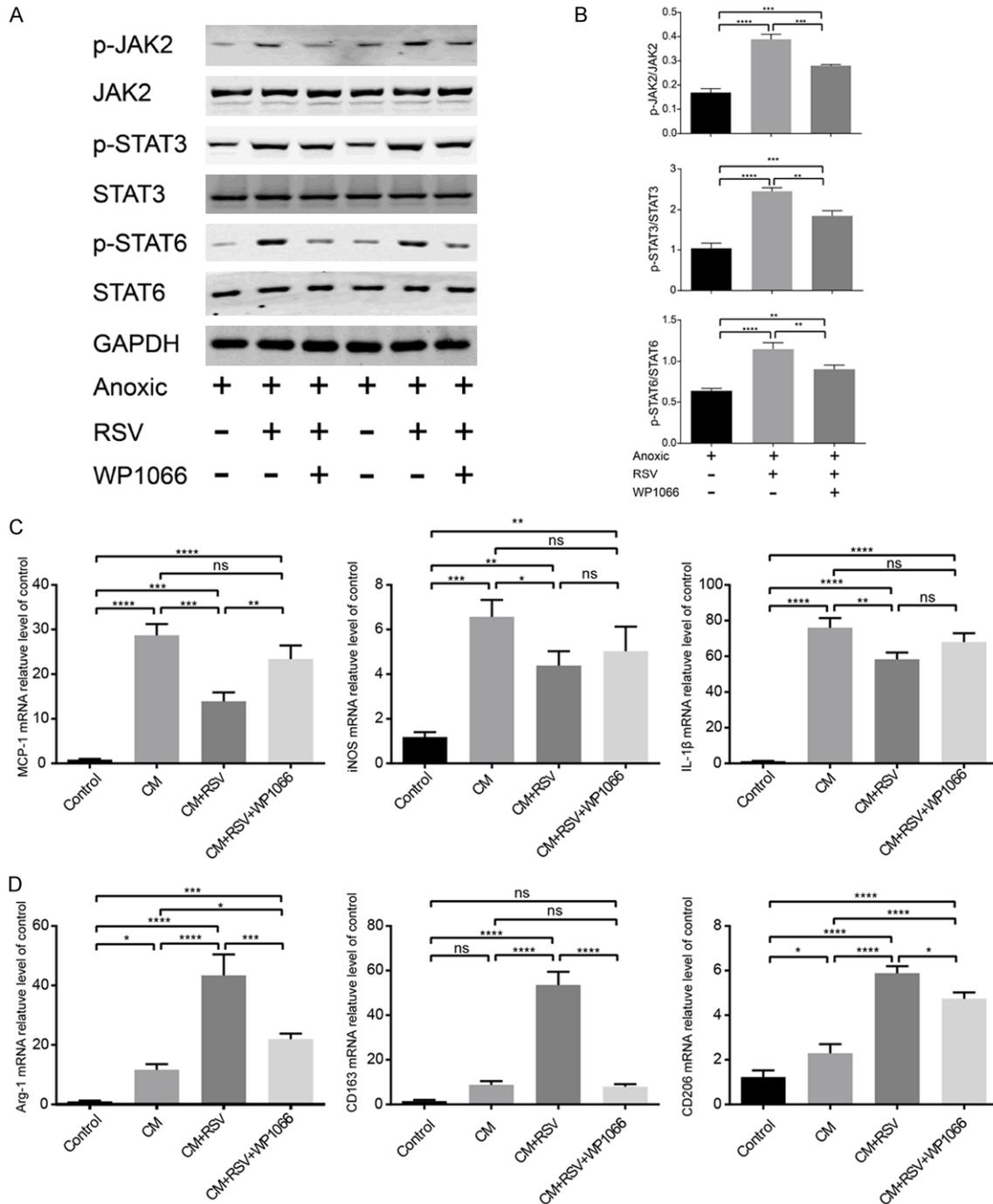
Figure 7. RSV promoted macrophage M2-like polarization under anoxic condition via JAK2/STAT3 pathway. After M0 macrophages were co-cultured with the supernatant (CM) from hypoxic cardiomyocytes with WP1066 or equivalent volume of DMSO 15 minutes before RSV (30 μM, 6 h) treatment, (A) the protein levels of JAK2, SATA3, STAT6, p-JAK2, p-SATA3, and p-STAT6 were measured by western blot, GAPDH was used as a loading control. (B) Quantification of western blotting results. (C) mRNA expression of M1-like macrophage-related MCP-1, iNOS, and IL-1β and (D) M2-like macrophage-related Arg-1, CD163, and CD206 were measured using qRT-PCR. All results are representative of three independent experiments. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$.

responses and help to engulf cellular debris [27-29]. In addition, considerable data has demonstrated that switching between subsets of macrophages is important for cardiac healing following MI [30, 31]. In this study, we showed that macrophages, when recruited to

the infarcted myocardium, and then, shifted toward the M2 phenotype driven by RSV, caused an increase in the synthesis of anti-inflammatory cytokines and a decrease in generation of proinflammatory cytokines. We also found that RSV promoted the proliferation of M2-like macrophages and this might be intrinsically related to M2-like polarization. These findings suggested a previously undiscovered mechanism underlying RSV-mediated cardioprotection.

It has been previously reported that macrophage accumulation in the infarcted myocardium is involved in cardiac rupture after MI [32, 33], playing a pivotal role in cardiac remodeling following MI. The main functions of M1-like macrophages include phagocytosis of cellular debris at sites of myocardial damage, secretion of inflammatory cytokines, and reorganization of tissue matrices by the production of metalloproteinases (MMPs) in the acute phase after MI [34]. In contrast, M2-like macrophages facilitate amelioration of inflammation and regeneration. In our study, MI resulted in cardiac fibrosis, which is similar to the results of a previous study [35]. We also found that, after RSV treatment, myocardial fibrosis indicators, including collagen I, collagen III, and fibronectin significantly decreased, suggesting that RSV could ameliorate cardiac fibrosis in mice.

Potential post-MI treatment strategies should consider macrophage recruitment, seeing that macrophages are involved in numerous pathways of tumorigenesis [36]. Previous studies have indicated that down-regulation of the prostaglandin STAT3 activation loop is essential for suppressing macrophage functions [37]. RSV has been observed to significantly improve STAT3 phosphorylation and iNOS and Arg-1 levels in BMDMs under anoxic condition. These changes were less pronounced after the blocking of the JAK2-STAT3 pathway. This finding suggested that RSV might promote M2-like polarization via JAK2-SATA3 phosphorylation. Further research on this mechanism is required for a conclusive correlation to be established. In conclusion, RSV could be a promising and effective drug for post-MI treatment.

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Disclosure of conflict of interest

None.

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References

[1] Cao Y, Lu L, Liu M, Li XC, Sun RR, Zheng Y and Zhang PY. Impact of epigenetics in the management of cardiovascular disease: a review. Eur Rev Med Pharmacol Sci 2014; 18: 3097-3104.

[2] Downey JM and Cohen MV. Why do we still not have cardioprotective drugs? Circ J 2009; 73: 1171-1177.

[3] Frangogiannis NG. The inflammatory response in myocardial injury, repair, and remodelling. Nat Rev Cardiol 2014; 11: 255-265.

[4] Frantz S, Hofmann U, Fraccarollo D, Schafer A, Kranepuhl S, Hagedorn I, Nieswandt B, Nahrendorf M, Wagner H, Bayer B, Pachel C, Schon MP, Kneitz S, Bobinger T, Weidemann F, Ertl G and Bauersachs J. Monocytes/macrophages prevent healing defects and left ventricular thrombus formation after myocardial infarction. FASEB J 2013; 27: 871-881.

[5] Gordon S and Taylor PR. Monocyte and macrophage heterogeneity. Nat Rev Immunol 2005; 5: 953-964.

[6] Nahrendorf M, Pittet MJ and Swirski FK. Monocytes: protagonists of infarct inflammation and repair after myocardial infarction. Circulation 2010; 121: 2437-2445.

[7] Schmidt T and Carmeliet P. Blood-vessel formation: bridges that guide and unite. Nature 2010; 465: 697-699.

[8] Mantovani A, Biswas SK, Galdiero MR, Sica A and Locati M. Macrophage plasticity and polarization in tissue repair and remodelling. J Pathol 2013; 229: 176-185.

[9] Nahrendorf M and Swirski FK. Monocyte and macrophage heterogeneity in the heart. Circ Res 2013; 112: 1624-1633.

[10] Escote X, Miranda M, Menoyo S, Rodriguez-Porrata B, Carmona-Gutierrez D, Jungwirth H, Madeo F, Cordero RR, Mas A, Tinahones F, Clotet J and Vendrell J. Resveratrol induces antioxidant defence via transcription factor Yap1p. Yeast 2012; 29: 251-263.

[11] Wang Z, Chen Y, Labinskyy N, Hsieh TC, Ungvari Z and Wu JM. Regulation of proliferation and gene expression in cultured human aortic smooth muscle cells by resveratrol and standardized grape extracts. Biochem Biophys Res Commun 2006; 346: 367-376.

[12] Bradamante S, Barenghi L and Villa A. Cardiovascular protective effects of resveratrol. Cardiovasc Drug Rev 2004; 22: 169-188.

[13] Das S and Das DK. Resveratrol: a therapeutic promise for cardiovascular diseases. Recent Pat Cardiovasc Drug Discov 2007; 2: 133-138.

[14] Fukuda S, Kaga S, Zhan L, Bagchi D, Das DK, Bertelli A and Maulik N. Resveratrol ameliorates myocardial damage by inducing vascular endothelial growth factor-angiogenesis and tyrosine kinase receptor Flk-1. Cell Biochem Biophys 2006; 44: 43-49.

[15] Das S, Alagappan VK, Bagchi D, Sharma HS, Maulik N and Das DK. Coordinated induction of iNOS-VEGF-KDR-eNOS after resveratrol consumption: a potential mechanism for resveratrol preconditioning of the heart. Vascul Pharmacol 2005; 42: 281-289.

[16] Brunner S, Winogradow J, Huber BC, Zaruba MM, Fischer R, David R, Assmann G, Herbach N, Wanke R, Mueller-Hoecker J and Franz WM. Erythropoietin administration after myocardial infarction in mice attenuates ischemic cardiomyopathy associated with enhanced homing of bone marrow-derived progenitor cells via the CXCR-4/SDF-1 axis. FASEB J 2009; 23: 351-361.

[17] Shen L, Gao Y, Qian J, Sun A and Ge J. A novel mechanism for endothelial progenitor cells homing: the SDF-1/CXCR4-Rac pathway may regulate endothelial progenitor cells homing through cellular polarization. Med Hypotheses 2011; 76: 256-258.

[18] Cheng W, Wu P, Du Y, Wang Y, Zhou N, Ge Y and Yang Z. Puerarin improves cardiac function through regulation of energy metabolism in streptozotocin-nicotinamide induced diabetic mice after myocardial infarction. Biochem Biophys Res Commun 2015; 463: 1108-1114.

[19] Gao S, Li C, Zhu Y, Wang Y, Sui A, Zhong Y, Xie B and Shen X. PEDF mediates pathological neovascularization by regulating macrophage recruitment and polarization in the mouse model of oxygen-induced retinopathy. Sci Rep 2017; 7: 42846.

[20] Cheng Y, Yang C, Zhao J, Tse HF and Rong J. Proteomic identification of calcium-binding chaperone calreticulin as a potential mediator for the neuroprotective and neuritogenic activities of fruit-derived glycoside amygdalin. J Nutr Biochem 2015; 26: 146-154.

[21] Bansal S, Biggar KK, Krivoruchko A and Storey KB. Response of the JAK-STAT signaling pathway to oxygen deprivation in the red eared slider turtle, trachemys scripta elegans. Gene 2016; 593: 34-40.

[22] Chuah SC, Moore PK and Zhu YZ. S-allylcysteine mediates cardioprotection in an acute myocardial infarction rat model via a hydrogen sulfide-mediated pathway. Am J Physiol Heart Circ Physiol 2007; 293: H2693-2701.

[23] Renga B. Hydrogen sulfide generation in mammals: the molecular biology of cystathionine-beta- synthase (CBS) and cystathionine-gamma-lyase (CSE). Inflamm Allergy Drug Targets 2011; 10: 85-91.

[24] Lambert JM, Lopez EF and Lindsey ML. Macrophage roles following myocardial infarction. Int J Cardiol 2008; 130: 147-158.

[25] Kakio T, Matsumori A, Ono K, Ito H, Matsushima K and Sasayama S. Roles and relationship of macrophages and monocyte chemotactic and activating factor/monocyte chemoattractant protein-1 in the ischemic and reperfused rat heart. Lab Invest 2000; 80: 1127-1136.

[26] Zamilpa R, Kanakia R, Cigarroa JT, Dai Q, Escobar GP, Martinez H, Jimenez F, Ahuja SS and Lindsey ML. CC chemokine receptor 5 deletion impairs macrophage activation and induces adverse remodeling following myocardial infarction. Am J Physiol Heart Circ Physiol 2011; 300: H1418-1426.

[27] Martinez FO, Helming L and Gordon S. Alternative activation of macrophages: an immunologic functional perspective. Annu Rev Immunol 2009; 27: 451-483.

[28] Nahrendorf M, Swirski FK, Aikawa E, Stangenberg L, Wurdinger T, Figueiredo JL, Libby P, Weissleder R and Pittet MJ. The healing myocardium sequentially mobilizes two monocyte subsets with divergent and complementary functions. J Exp Med 2007; 204: 3037-3047.

[29] Tabas I. Macrophage death and defective inflammation resolution in atherosclerosis. Nat Rev Immunol 2010; 10: 36-46.

[30] Cho DI, Kim MR, Jeong HY, Jeong HC, Jeong MH, Yoon SH, Kim YS and Ahn Y. Mesenchymal stem cells reciprocally regulate the M1/M2 balance in mouse bone marrow-derived macrophages. Exp Mol Med 2014; 46: e70.

[31] Zhou LS, Zhao GL, Liu Q, Jiang SC, Wang Y and Zhang DM. Silencing collapsin response mediator protein-2 reprograms macrophage phenotype and improves infarct healing in experi-

mental myocardial infarction model. J Inflamm (Lond) 2015; 12: 11.

[32] van den Borne SW, Cleutjens JP, Hanemaaijer R, Creemers EE, Smits JF, Daemen MJ and Blankesteijn WM. Increased matrix metalloproteinase-8 and -9 activity in patients with infarct rupture after myocardial infarction. Cardiovasc Pathol 2009; 18: 37-43.

[33] Nagai T, Honda S, Sugano Y, Matsuyama TA, Ohta-Ogo K, Asaumi Y, Ikeda Y, Kusano K, Ishihara M, Yasuda S, Ogawa H, Ishibashi-Ueda H and Anzai T. Decreased myocardial dendritic cells is associated with impaired reparative fibrosis and development of cardiac rupture after myocardial infarction in humans. J Am Heart Assoc 2014; 3: e000839.

[34] ter Horst EN, Hakimzadeh N, van der Laan AM, Krijnen PA, Niessen HW and Piek JJ. Modulators of macrophage polarization influence healing of the infarcted myocardium. Int J Mol Sci 2015; 16: 29583-29591.

[35] Ishikawa S, Noma T, Fu HY, Matsuzaki T, Ishizawa M, Ishikawa K, Murakami K, Nishimoto N, Nishiyama A and Minamino T. Apoptosis inhibitor of macrophage depletion decreased M1 macrophage accumulation and the incidence of cardiac rupture after myocardial infarction in mice. PLoS One 2017; 12: e0187894.

[36] Coussens LM, Zitvogel L and Palucka AK. Neutralizing tumor-promoting chronic inflammation: a magic bullet? Science 2013; 339: 286-291.

[37] Hagemann T, Lawrence T, McNeish I, Charles KA, Kulbe H, Thompson RG, Robinson SC and Balkwill FR. "Re-educating" tumor-associated macrophages by targeting NF-kappaB. J Exp Med 2008; 205: 1261-1268.