HIMF deletion ameliorates acute myocardial ischemic injury by promoting macrophage transformation to reparative subtype

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Abstract
Appropriately manipulating macrophage M1/M2 phenotypic transition is a promising therapeutic strategy for tissue repair after myocardial infarction (MI). Here we showed that gene ablation of hypoxia-induced mitogenic factor (HIMF) in mice (Himf−/− and HIMFflx/flx;Lyz2-Cre) attenuated M1 macrophage-dominated inflammatory response and promoted M2 macrophage accumulation in infarcted hearts. This in turn reduced myocardial infarct size and improved cardiac function after MI. Correspondingly, expression of HIMF in macrophages induced expression of pro-inflammatory cytokines; the culturing medium of HIMF-overexpressing macrophages impaired the cardiac fibroblast viability and function. Furthermore, macrophage HIMF was found to up-regulate C/EBP-homologous protein (CHOP) expression, which exaggerated the release of pro-inflammatory cytokines via activating signal transducer of activator of transcription 1 (STAT1) and 3 (STAT3) signaling. Together these data suggested that HIMF promotes M1-type and prohibits M2-type macrophage polarization by activating the CHOP–STAT1/STAT3 signaling pathway to negatively regulate myocardial repair. HIMF might thus constitute a novel target to treat MI.

Keywords Myocardial infarction · Macrophage polarization · Hypoxia-induced mitogenic factor · Tissue repair · Cardiac fibroblast · C/EBP-homologous protein

Introduction
Inflammation and cytokine elaboration promotes the clearance of damaged tissue and have active roles in left ventricular (LV) remodeling after myocardial infarction (MI) [31]. However, when the inflammatory response is overheated, it exaggerates infarct expansion and prohibits wound repair, leading to adverse LV remodeling and long-term heart failure [6, 21, 41]. Continuous efforts are being made to modulate MI-induced inflammation [6, 26]. Macrophages are the predominant immune cell type to infiltrate the infarcted myocardium. They orchestrate the inflammatory response by polarizing into distinct pro-inflammatory (Ly6Chigh, M1-like) or pro-healing (Ly6Clow, M2-like) subpopulations [22, 23]. Initial ischemic injury triggers macrophage infiltration and M1 polarization in the first 3 days post MI [23]. These cells display phagocytic and pro-inflammatory properties, highly express pro-inflammatory genes [e.g. interleukin-1β, inducible nitric oxide synthase (NOS2)], meanwhile release cytotoxic molecules [e.g. nitric oxide, reactive oxygen species], to clear damaged myocardial cells and set the stage for wound healing [40]. Besides, exosomes derived from M1 inflammatory macrophage were found to suppress angiogenesis and exacerbate cardiac dysfunction post MI [19]. Around 4–7 days post MI, M2 macrophages increase in number and become the predominant cell type. M2 macrophages...
express anti-inflammatory cytokines, such as IL-10 that suppresses pro-inflammatory cytokine synthesis. They also express reparative cytokines and enzymes, such as arginase 1 (Arg1) and transforming growth factor (TGF) β to promote collagen and extracellular matrix protein deposition [3, 40]. It has been reported that transplantation of M2 reparative macrophage evidently improves the cardiac function recovery of MI [30]. Thus, the predominance and activity of M2 macrophages in the infarcted myocardium facilitates inflammation resolution and tissue repair. Despite advances in our understanding on the role of macrophages during MI repair, the mechanisms regulating M1/M2 phenotypic transformation are unclear.

Hypoxia-induced mitogen factor (HIMF)—also known as resistin-like molecules (RELMα) or found in inflammatory zone (FIZZ1)—belongs to a cysteine-rich RELM family that is highly conserved in mammals [5]. There are four murine isoforms (i.e. HIMF/RELMα/FIZZ1, RELMβ/FIZZ2, Resistin/FIZZ3, and RELMy/FIZZ4) and two human isoforms (i.e. RELMβ/FIZZ2, Resistin/FIZZ3). Each isoform shows unique tissue distribution and expression pattern, exerting different biological roles [37]. Strikingly, HIMF can be stimulated in immune cells [5, 13, 24, 27], especially in macrophages where HIMF is highly inducible by T helper (Th) 2 cytokines. HIMF is thus considered as an M2 macrophage biomarker, despite its function in macrophages is largely unknown [32]. Previous studies found distinct roles of HIMF in the immune response during different pathologies. For example, HIMF expression suppresses Th2 immune responses in mouse models of helminth infection and Schistosoma mansoni egg challenge [24]; while it has no effect on the Th2 response yet promotes the Th17 response in a mouse model challenged with intermittent ovalbumin and a colitis model induced by bacterial infection [5, 27]. As Th17 cytokines are pro-inflammatory and Th2 cytokines mediate tissue remodeling and fibrosis [1, 28], these immune responses share functional similarities with the respective M1- and M2-macrophage-dominated responses after MI. Thus, it is intriguing to know whether HIMF participates in macrophage transformation and its possible role in modulating the inflammation response and tissue repairing during MI.

Here, we conducted our studies in Himf−/− and WT mouse models of MI induced by coronary artery ligation, and compared the cardiac outcomes, M1/M2 macrophage transformation and M1/M2-related cytokines after MI. The in vivo outcomes were further examined in the macrophage-specific knockout mouse (HIMFfloxFloxLyz2-Cre) and their littermate Flox mouse models of MI. The direct effect of HIMF on macrophage transformation and the underlying mechanisms were studied in cultured mouse bone marrow-derived macrophages (BMDMs) and RAW 264.7 murine macrophages. Furthermore, we investigated the crosstalk between HIMF-overexpressing macrophages and cardiac fibroblasts (CFs).

Materials and methods

Animal care

Animals were purchased from the Animal Center of Guangdong Province, China, and housed under pathogen-free conditions with free access to food and water. All animal procedures were approved by the Institutional Care and Ethical Committee of Shenzhen University, China and conformed to Guide for the Care and Use of Laboratory Animals (National Institutes of Health publication No. 85-23, revised 1996).

Generation of the HIMF Knockout (Himf−/−) and HIMFfloxFloxLyz2-Cre (Himf-CKO) mice

Himf−/− mice were generated via a CRISP/Cas9 system, which resulted in the deletion of all four exons of HIMF gene, as previously described [14]. The genotyping was performed by PCR with 5′–GTGCTGATGCTGACTGTA–3′ and 5′–GTGACACTGCTTCCATAAG–3′ primers to identify the HIMF allele (504 bp) or 5′–CTCTTGAACACACC TCTT–3′ and 5′–CTACCCAGGCTCCTCACAT–3′ primers to identify the Himf−/− allele (239 bp). Himf-CKO mice were generated using CRISP/Cas9/Cre method by Cyagen Biosciences (Guangzhou, China, Supplementary Fig. 1a). Briefly, the gRNA targeting HIMF gene, the donor vector-containing loxP sites, and Cas9 mRNA were co-injected into fertilized mouse eggs to generate HIMF-floxed offspring. The macrophage-specific deletion of HIMF was achieved by crossing HIMF-floxed mice with the Lyz2-Cre mice. The genotyping was performed by PCR with 5′–CTCTTCCCTG TCTCTCAAGTGCTCGG–3′ and 5′–GGATCTTAACTG TTCGGTCTTT CTT–3′ primers to identify the Flox insertion (334 bp, or 266 bp for WT band), or 5′–CCAGAAAT GCCAGATTAG–3′ and 5′–CTTGGGCTGCCAGATTT CTC–3′ to identify the Cre allele (~700 bp) (Supplementary Fig. 1b).

Model of myocardial infarction

Myocardial infarction (MI) was induced in C57BL/6 J male mice (10–12 weeks old, 25 ± 2 g) by permanent ligation of the left anterior descending coronary artery (LAD). The mice were anesthetized with 3% pentobarbital sodium, intubated and mechanically ventilated (100 strokes/min, 250 μL stroke volume, Hugo Sachs Elektronik-Harvard Apparatus). Each mouse was placed on a heating plate to maintain the body temperature, and a left thoracotomy was performed in the third left intercostal space to expose the heart. The LAD...
was ligated with a 7–0 silk suture at a depth of 1 mm and a width of 1–1.5 mm. The ischemia and whitening of the area between the ligation position and the heart apex suggested successful LAD ligation. The chest and skin were closed in layers with a 7–0 nylon suture and the air was removed from the thorax with a pleural catheter, followed by subcutaneous injection of 0.2 ml 0.9% saline for rehydration. For the sham operation, mice underwent the same procedure except for LAD ligation. The same surgeon blinded to genotypes performed MI and sham operations. The mice were sacrificed on day 3 post MI/sham operation to analyze the inflammatory phase, or on day 7 to analyze the transition from the inflammatory to the reparative phase. The left ventricle of MI mice was either collected as a whole sample or separated into the remote zone, border zone and infarcted zone according to the specific experiment aims. For comparison, left ventricle were collected from sham mice as a control for the surgical procedure. Tissue samples were immediately placed into TRIzol (Cat#15596018, Invitrogen, Carlsbad, CA) or RIPA lysis buffer (Cat#R0010, Solarbio, Beijing, China) for subsequent RNA or protein extraction, respectively, or snap frozen in liquid nitrogen and stored at −80 °C for further processing. For sampling of different region of MI heart, we collected whitening papery region at 3 day and 7 day post MI as infarct area, collected the border zone in 1 mm distance to the infarcted area from the left ventricle, collected the remote zone 2 mm away from the infarcted area from the left ventricle.

**Echocardiography**

Echocardiography was performed on anesthetized mice (with 1.0% isoflurane) using a Vevo 2100 system (Visual Sonics, Toronto, Ontario, Canada), as previously described [15]. Briefly, the heart image was captured in the two-dimensional (2-D) mode in the parasternal short-axis view. M-mode tracings were recorded at the papillary muscle level and the following parameters were measured: the left ventricular (LV) internal dimensions at diastole (LVIDd) and systole (LVIDs), LV posterior wall dimensions at diastole (LVPWd) and systole (LVPWs). The LV fractional shortening (FS, %) was calculated as \( \frac{(LVIDd^2 - LVIDs^2)}{LVIDd^2} \times 100 \), and the LV ejection fraction (EF, %) was calculated as \( \frac{(LVIDd^2 - LVIDs^2)}{LVIDd^2} \times 100 \). An individual observer blinded to mice genotypes performed the echocardiography and subsequent data analysis.

**Flow cytometry**

Mice were anesthetized with 3% pentobarbital sodium and the heart was quickly exposed and perfused with 10 ml sterile cold phosphate-buffered saline (PBS, Cat#AAPR52, PythonBio, Guangzhou, China). After removal, the heart was minced in 25 μl PBS, and digested in Dulbecco’s modified eagle’s medium (DMEM, Cat#C11995500BT, Gibco, Hyclone) solution with 60 U/ml hyaluronidase (Cat#H3506, Sigma-Aldrich, Burlington, MA), 60 U/ml DNase1 (Cat#18047019, Invitrogen, Carlsbad, CA), and 450 U/ml collagenase type I (Cat#C0130, Sigma-Aldrich, Burlington, MA), for 1 h at 37 °C with gentle rotation. The digested solution was vortexed for 20 s, filtered through a 40-μm nylon cell strainer (WJB, Shanghai, China) and topped up to 10 ml with Hank’s Balanced Salt Solution (HBSS, Cat#AAPR25-1, PythonBio, Guangzhou, China) containing 2% fetal bovine serum (FBS, Cat#A3160802, Gibco, South American) and 0.2% BSA (Cat#AAPR615, PythonBio, Guangzhou, China). The cells were pelleted by centrifugation at 400 rcf for 5 min (4 °C) and washed in 1 ml Stain Buffer (FBS) (Cat#554656, BD Biosciences, San Jose, CA). After a second centrifugation (400 rcf, 5 min, 4 °C), the cell pellet was resuspended in 100 μl stain buffer (FBS) containing 1% anti-CD16/CD32 (Cat#553141, BD Biosciences, San Jose, CA) and incubated for 15 min at room temperature prior to staining. For cell sorting, the prepared samples were incubated for 30–60 min on ice with the following antibodies from BD Biosciences (San Jose, CA): anti-CD45 APC-Cy7 (Cat#557659 1:100), anti-CD11b FITC (Cat#553310, 1:100), Ly-6G PE-Cy7 (Cat#560601, 1:100), anti-Ly-6C APC (Cat#560595, 1:40); or antibodies from Biolegend (San Diego, California): anti-CD45 BV605 (Cat#103137, 1:100), anti-CD11b FITC (Cat#101205), anti-F4/80 PE (Cat#123110), anti-Ly6G APC (Cat#127613), anti-MHCII APC/Cy7 (Cat#107627), anti-CD163 BV421 (Cat#155309). The cells were sorted on a FACS Aria II cell sorter (BD Biosciences) directly into PBS for subsequent RNA isolation. At least 50,000 events were acquired and analyzed with FlowJo™ version 10.4.0 (Ashland, OR, USA: Becton, Dickinson and Company; 2019).

**Immunofluorescence**

Infarcted hearts were fixed in 4% paraformaldehyde (PFA), embedded in paraffin, and sectioned at 5 μm intervals from the level of coronary artery ligation to the heart apex. After routine hydration, the heart section was microwaved for 3 min in citrate buffer (0.4 g/L citric acid and 3 g/L sodium citrate) for antigen retrieval. Permeabilization and blocking were performed in PBS with 5% bovine serum albumin (BSA) and 0.2% Triton X-100, at room temperature for 30 min. The sections were incubated overnight at 4 °C with the following primary antibodies: anti-HIMF (Cat#ab39626, Abcam, Cambridge, MA, USA), anti-CD68 (Cat#NBP-33337, Novus, CO, USA), anti-NOS2 (Cat#2D2-B2, R&D system, Minneapolis, MN), and anti-Arg1 (Cat#93668, Cell Signaling Technology, Danvers,
MA, USA. Then the samples were incubated for 1 h at 30 °C with the following respective secondary antibodies: A-21070 (1:400), A-11006 (1:400), A11030 (1:400) (Invitrogen, Carlsbad, CA) and 4,6-Diamidino-2′-phenylindole dihydrochloride (DAPI, Cat#10236276001, Sigma-Aldrich, Burlington, MA, USA). Confocal images were captured under a Zeiss LSM880 microscope (Carl Zeiss, Germany). A total of 3–4 animals were examined per group.

**Histological analysis of infarct size and collagen deposition**

To assess the overall infarct extent, 5 μm paraffin sections of infarct heart tissue were prepared at five equal intervals from the ligation line to the heart apex, and stained with Masson trichrome (Cat#G1345, Solarbio, Beijing, China) following the manufacturer’s protocol. The percentage of the blue-stained area versus the total area was used to indicate the extent of the infarction. To assess the collagen deposition, three paraffin sections per heart containing the infarct region were randomly selected and stained with Picrosirius red (Cat#36324ES60, Yeasen, Shanghai, China) following the manufacturer’s protocol. The percentage of the red-stained area versus the total area was applied and used to indicate the collagen density. Data analysis was performed with Image-J software (NIH, USA). A total of 3–4 animals were examined per group.

**Cell culture, cytokine stimulation and inhibitor treatment**

For isolation and culture of murine bone marrow-derived macrophages (BMDMs), C57BL/6 J male mice (6–8 weeks old) were sacrificed and the femur and tibia were isolated under sterile conditions. The marrow was collected by repeatedly flushing the bone cavities of the femur and tibia with ice-cold PBS. The solution was then filtered through a 38-μm nylon cell strainer (WHB, Shanghai, China) and centrifuged at 1000 rcf for 5 min. The marrow pellet was resuspended in DMEM containing 10 ng/mL M-CSF (Cat#NBP2-35165, Novus, Novus, CO, USA), 10% FBS (Cat#A3160802, Gibco, Carlsbad, CA, USA) and 1% penicillin/streptomycin (Cat#15140122, Invitrogen, Carlsbad, CA, USA), then seeded in 35 mm plates and cultured in a 37 °C cell incubator (5% carbon dioxide and 95% air). The plates were supplemented with equal amounts of fresh DMEM medium (10 ng/mL M-CSF, 10% FBS, 1% penicillin/streptomycin) on day 3, and washed with PBS on day 7 to remove non-adherent or dead cells. The adherent BMDMs were kept in complete growth medium (DMEM containing 10% FBS and 1% penicillin/streptomycin) and tested by M1 and M2 type cytokine stimulation. Here, recombinant murine IL-4 (10 ng/ml), IFNγ (5 ng/ml) and LPS (1 μg/ml; all from R&D systems, Minneapolis, MN, USA) were applied for 24 h to promote the M1 and M2 polarization of BMDMs. Then, qRT-PCR analysis of M1 and M2 maker genes (NOS2 and Arg1, respectively) was performed in the stimulated cell samples. Primary cultured BMDMs were used without passaging. To inhibit STAT3 activity, 50 μM STAT3-specific inhibitor S3I-201 (Cat# HY-15146, MCE, Monmouth Junction, NJ, USA) was administrated 8 h before ad-HIMF/ad-GFP adenoviral infection. Equal amounts of DMSO or H2O were added as necessary and served as carrier controls.

The murine macrophage RAW264.7 cells were purchased from the Cell Bank of Chinese Academy of Sciences (Shanghai, China) and amplified though passaging. When ready to use, the RAW264.7 cells were grown in complete DMEM (supplemented with 10% FBS and 1% penicillin/streptomycin), and treated with cytokine stimuli or the STAT3 inhibitor S3I-201 as described above, when the cells reached ~60% confluence. The THP-1 cells were purchased from the Procell (Wuhan, China) and amplified though passaging. When ready to use, the THP-1 cells were grown in Roswell Park Memorial Institute (RPMI) 1640 medium (supplemented with 10% FBS, 1% penicillin/streptomycin and 0.05 mM β-mercaptoethanol), and treated with 100 ng/ml PMA (Phorbol 12-myristate 13-acetate, Cat# HY-18739, MCE, Shanghai, China) for 24 h to obtain adherent macrophages.

Cardiac fibroblast (CFs) were isolated from 1–2-day-old Sprague–Dawley (SD) rats, as previously described [15]. In brief, the hearts were removed from decapitated neonatal SD rats, immersed in PBS and minced with scissors into small pieces. The minced tissue fragments were digested in PBS containing 0.25% trypsin–EDTA at 37 °C and the isolated cells were added to fetal bovine serum (FBS) for subsequent centrifugation at 106 rcf for 5 min. The cells were resuspended in DMEM growth medium, and then pre-plated for 30 min at 37 °C to allow the fibroblasts to adhere to the plate. The cardiomyocytes were removed by changing the supernatant for fresh DMEM medium to allow the fibroblasts to adhere to the plate. The Cardiomyocytes were removed by changing the supernatant for fresh DMEM growth medium within 2 h after plating. Then, the CFs were allowed to recover and grow for 48 h in a 37 °C cell incubator prior to being seeded in experiment plates.

**Transfection of small interfering RNA**

Small interfering (si)-HIMF, si-CHOP and the corresponding negative control RNAs were chemically synthesized by Ribobio (Guangzhou, China). The siRNA sense sequences designed were as follows: si-HIMF, 5′-GCACTAGTGCTCA AGACTAT-3′; si-CHOP, 5′-GAAGAGCAGGAAGAAGA A-3′. Transient transfection of si-HIMF and si-CHOP into RAW264.7 and BMDM cells was accomplished with either Lipofectamine™ RNAiMAX Transfection Reagent (Cat#:
uptake of MTT ([4,5–dimethylthiazol–2–yl]–2,5-diphenyl
exposed to GFP-conditioned medium. 24 h, and expressed as a fold change compared with cells
as the mean linear movement of CFs over wound edges at
microscope. The speed of the cell migration was calculated
(0 h) and at 24 h after culturing under an Olympus inverted
pipette tip. Photos of the scratch were taken immediately
was made in the monolayer of cells using a sterile p200
cells in DMEM containing 0.5% FBS for 12 h. A line scratch
formed. Here, CFs were seeded in 6-well plates and grown
up to confluence, then driven into quiescence by culturing the

Adenoviral transduction of macrophages

A green fluorescent protein (GFP)-tagged recombinant ade-
ovirus encoding mouse HIMF (Ad-HIMF; Weizhen Bio-
tech, Shandong, China) was used to induce controlled HIMF
over-expression in BMDMs and RAW264.7 cells. A recom-
binant adenovirus-expressing GFP (Ad-GFP) was used as a
control. BMDMs and RAW264.7 cells were infected with
adenoviruses (MOI-30) for 48 h. Then, the DMEM culture
medium was either replaced with fresh virus-free medium
at 4 h after infection for analysis of the crosstalk between
BMDMs or RAW264.7 cells and CFs, or not changed until
harvesting.

Cell death, migration, viability and proliferation
analysis of CFs

To analyze the influence of HIMF expression on the cross-
talk between macrophages and CFs, the culture media
derived from HIMF-overexpressing or GFP-overexpressing
BMDMs and RAW264.7 cells was used to grow CFs. briefly,
the microphages were infected with ad-GFP or ad-HIMF
virus for 4 h and washed with normal incubation medium,
after another 48 h-incubation, the supernatants were har-
vested for the subsequent incubation of CFs. After 96 h
incubation, the CFs were collected for subsequent func-
tion analyses. To detect cell death, CF slides were prepared
for TUNEL assay as recommended (In Situ Cell Death
Detection Kit, Roche, Basel, Switzerland). The percent-
age of dead cells was calculated as the number of TUNEL-
stained cells (red) divided by number of DAPI-stained cells
(blue) × 100%.

To analyze CF migration, a wound-healing assay was per-
formed. Here, CFs were seeded in 6-well plates and grown
to confluence, then driven into quiescence by culturing the
cells in DMEM containing 0.5% FBS for 12 h. A line scratch
was made in the monolayer of cells using a sterile p200
pipette tip. Photos of the scratch were taken immediately
(0 h) and at 24 h after culturing under an Olympus inverted
microscope. The speed of the cell migration was calculated
as the mean linear movement of CFs over wound edges at
24 h, and expressed as a fold change compared with cells
exposed to GFP-conditioned medium.

Cellular viability was measured according to the
uptake of MTT ([(4,5-dimethylthiazol–2–yl)–2,5-diphenyl
tetrazolium bromide) (Sigma-Aldrich, Burlington, MA,
USA). CFs were seeded in 96-well plates overnight and
then treated with 110 μl DMEM-containing 0.5% FBS and
10 μl MTT solution (5 mg/ml). After incubation for a fur-
ther 2 h, the MTT reaction were terminated by replacing
the DMEM with 100 μl DMSO and subsequent shaking for
15 min. The absorbance was measured at 570 nm wave-
length using an ELISA reader (Infinite 200 PRO, Tecan,
Austria) and subtracted from that of a blank-containing
medium buffer alone. The CF viability was defined as the
relative absorbance of the treated versus the untreated con-
trol cells and expressed as fold change compared with cells
with GFP conditioned medium.

The CF proliferation was determined using the colori-
metric nonradioactive reagent bromodeoxyuridine (BrdU,
Roche/ Sigma-Aldrich, Burlington, MA), following the
manufacturer’s protocol. In brief, CFs were seeded and
grown in 96-well plates overnight, starved in DMEM with
0.5% FBS for 24 h, then incubated with 10 μM BrdU-labe-
ling solution for 24 h. After fixation for 30 min, the CFs
were re-incubated with an HRP-coupled anti-BrdU-anti-
body for 90 min at room temperature, and 100 μL tetra-
ethyl-benzidine solution was added for a further 5–30 min
incubation until a blue color developed. The absorbance
was measured at 370 nm (reference wavelength, 492 nm)
in an ELISA plate reader and subtracted from that of a
blank-containing medium buffer alone. CF proliferation
was defined as the relative absorbance of treated versus
untreated control cells, and expressed as fold change com-
pared with cells treated with GFP conditioned medium.

RNA reverse transcription and gene expression
analysis

The total RNA from heart tissue and cell samples was
extracted with TRIzol reagent (Invitrogen, Carlsbad,
CA, USA) according to standard protocol, and quanti-
fied using a spectrophotometer at 260 nm. The qual-
ity was evaluated according to the A260/A280 and
A260/A230 ratios (Molecular Devices, Holliston, MA,
USA). Then, 500 ng–1 μg total RNA was treated with
gDNA wiper mix (Vazyme, Nanjing, China) to remove
genomic DNA contamination, and reverse transcribed
using HiScript III qRT Supermix (Vazyme, Nanjing,
China) according to the manufacturer’s instructions. For
gene expression analysis, qRT-PCR was performed with
ChamQ Universal SYBR qPCR master mix and gene-
specific primers (Supplementary Table 1) on an Quant-
Studio 3 real-time PCR System (Applied Biosystems,
Waltham, MA, USA). The expression level of each gene
was normalized to internal control GAPDH gene and
calculated using the 2−ΔΔCt method.
Western blot analysis

Heart tissues and cells were lysed in RIPA lysis buffer (Solarbio, Beijing, China) supplemented with a protease inhibitor cocktail (Cat#HY-K0010, MCE, Monmouth Junction, NJ) and Phosphatase Inhibitor Cocktail II&III (Cat#HY-K0022& HY-K0023, all from MCE, Monmouth Junction, NJ, USA). After centrifugation at 15,294 rcf, 4 °C for 20 min, the supernatant was analyzed by BCA assay (Pierce, Thermo Scientific, Rockford, IL) to quantify the protein concentration. SDS-PAGE was performed by loading equal amounts of the proteins into the gel, which were then transferred to PVDF membranes (Merck Millipore, Bedford, MA, USA). The membrane was blocked with TBST solution containing 5% BSA. The target proteins were incubated overnight at 4 °C with the corresponding primary antibodies.
(Supplementary Table 2). Then horseradish peroxidase-conjugated anti-Rabbit (Cat#7074) or anti-Mouse (Cat#7076) secondary antibodies were used (Cell Signaling Technology, Danvers, MA). The blots were developed using Western Chemiluminescent HRP Substrate (Merck Millipore, Bedford, MA) and visualized using a UVP ChemStudio PLUS
HIMF deficiency results in a suppressed M1 inflammatory response in MI hearts. a Co-immunostaining of MI heart sections stained with the macrophage surface marker CD68 (green), HIMF (red) and DAPI (blue). The percentage of HIMF+CD68+cells in the total HIMF+cells was calculated on the right. The yellow line indicates the boarder of the infarct region. IZ infarct zone. Scale bar = 50 μm. The lower panel represents a close-up of the staining, magnification=×400. b mRNA expression analysis of pro-inflammatory and pro-reparative genes in the left ventricle collected on day 3 post-MI. For sham hearts, the left ventricles were collected as a control. For MI hearts, the infarct zone (IZ), border zone (BZ) and remote zone (RZ) were separately collected from each left ventricle. n=5 mice for sham control, n=6–9 mice for MI. c Same analysis was performed on day 7 post-MI. n=5–6 mice for sham control, n=7–8 mice for MI. *or # p<0.05, ** or ## p<0.01, ### p<0.001, N.S. not significant.

**Fig. 3** Himf deficiency results in a suppressed M1 inflammatory response in MI hearts. a Co-immunostaining of MI heart sections stained with the macrophage surface marker CD68 (green), HIMF (red) and DAPI (blue). The percentage of HIMF+CD68+cells in the total HIMF+cells was calculated on the right. The yellow line indicates the boarder of the infarct region. IZ infarct zone. Scale bar = 50 μm. The lower panel represents a close-up of the staining, magnification=×400. b mRNA expression analysis of pro-inflammatory and pro-reparative genes in the left ventricle collected on day 3 post-MI. For sham hearts, the left ventricles were collected as a control. For MI hearts, the infarct zone (IZ), border zone (BZ) and remote zone (RZ) were separately collected from each left ventricle. n=5 mice for sham control, n=6–9 mice for MI. c Same analysis was performed on day 7 post-MI. n=5–6 mice for sham control, n=7–8 mice for MI. *or # p<0.05, ** or ## p<0.01, ### p<0.001, N.S. not significant.

**Results**

**HIMF is upregulated after MI and exaggerates myocardium ischemic injury**

HIMF expression was examined in the infarct zone (IZ), border zone (BZ) and remote zone (RZ) in the left ventricles of WT mice at 3 and 7 days post MI and WT sham-operated mice. HIMF protein level was significantly increased in IZ at 3 days post MI and in BZ at 7 days post MI (Fig. 1a). Then the cardiac response to MI was compared between WT and Himf<sup>−/−</sup> mice. Echocardiography demonstrated a significant increase in the thickness of left ventricular posterior wall at systole (LVPWs) in Himf<sup>−/−</sup> MI hearts compared with WT MI hearts (Fig. 1b, c). The left ventricular dilatation, evaluated by LVIDs, was significantly reduced in MI hearts from Himf<sup>−/−</sup> mice compared to WT mice (Fig. 1c). Accordingly, the cardiac ejection fraction (EF) and fractional shortening (FS) were significantly higher in Himf<sup>−/−</sup> mice than WT mice post MI (Fig. 1c), suggesting that an Himf deficiency improved the contractile performance of MI hearts.

The infarct size in Himf<sup>−/−</sup> hearts was observed smaller than in WT hearts at 7 days post MI (Fig. 2a). This was verified by Masson trichrome staining of transverse sections of the infarcted hearts (Fig. 2b, c). As adequate collagen deposition driven by cardiac fibroblasts (CFs) limits infarct expansion, picrosirius red (PSR)-staining was performed to examine the collagen deposition. The PSR results showed that the infarct region of Himf<sup>−/−</sup> hearts was filled with denser collagen fibers (Fig. 2d). Consistently, type I collagen (COL1α2) protein expression in the infarct region of Himf<sup>−/−</sup> hearts was found higher than that in WT hearts (Fig. 2e). Finally, we compared the survival rate between WT and Himf<sup>−/−</sup> mice from day 1 to day 7 post MI. Himf<sup>−/−</sup> mice displayed a significantly lower mortality rate than WT (Fig. 2f). Here, we observed that more WT mice died on day 3–5 post MI, likely due to observed cardiac rupture, than Himf<sup>−/−</sup> mice. This is consistent with the report that exaggerated thinning of the LV wall and infarction expansion are often associated with cardiac rupture, a leading cause of mortality between day 3–5 post LAD coronary ligation [7].

**HIMF promotes M1 macrophage-mediated inflammatory response after MI**

Co-immunostaining for HIMF and the macrophage surface marker CD68 in transverse sections of the infarcted left ventricle identified higher HIMF protein levels in CD68+ cells than any surrounding area (Fig. 3a), suggesting that macrophages are a major source of HIMF production. The mRNA levels of M1-type inflammatory cytokines, including IL-6, TNFα, IL-1β, and the enzyme NOS2 increased to milder extents in the BZ and IZ regions of Himf<sup>−/−</sup> LV than WT LV at 3- and 7-day post MI (Fig. 3b, c). The M2-type reparative genes show different expression patterns. Namely the expression of Arg1 (encodes an enzyme for collagen production) in the IZ region of the Himf<sup>−/−</sup> left ventricle was significantly higher compared to WT at day 7 post MI (Fig. 3c). Except that, the mRNA levels of the anti-inflammatory M2 cytokines, including IL-10, TGFβ, CX3CR1 were comparable in the BZ and IZ regions of Himf<sup>−/−</sup> and WT hearts at 3- and 7-day post MI (Fig. 3b, c). These data suggest that HIMF ablation tempers the macrophage M1 pro-inflammatory response and facilitates M2 reparative activity.

Therefore, to examine if the improved healing outcome of Himf<sup>−/−</sup> MI heart is due to the ablation of HIMF in
**Fig. 4** HIMF influences M1/M2-like macrophage polarization.

- **a** Co-immunostaining of CD68 (green) and NOS2 (M1 macrophage marker, red) in MI-heart sections from WT and Himf−/− mice. Scale bar = 100 μM. White box: a close-up of staining, magnification = ×700. The nuclei counterstained with DAPI (blue). Transverse heart sections were prepared on day 7 post MI.

- **b** Co-immunostaining of CD68 and Arginase 1 (Arg1, M2 macrophage marker, red) in MI-heart sections from WT and Himf−/− mice.

- **c** Flow cytometry sorting of M1-like and M2-like macrophages/monocytes from hearts on day 7 post MI. CD45 + CD11b + Ly6G + cells as shown in the middle panel were classified as neutrophiles, while the circled CD45 + CD11b + Ly6G− cells were classified as monocytes/macrophage population (Mos/Mps).

- **d** Comparison of Himf mRNA expression in isolated heart macrophages/monocytes (CD45+CD11b+Ly6G−) and the homogenized left ventricle samples of MI and sham hearts. n = 5–9 mice per group.

- **e** The percentages of M1-like (CD45+CD11b+Ly6G−Ly6C+) and M2-like (CD45+CD11b+Ly6G−Ly6C−) cells sorted in **c**. n = 3–4 mice per group.

- **f** mRNA expression analysis of pro-inflammatory (left panel) and reparative (right panel) genes in macrophages/monocytes (CD45+CD11b+Ly6G−) sorted from hearts on day 7 post MI. n = 13–14 mice per group.

- **g** mRNA expression analysis of pro-inflammatory (left panel) and reparative (right panel) genes in RAW264.7 cells and bone marrow-derived macrophages (BMDMs) with or without adenoviral HIMF overexpression (ad-HIMF). A GFP-carrying (ad-GFP) adenovirus was used as an infection control. n = 7–9 replicates per group.

*p < 0.05, **p < 0.01, ***p < 0.001, N.S. not significant.
Fig. 4 (continued)
a. Add virus  →  Change with normal medium  →  Transfer the supernatant into CF plate

Macrophage  →  Cardiac fibroblast

b. MTT assay

- CF viability
  - CF viability of ad-GFP sup
  - CF viability of ad-HIMF sup

- Graph showing CF viability comparison

TUNEL assay

- CF apoptosis (%)
  - Graph showing CF apoptosis comparison

b. CCK8 assay

- CF viability
  - CF viability of ad-GFP sup
  - CF viability of ad-HIMF sup

- Graph showing CF viability comparison

c. Wound healing

- CF 24h-migration rate
  - Graph showing CF migration rate comparison

d. COL1α1, COL1α2

- RNA fold change
  - Graph showing RNA fold change comparison

- Protein fold change
  - Graph showing protein fold change comparison

- Western blot for COL1α2 and α-SMA

- Western blot for cleaved-cas3 and GAPDH
macrophages, we constructed macrophage-specific knock-out of HIMF mice (HIMF^floxed/floxed; Lyz2-Cre (hereafter Himf-CKO, Supplementary Fig. 1). The hearts of Himf-CKO and HIMF-Floxed mice were collected at day 7 post MI (Supplementary Fig. 1). The hearts of Himf−/− mice and HIMF-floxed mice at day 7 post MI. With alternative gating methods, we also identified a significantly decreased portion of M1 macrophages (CD45^+ CD11b^+ F4/80^+ Ly6G^− MHCII^− CD163^− ) and increased portion of M2 macrophages (CD45^+ CD11b^+ F4/80^+ Ly6G^− MHCII^− CD163^+ ) in the absence of macrophage-HIMF (Supplementary Fig. 3). We thus conclude that an Himf deficiency suppresses macrophage polarization into M1-like cells, and promotes the transition to a M2-dominant type following MI.

**HIMF promotes M1 inflammatory gene expression in BMDMs and RAW264.7 cells**

To investigate the direct effect of HIMF on macrophage transformation, we manipulated Himf expression in RAW264.7 macrophages and BMDMs (Supplementary Fig. 4a, b). Adenoviral-mediated HIMF overexpression significantly activated the expression of M1 marker genes in both RAW264.7 cells and BMDMs compared with GFP controls (Fig. 4g, h). By contrast, HIMF overexpression resulted in a decrease in Arg1 (Fig. 4g, h). siRNA-mediated Himf down-expression in BMDMs and RAW264.7 cells decreased the mRNA level of M1 marker NOS2 (Supplementary Fig. 4c, d), mirroring the effects of HIMF overexpression. These data indicate that HIMF directly promotes macrophage transformation to an M1 phenotype, which coincides with the expression of a series of pro-inflammatory genes and suppression of M2 transformation.

**HIMF expression in macrophages impaired cardiac fibroblast activity through a paracrine effect**

Himf deficiency elevates collagen deposition in infarct region of hearts (Fig. 2d). However, Himf expression is not induced in cardiac fibroblasts (CFs) [15]. Hence, we explored the crosstalk between macrophages overexpressing HIMF and CFs. CFs were cultured in conditioned medium derived from macrophages overexpressing HIMF (ad-HIMF sup, Fig. 5a). MTT and CCK8 assays demonstrated that ad-HIMF medium impaired the CF viability (Fig. 5b, c). The level of the apoptosis marker cleaved-caspase 3 increased in CFs by ad-HIMF medium, and TUNEL staining also indicated increased CF cell death (Fig. 5d). Further study of the role of Himf deficiency in fibrosis will contribute to the understanding of cardiac remodelling and fibrosis.
HIMF regulates macrophage polarization by activating CHOP expression

Previous studies have demonstrated a critical role for the cellular stress sensor, C/EBP-homologous protein (CHOP) in determining macrophage polarity [33, 38, 45]. We found that the CHOP mRNA and protein levels were upregulated in MI hearts of WT mice, and this upregulation was inhibited in Himf−/− mice (Fig. 6a-e). HIMF overexpression directly increased CHOP expression in BMDM and RAW264.7 cells (Fig. 6f, g, and Supplementary Fig. 6a, b). Decreasing CHOP expression by siRNA partially suppressed HIMF-induced M1 inflammatory cytokine production (Fig. 6h), and the decreased Arg1 expression (Fig. 6h). Therefore, CHOP seems to mediate HIMF-induced M1 macrophage polarization and transformation in the suppression of M2 transformation.

CHOP–STAT1/STAT3 signaling pathway mediates HIMF-induced M1 polarization

Signal transducer of activator of transcription (STAT) 1 is a well-established driver of M1 polarization [16]. We found that STAT1 activation correlated with the changes in CHOP expression in infarcted hearts, where the STAT1 phosphorylation level was increased in MI hearts of WT mice at 3 days and 7 days post MI; while Himf deficiency inhibited this effect (Fig. 7a, b). Over-expressing HIMF in BMDM and RAW264.7 cells consistently increased both CHOP expression (Fig. 6f, g) and STAT1 phosphorylation (Fig. 7c). siRNA-mediated CHOP knockdown suppressed STAT1 activation upon HIMF expression (Fig. 7e), suggesting STAT1 is a downstream signal of CHOP mediating HIMF-induced M1 polarization.

HIMF can activate STAT3 [15], and STAT3 might also be associated with M1/M2 cytokine production [8, 46]. We found the STAT3 activation pattern was quite similar to that of STAT1 in heart tissues (Fig. 7a, b) and cultured macrophages (Fig. 7d). siRNA-mediated CHOP down-expression inhibited STAT3 phosphorylation in HIMF-overexpressing cells (Fig. 7e), and STAT3 inhibitor S3I-201 suppressed HIMF-induced pro-inflammatory cytokine expression in BMDMs (Supplementary Fig. 7a, b). Therefore, HIMF promotes M1 polarization at least partially through CHOP–STAT1/STAT3 signaling pathways.

Discussion

Monocyte and macrophage numbers expand rapidly in the heart after acute MI, driving post-MI repair and long-term ventricular remodeling [17]. The initial infiltrated monocytes/macrophages transform from a pro-inflammatory phenotype to a reparative phenotype, coordinating clearance of necrotic tissue and scar deposition to prevent myocardial rupture and limit functional deterioration. Manipulations to facilitate this monocyte/macrophage transformation are highly valued, but designing an effective intervention is difficult because the mechanisms are largely unknown [17, 22]. Here, we have established a novel mechanism whereby HIMF negatively regulates M1-to-M2 transformation post MI. Specifically, HIMF is upregulated in macrophages post MI, promoting macrophage M1 polarization and pro-inflammatory cytokine production. HIMF ablation inhibits the M1 inflammatory response and facilitates M2 transformation and reparative function, resulting in enhanced collagen deposition and tissue repair, reduced infarct expansion, and improved cardiac function and survival post-MI.

HIMF is a M2 macrophage marker, but its expression could also be induced by M1 type stimuli (LPS) (Supplementary Fig. 8). We found that HIMF is upregulated in macrophages throughout the M1-dominated pro-inflammatory and M2-dominated reparative phases of MI. More interestingly, HIMF was found to promote macrophage transformation to a M1 phenotype. The in vivo data show that HIMF expression levels positively correlate with M1 pro-inflammatory cytokine production, and that an Himf deficiency decreases the M1-type population (Ly6Chigh) and pro-inflammatory cytokine production post MI. The in vitro data provide direct evidence showing that HIMF stimulates
macrophage transformation to a M1 phenotype. These data collectively indicate the critical role of HIMF in promoting macrophage polarization into the M1-like subgroup, exaggerating the inflammatory response and tissue damage post MI. Of note, this M2-like transition had negligible effects on anti-inflammatory M2 marker genes, such as IL-10, CX3CR1; and the function of CD163+ M2 macrophage in MI heart is currently elusive, especially considering quite
distinct roles in the context of different diseases and conditions [9, 12, 34, 42]. Here, we can only speculate that an Himf deficiency likely tunes macrophages to exhibit a more reparative-prone status mainly by upregulating Arg1 expression.

One of the most prominent beneficial changes that we observed in Himf−/− and himf-CKO mice after MI was enhanced scar deposition at the infarction site. Scar deposition indicates adaptive remodeling that is necessary to prevent myocardial rupture, limit infarct expansion and ameliorate functional deterioration following acute MI [31]. We found that an Himf deficiency increases collagen density and collagen fibril assembly in the infarct region. However, exogenously applied HIMF induces CF proliferation, migration and myofibroblast differentiation [15, 20], suggesting that the effect of HIMF deficiency on promoting scar deposition in MI hearts is not due to the direct effect of HIMF on CFs. Interestingly, we found that the conditioned medium derived from HIMF-overexpressing macrophages decreased CF vitality and inhibited CF activation. Therefore, we conclude that the cytotoxic effects of HIMF-induced macrophage M1 polarization on CFs is overwhelming during the early stages of MI. Himf ablation facilitates M2 macrophage transformation, promoting scar deposition and limiting infarct expansion. It is worth mentioning that the infiltration of monocytes/macrophages may also contribute to the altered inflammation/repair outcome in the MI hearts of Himf−/− and himf-CKO mice. This possibility should not be excluded, as the blood cell counts were not analyzed in this study.

Recent studies discovered a novel pro-inflammatory function of cellular stress protein CHOP in adipose tissue macrophages, promoting M1 polarization in the context of high-fat-diet-induced metabolic disorders [38]. Here, we found that CHOP was upregulated in heart tissues and macrophages after MI, positively correlating with HIMF expression and macrophage M1 polarization. CHOP knock-down prohibited M1 pro-inflammatory cytokine production. Besides that, STAT1 and STAT3 activation can regulate macrophage M1/M2 polarization and inflammatory responses [16, 35]. We found STAT1 and STAT3 were activated in WT but not Himf−/− hearts during MI. STAT1 and STAT3 were also activated upon HIMF overexpression in BMDM and RAW264.7 cells, while CHOP knock-down inhibited STAT1 and STAT3 activation and pro-inflammatory cytokine production. Therefore, we conclude that HIMF upregulates CHOP expression to drive macrophage M1 transformation and a pro-inflammatory response via activation of STAT1 and STAT3 signaling (Supplementary Fig. 9). In addition, STAT3 was reported to exert cardioprotective effects in the context of myocardial ischemic or ischemia/reperfusion injury, by actively involving in the signal transduction of remote ischemic preconditioning, upregulating the expression of cardioprotective genes or improving the mitochondrial function [3–6]. Here, we proved that STAT3 mediates HIMF-induced expression of pro-inflammatory genes, suggesting a detrimental role of STAT3 may exist and complex the MI repair outcome. Since our study demonstrated HIMF was predominantly expressed in macrophages after MI, and its pro-inflammatory property played a causal role in the reduced scar deposition and infarct expansion, we hypothesize that STAT3 in macrophages partially mediates the detrimental role of HIMF [10, 11, 18, 36].

Rodent HIMF has two human analogues: resistin and RELMβ. HIMF might be functionally more similar to human resistin because they share similar expression patterns [25]. Specifically, mouse HIMF and human resistin are expressed in myeloid cells and are highly inducible. Clinical investigations have demonstrated a positive correlation between the circulating levels of resistin and the risk of MI [29, 43]. Others have suggested that resistin has pro-inflammatory properties, as recombinant resistin can activate pro-inflammatory cytokine expression in human peripheral blood mononuclear cells and monocytic THP-1 cells [2]. For the translational purpose, we infected THP-1 cells with adenovirus overexpressing human resistin (ad-RETN, Supplementary Fig. 10). By comparing the expression of M1 and M2 genes, we found the increased expression of M1 proinflammatory genes (i.e. NOS2, TNFα, IL-1β) and decreased M2 reparative gene (i.e. IL-10) upon RETN expression. This supports resistin shares the function similarity with HIMF. We thus speculate that our findings on HIMF might be extended to resistin, and resistin might serve as a novel promising target for the treatment of MI. Since no such studies investigating whether the resistin is majorly expressed from macrophage of MI patients, the clinical significance of macrophage-resistin under the MI background worth being further explored.

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Availability of data and materials All data needed to evaluate the conclusions in the paper are present in the paper and/or the Supplementary Materials. Additional data related to this paper may be requested from the authors.

Declarations

Conflict of interest The authors declare that they have no conflict of interest.

Ethics approval All animal procedures were approved by the Institutional Care and Ethical Committee of Shenzhen University, China and conformed to Guide for the Care and Use of Laboratory Animals (National Institutes of Health publication No. 85-23, revised 1996).

Consent to participate/Consent for publication The manuscript does not contain clinical studies or patient data.

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