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Growth differentiation factor 11 promotes macrophage polarization towards M2 to attenuate myocardial infarction *via* inhibiting Notch1 signaling pathway

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Abstract

Background: Myocardial infarctions (MI) is a major threat to human health especially in people exposed to cold environment. The polarization of macrophages towards different functional phenotypes (M1 macrophages and M2 macrophages) is closely related to MI repairment. The growth differentiation factor 11 (GDF11) has been reported to play a momentous role in inflammatory associated diseases. In this study, we examined the regulatory role of GDF11 in macrophage polarization and elucidated the underlying mechanisms in MI. **Methods:** *In vivo*, the mice model of MI was induced by permanent ligation of the left anterior descending coronary artery (LAD), and mice were randomly divided into the sham group, MI group, and MI+GDF11 group. The protective effect of GDF11 on myocardial infarction and its effect on macrophage polarization were verified by echocardiography, triphenyl tetrazolium chloride staining and immunofluorescence staining of heart tissue. *In vitro*, based on the RAW264.7 cell line, the effect of GDF11 in promoting macrophage polarization toward the M2 type by inhibiting the Notch1 Signaling pathway was validated by qRT-PCR, Western blot, and flow cytometry. **Results:** We found that GDF11 was significantly downregulated in the cardiac tissue of MI mice. And GDF11 supplementation can improve the cardiac function. Moreover, GDF11 could reduce the proportion of M1 macrophages and increase the accumulation of M2 macrophages in the heart tissue of MI mice. Furthermore, the cardioprotective effect of GDF11 on MI mice was weakened after macrophage clearance. At the cellular level, application of GDF11 could inhibit the expression of M1 macrophage (classically activated macrophage) markers iNOS, interleukin (IL)-1β, and IL-6 in a dose-dependent manner. In contrast, GDF11 significantly increased the level of M2 macrophage markers including IL-10, CD206, arginase 1 (Arg1), and vascular endothelial growth factor (VEGF). Interestingly, GDF11 could promote M1 macrophages polarizing to M2 macrophages. At the molecular level, GDF11 significantly down-regulated the Notch1 signaling pathway, the activation of which has been demonstrated to promote M1 polarization in macrophages. **Conclusions:** GDF11 promoted macrophage polarization towards M2 to attenuate myocardial infarction *via* inhibiting Notch1 signaling pathway.

Keywords

myocardial infarction; growth differentiation factor 11; M1 macrophage; M2 macrophage; Notch1

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1 Introduction

Myocardial infarction (MI) is one of the most serious cardiovascular diseases. Studies have shown that exposure to cold environment would increase the risk of MI and the highest incidence of ST-segment elevation myocardial infarction (STEMI) was reported in winter[1-2]. However, the exact trigger of MI may not always be readily apparent. Massive loss of cardiomyocytes due to MI triggers malignant cardiac remodeling. The recruitment of immune cells and the secretion of inflammatory factors promote clearance of damaged tissue and play an active role in the recovery of cardiac function after MI[3]. When the inflammatory response is overdone, it prevents wound repair and leads to adverse left ventricle remodeling

and long-term heart failure[4-5]. Therefore, it is important to understand the cellular and molecular mechanisms of inflammatory regulation in MI and to establish effective targeted therapy accordingly. As an essential part of innate immune system, macrophages play an important role in defending against microbial invasion and participate in regulating tissue repair[6].The highly plastic macrophages can transform into M1 phenotype and M2 phenotype in response to different pathological conditions or various environmental factors (microbial products, damaged cells, activated lymphocytes). The imbalance of M1/M2 polarization in macrophages is usually associated with various inflammatory diseases[7]. M1 macrophages are mainly involved in the occurrence and development of inflammatory responses, and M2 macrophages are mainly involved in inflammation resolution and tissue repair[8]. Therefore, in-depth studies of the molecular mechanisms associated with macrophage polarization and clarifying their interactions are essential to elucidate the pathogenesis of inflammatory diseases and discovering new therapeutic strategies. Growth differentiation factor 11 (GDF11), a member of the transforming growth factor-β (TGF-β) superfamily, plays an important role in embryonic development, bone, and muscle formation[9]. GDF11 has attracted considerable attention to the research field because of its contradictory relationship with aging[10-13]. Recently, researchers found that GDF11 participated in inflammatory reactions[14]. Wang *et al.*[15] discovered that GDF11 could inhibit the activation of NLRP3 inflammatory bodies *via* up-regulating the Smad2/3 signaling pathway, thereby inhibiting the release of inflammatory factors and regulating the development of ulcerative colitis. In the study of inflammatory arthritis, GDF11 was found to suppress the nuclear factor kappa-light-chain-enhancer of the NF-κB signaling pathway to suppress the persistent state of inflammation[16].

Our previous study had shown that GDF11 could significantly inhibit the level of inflammatory factors at the wound area during the healing process of diabetic wounds[17]. In this process, macrophage-specific polarization may be involved in the healing process of diabetic wounds. Therefore, we explored the role and mechanism of GDF11 in macrophage polarization after MI.

The polarization of macrophages is regulated by various signaling pathways and the Notch signaling pathway is one of them. There are 4 types of Notch receptors in mammals including Notch1, Notch2, Notch3, and Notch4, which are expressed in a variety of tissues and organs[18]. Among them, Notch1 is the most widely explored and studies have shown that the binding of the ligand DLL4 to the Notch1 receptor promotes its downstream effectors activation. The regulation of proteases and γ-secretase causes NICD to enter the nucleus interacting with RBP-J and ultimately promoting macrophages to M1 polarization[19].

Therefore, we speculated that GDF11 might promote the polarization of macrophages towards M2 *via* regulating the Notch signaling pathway and therefore improve myocardial repair after MI.

2 Methods

2.1 Mouse model of myocardial infarction

Myocardial infarction was induced in C57BL/6 J male mice aged 6 to 8 weeks by permanent ligation of the left anterior descending coronary artery (LAD), and mice were randomly divided into the sham group, MI group, and MI+GDF11 group. Briefly, mice were anesthetized by Avertin (2%, 10 μL/g) *via* intraperitoneal injection. In the presence of a ventilator, a left thoracotomy is performed in the third left intercostal space to expose the heart. The left anterior descending coronary artery was permanently ligated using a 7-0 silk suture. For sham mice, performed the same surgical procedure without LAD ligation. The mice in the MI+GDF11 group underwent MI surgery and GDF11 (Cat.120-11, Peprotech, New Jersey, USA) was administered by tail vein injection at a dosage of 0.1 mg/kg/day for 7 consecutive days. In macrophage depletion experiments, the clodronate liposomes (CLs) (100 μL/10 g) (Cat.SN-MLE04, SunLipo NanoTech, Shanghai, China) were administrated *via* tail vein injections for two days after myocardial infarction surgery.

C57BL/6 mice ranging from 8 to 10 weeks in age and weighing between 22-25 g each were used for animal studies (Animal Experimental Ethical Inspection Protocol No. HMUIRB3021619). Use of animals was approved by the Ethic Committees of Harbin Medical University and conformed to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996).

2.2 Echocardiography

Echocardiography was performed on anesthetized mice, using an echocardiographic imaging system (Visualsonics, Toronto, Canada). Mice were anesthetized with intraperitoneal injection of avertin (2%, 10 μL/g) and fixed in a supine position on an ultrasound table. After the heart rate was stable, the complete cardiac morphology and function were measured by parasternal short-axis continuous M-mode transthoracic echocardiography. Analysis was performed with a Vevo 2100 high-resolution imaging system. Ventricular ejection fraction (EF) was calculated as (ventricular end-diastolic volume [EVD] - ventricular end-systolic volume ES) / EVD; ventricular fractional shortening (FS) was calculated as (left

ventricular diastolic diameter [LVIDd] - Systolic left ventricular diameters [LVIDs])/diastolic LVIDd. All measurements represent the average of five consecutive cardiac cycles.

2.3 Cell culture

RAW264.7 cells were cultured in DMEM (Thermo Fisher Scientific, Waltham USA) containing fetal bovine serum (10%) (Cat.10100147, Thermo Fisher Scientific, Waltham, USA), Penicillin-Streptomycin Solution(1%) (Cat.SV30010, HyClone, Logan, USA). Cells were maintained at 37°C in a 5% CO_2 incubator. The agents were dissolved in 0.1% BSA buffer (1 g/L, pH 4.5). RAW 264.7 macrophages were incubated with GDF11 (1, 10 or 50 ng/mL, Cat.120-11, Peprotech, New Jersey, USA) for 24 hours (or 48 hours) or pre-incubated with LPS (100 ng/mL, Cat.L2880, Sigma-Aldrich, Saint Louis, USA) for 6 hours and then added with GDF11 (50 ng/mL) for 24 hours (or 48 hours) for subsequent experiments.

2.4 Reverse transcription and quantitative real-time PCR (qRT-PCR)

Total RNA samples were extracted from macrophages using Trizol reagent (Cat.15596026, Invitrogen, Carlsbad, USA), according to the manufacturer's protocol. The quality of the samples was determined using an Nano Drop 8000 Bioanalyzer (Thermo Fisher Scientific, Waltham, USA). Complementary DNA synthesis was performed with random primers according to the manufacturer's instructions. The SYBR Green PCR Master Mix Kit (ABI, Boston, USA) was used for real-time PCR to quantify the target genes on the ABI 7500 fast Real-Time PCR system (ABI, Boston, USA). β-actin was used as an internal control in sample. The following specific primer sequences were used: vascular endothelial growth factor (VEGF) forward: 5′-CTCGCAGTCCGAGCCGGAGA-3′, reverse: 5′-GCAGCCTGGGACCACTTGGC-3′; arginase 1 (Arg1) forward: 5′-AGCTCTGGGAATCTGCATGG-3′, reverse: 5′-ATGTACACGATGTCTTTGGCAGATA-3′; Notch1 forward: 5′-CCTGAGGGCTTCAAAGTGTC-3′, reverse: 5′-CGGAACTTCTTGGTCTCCAG-3′; iNOS forward: 5′-CCAAGCCCTCACCTACTTCC-3′, reverse: 5′-GGCAGTGTAACTCTTCTGCAT-3′; interleukin (IL)-1β forward: 5′-AATCTATACCTGTCCTGTGTAATGAAA GAC-3′, reverse: 5′-TGGGTATTGCTTGGGATCCA-3′; IL-6 forward: 5′-CCTCTGGTCTTCTGGAGTACC-3′, reverse: 5′-GGAGAGCATTGGAAATTGGGG-3′; IL-10 forward: 5′-GCTCTTACTGACTGGCATGAG-3′, reverse: 5′-CGCAGCTCTAGGAGCATGTG-3′; CD206 forward: 5′-CTCGTGGATCTCCGTGACAC-3′, reverse: 5′-GCAAATGGAGCCGTCTGTGC-3′; Hes1 forward: 5′-AACTGCATGACCCAGATCAA-3′, reverse: 5′-TGATCTGGGTCATGCAGTTG-3′; Hey1 forward: 5′-TTTTCCTTCAGCTCCTTCCA-3′, reverse: 5′-ATCTCTGTCCCCCAAGGTCT-3′.

2.5 Immunofluorescence

Infarcted hearts sectioned at 5 μm intervals using freezing microtome were fixed in 4% formaldehyde, permeabilized with 0.1% Triton X-100, and blocked with goat serum for 30 min. Heart tissue sections were incubated with CD86 (1:200, Cat.ab119857, Abcam, Boston, UK) or CD206 (1:200, Cat.ab8918, Abcam, Boston, UK) overnight at 4°C and incubated with fluorescence secondary antibody (1:1000) for 2 hours at room temperature. DAPI was used for nuclei staining. The slides with cells sections were fixed with 4% para formaldehyde, washed and blocked with goat serum and then incubated with iNOS (1:200, Cat. GTX130246, GeneTex, Irvine, USA) primary antibody, fluorescent secondary antibody (Cat.115-545-003 and 115-585-003, Jackson Immuno Research, Lancaster, USA) and DAPI (Cat.C1006, Beyotime, Shanghai, China). Images were observed using laser confocal microscopy.

2.6 TTC staining

The mice were euthanized on the twenty-eighth day of myocardial infarction surgery. Intact heart tissue was immediately frozen at -80°C and removed after 2 minutes. We immediately divided the section below the ligature into 4 pieces with same thickness. The heart slices were immersed in a 2% triphenyl tetrazolium chloride (TTC) solution and incubated at 37°C for 20 minutes in the dark. The tissues were arranged in order and photos were taken under a stereo microscope.

2.7 Western Blot

Total protein was extracted from cultured RAW264.7 cells for immunoblotting analysis. The protein concentration was determined by BCA Protein Assay Kit (Cat.P0010S, Beyotime, Shanghai, China). The protein was separated by polyacrylamide gel electrophoresis with different concentrations according to the molecular weight of the target protein. The wet electrotransfer method was used to transfer the protein on the gels to the NC membrane. The primary antibodies used were anti-GDF11 (1:1000, Cat.DF8364, Affinity, Shanghai, China), anti-Arg1 (1:1000, Ca.GTX109242, GeneTex, Irvine, USA), anti-iNOS (1:1000, Cat.GTX130246, GeneTex, Irvine, USA), anti-Notch1 (1:1000, Cat.4380T, Cell Signaling, Danvers, USA), anti-β-actin (1:1000, Cat.sc-47778, Santa, Dallas, USA). The NC membrane was incubated with the secondary antibody (1:10 000, Cell Signaling, Danvers, USA), scanned with an Odessey infrared imaging scanner, and the blot was analyzed by the supporting software.

2.8 Flow cytometry

The RAW264.7 cells were incubated with fluorescein isothiocyanate-conjugated antibodies against mouse CD11b (Cat.101206, Biolegend, California, USA) and phycoerythrin-conjugated antibodies against mouse CD206 (Cat.141706, Biolegend, California, USA). All antibodies were used at 1.25 μg/mL. Cells were incubated with the antibodies for 30 min at 4°C and washed with phosphate-buffered saline (PBS). Samples were analyzed by using a Beckman Coulter flow cytometer. The results were analyzed by using FACScan flow cytometer with Cell Quest software (Beckman Coulter, Bria, USA).

3. Results

3.1 GDF11 improves the cardiac function of MI mice *via* regulating macrophages.

To explore the role of GDF11 in MI, we performed Western blotting experiments to detect the protein level of GDF11 in the infarct border zone of the cardiac tissue. Compared with sham groups, GDF11 protein level was significantly decreased at 7 days post MI (Fig. 1A). To determine the therapeutic effect of GDF11 on MI, the echocardiography were performed. Compared with MI groups, GDF11 could significantly improve the cardiac function of MI mice (Fig. 1B). It has been known that macrophages play an irreplaceable role in the repair of MI. In the first three days after MI, M1 macrophages proliferate massively, release a large number of inflammatory factors, and aggravate myocardial injury. Until the seventh day, M1 macrophages are gradually replaced by M2 macrophages with repair functions. Macrophages can secrete cytokines and participate in the repair of ischemic myocardial tissue damage. Therefore, we detected the infiltration of M1 macrophages on the third day after MI and the infiltration of M2 macrophages on the seventh Day after MI. As shown in Fig. 1C, the expression of CD86, a surface marker of M1 macrophages, was increased in the cardiac tissue of MI mice and GDF11 administration significantly decreased the level of CD86. In addition, GDF11 improved the expression of CD206, a surface marker of M2 macrophages, on the infarct margin tissue of mice on the 7^{th} day after MI (Fig. 1D). To verify whether the therapeutic effect of GDF11 on MI was attributable to its regulation on macrophages, we used the CLs to eliminate macrophages in mouse hearts and explored the long-term effect of GDF11 on MI mice. As shown in Fig. 1E, after 7 days of GDF11 administration, the protective effect of GDF11 on the cardiac function of MI mice lasted until 28 days. Similarly, TTC staining demonstrated a larger infarct size in the MI group compared with the Sham group. Moreover, the protective effect of GDF11 on cardiac function was disappeared after macrophage clearance. GDF11 could not improve the infarct size of MI mice after macrophage clearance (Fig. 1F). These results indicated that GDF11 improves cardiac function and reduces infarct size in MI mice *via* targeting macrophages.

3.2 GDF11 inhibits the polarization of macrophages to M1 phenotype

To explore the potential role of GDF11 on macrophage polarization, we firstly examined the expression of markers that representing M1 macrophages (IL-6, iNOS, and IL-1β) in the RAW264.7 cell line. Real-time PCR analysis showed that GDF11 inhibited the expression of IL-6, IL-1β and iNOS in a dose-dependent manner (1, 10, and 50 ng/mL) compared with the control group (Fig. 2A-C). Consistently, western blotting analysis demonstrated that GDF11 significantly down-regulated the expression of M1 macrophages marker iNOS (Fig. 2D). The above results preliminarily indicated that GDF11 could inhibit the polarization of macrophages to M1 phenotype by suppressing the expression of M1 macrophage markers.

3.3 GDF11 promotes macrophages to M2 polarization

When macrophages switch from a pro-inflammatory to a repairing phenotype, they can inhibit inflammation and contribute to myocardial infarction healing, thereby improving cardiac remodeling and prognosis after myocardial infarction. M1 macrophages are mainly involved in initiating and sustaining the inflammatory response, and M2 macrophages mainly participate in the process of inflammation regression and tissue repair. To clarify whether GDF11 could promote the macrophages to M2 polarization, we detected the effect of GDF11 on the expression of M2 macrophages markers. As expected, different concentrations of GDF11, especially at 50 ng/mL, could significantly upregulated the expression of M2 macrophagesrelated genes (Arg1, IL-10, and CD206) (Fig. 3A-C). Consistently, western blotting analysis demonstrated that GDF11 significantly up-regulated the expression of M2 macrophage marker Arg1 at the concentration of 50 ng/mL (Fig. 3D). Moreover, GDF11 increased the number of CD206+ macrophages obviously (Fig. 3E). The above data suggested that GDF11 promoted macrophages to M2 polarization.

3.4 GDF11 inhibits the expression of inflammatory factors in macrophages induced by LPS

It has been known that excessive or persistent promotion of M1 polarization can cause pathological damage and disease. Evidence has shown that LPS can induce the polarization of macrophages into M1 macrophages and promote the expression of M1 macrophage-related inflammatory factors[20]. Therefore, we next explored the effects of GDF11 on LPS treated macrophages. Compared with the LPS group, GDF11 treatment

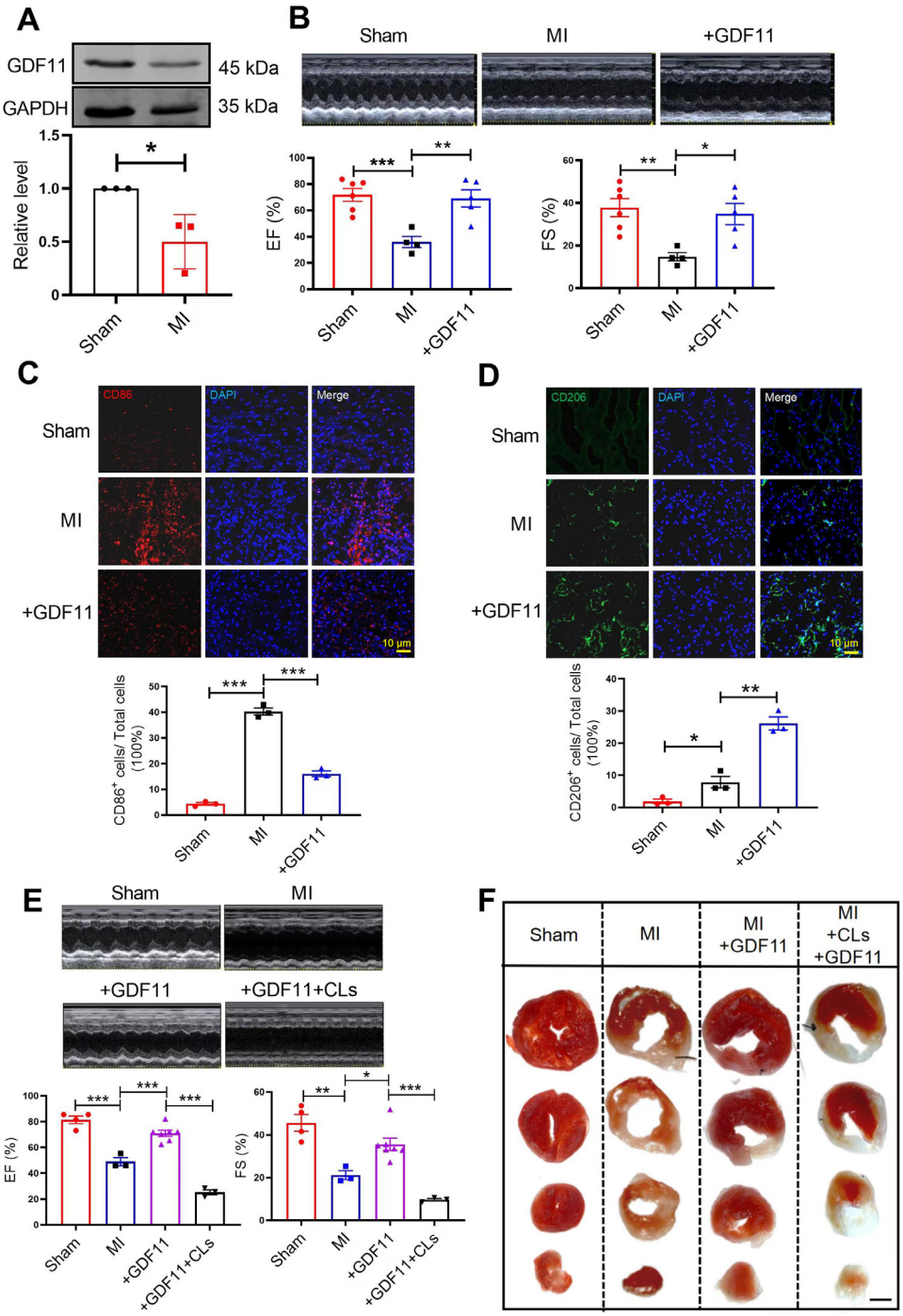

Fig. 1 GDF11 regulates macrophage polarization to improve cardiac function in MI mice

(A) Western blotting detected the protein expression of GDF11 in MI, *N* = 3 per group. (B) Echocardiography of MI mice and GDF11-treatment mice at 7 days after MI or sham operation, *N* = 4-6 per group. (C) Co-immunostaining of cardiac tissue sections at 3 days after MI stained with the M1 macrophage surface marker CD86 (red) and DAPI (blue). *N* = 3 per group. (D) Co-immunostaining of cardiac tissue sections at 7 days after myocardial infarction stained with the M2macrophage surface marker CD206 (red) and DAPI (blue). *N* = 3 per group. (E) Echocardiography was measured, EF% and FS% were detected in sham mice, MI mice, MI+GDF11+CLs mice and MI+GDF11 mice, *N* = 3-7 per group. (F) TTC staining to verify the infarct area of myocardial infarction mice. Data are described as means ± SEM. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; GDF11, growth differentiation factor 11; MI, myocardial infarctions; CLs, clodronate liposomes; EF, ejection fraction; FS, fractional shortening.

significantly inhibited the mRNA levels of M1 macrophage-related inflammatory factors (iNOS, IL-6 and IL-1β) (Fig. 4A-C). Likewise, western blotting and immunofluorescence analysis showed that LPS induced the upregulation of iNOS, whereas such up-regulated effects were largely reversed by GDF11 (Fig. 4D-F). Additionally, the repressed number of CD206+ macrophages induced by LPS was significantly rescued by GDF11 (Fig. 4G). These findings indicated that GDF11 inhibited the polarization of macrophages to M1 type, and inhibits the release of inflammatory factors, ultimately reducing the persistent inflammatory state of the microenvironment during myocardial infarction.

3.5 GDF11 promotes the polarization transition of M1 macrophages to M2 macrophages.

To investigate whether GDF11 promotes the transition of M1 macrophages to M2 macrophages, we detected the effect of GDF11 on the expression of M2 macrophages related anti-inflammatory factors. As shown in Fig. 5A-C, comparing with the LPS group, GDF11 significantly increased the expression of M2 macrophage-related anti-inflammatory factors (Arg1, VEGF, and IL-10). Moreover, incubation with GDF11 significantly reversed the LPS-induced decrease in Arg1 expression (Fig. 5D). Taken together, GDF11 induced M1 macrophages to M2 macrophages polarization.

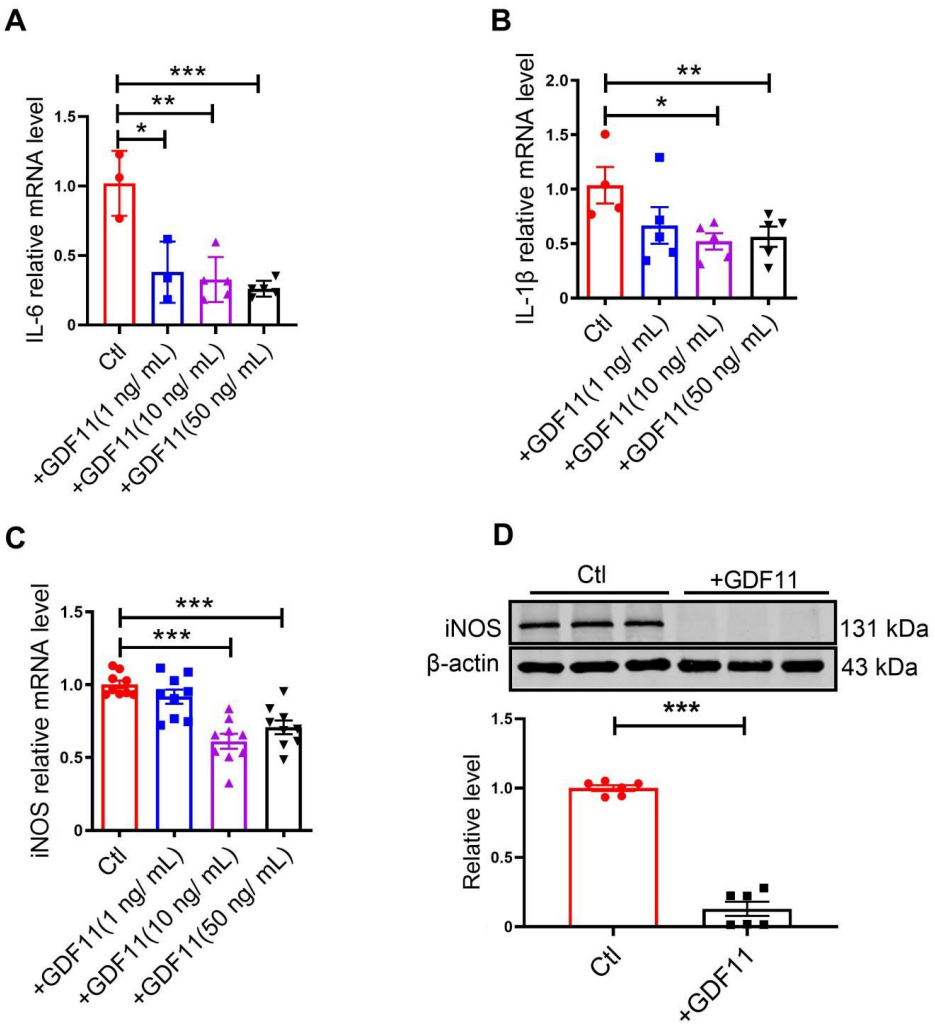

Fig. 2 Concentration gradient studies of GDF11-induced expression of iNOS, IL-1β and IL-6 in RAW264.7 macrophages

(A-C) Cultured RAW264.7 macrophages were treated with GDF11 (1, 10, 50 ng/mL) for 24 hours. GDF11-induced the expression of several genes labeled M1 macrophages, including iNOS, IL-1β, and IL-6 were analyzed by qRT-PCR. *N* = 3-9 per group. (D) Cultured RAW264.7 macrophages were treated with GDF11 (50 ng/mL) for 48 hours. iNOS protein levels were measured using western blotting analysis. *N* = 6 per group. Data are described as means ± SEM. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ *vs.* control group; GDF11, growth differentiation factor 11; IL, interleukin.

3.6 GDF11 regulates macrophage polarization by regulating the Notch1 signaling pathway.

Next, we sought to elucidate the cellular signaling mechanisms by which GDF11 led to the M2 polarization of macrophages. It has been reported that the Notch signaling pathway is essential for the development of inflammatory disease[21-22] and Notch signaling activation could promote M1 polarization of macrophages[23-24]. Our results demonstrated that GDF11 could repress the up-regulation of Notch1 induced by LPS at mRNA level (Fig. 6A). It indicated that GDF11 could not only inhibit the expression of M1 macrophages related inflammatory factors but also showed effects on the repression of Notch1 signaling. To further explore whether GDF11 regulated macrophage polarization by regulating the Notch1 signaling pathway, we used valproic acid (VPA), an agonist of Notch1, to activate the Notch1 signaling pathway. As shown in Fig. 6 B-D, GDF11 could reverse the VPA-induced up-regulation of Notch1, Hes1 and Hey1 (Fig. 6B-D). Moreover, we found that VPA could increase the expression of M1 macrophage cytokines iNOS, which could be significantly inhibited by GDF11 (Fig. 6E). These findings indicated that GDF11 regulated macrophage polarization by regulating the Notch1 signaling pathway.

4 Discussion

Monocytes are rapidly recruited to the ischemic zone and differentiate into macrophages in large numbers in the heart after myocardial infarction, promoting reparative cytokine release and cardiac repair[25]. Macrophages transform from a pro-inflammatory phenotype to a repairing phenotype, participating in the clearance of necrotic tissue and angiogenesis to restore cardiac function as much as possible. Manipulations that promote phenotypic transformation of

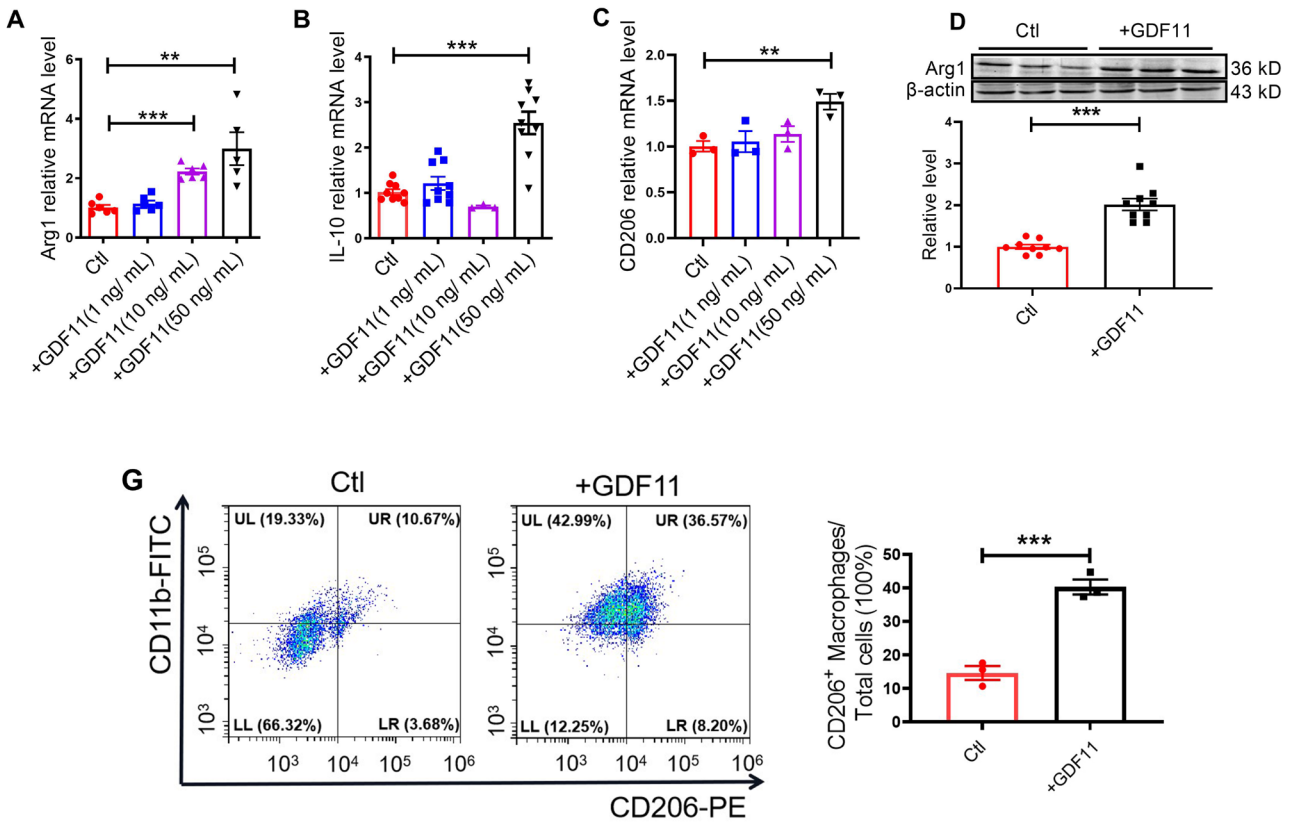

Fig. 3 Concentration gradient studies of GDF11-induced expression of Arg1, IL-10 and CD206 in RAW264.7 macrophages

(A-C) Cultured RAW264.7 macrophages were treated with GDF11 (1, 10, 50 ng/mL) for 24 hours. GDF11-induced expression of several genes labeled M2 macrophages, including Arg1, IL-10, and CD206 were analyzed by Real-time PCR. *N* = 3-10 per group. (D) Cultured RAW264.7 macrophages were treated with GDF11 (50 ng/mL) for 48 hours. Arg1 protein levels were measured using western blotting analysis. *N* = 9 per group. (E) Cultured RAW264.7 macrophages were treated with GDF11 (50 ng/mL) for 48 hours and the number of CD206+ cells was measured by flow cytometry analysis. *N* = 3 per group. Data are described as means ± SEM. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ *vs.* control group; GDF11, growth differentiation factor 11; IL, interleukin; Arg1, arginase 1.

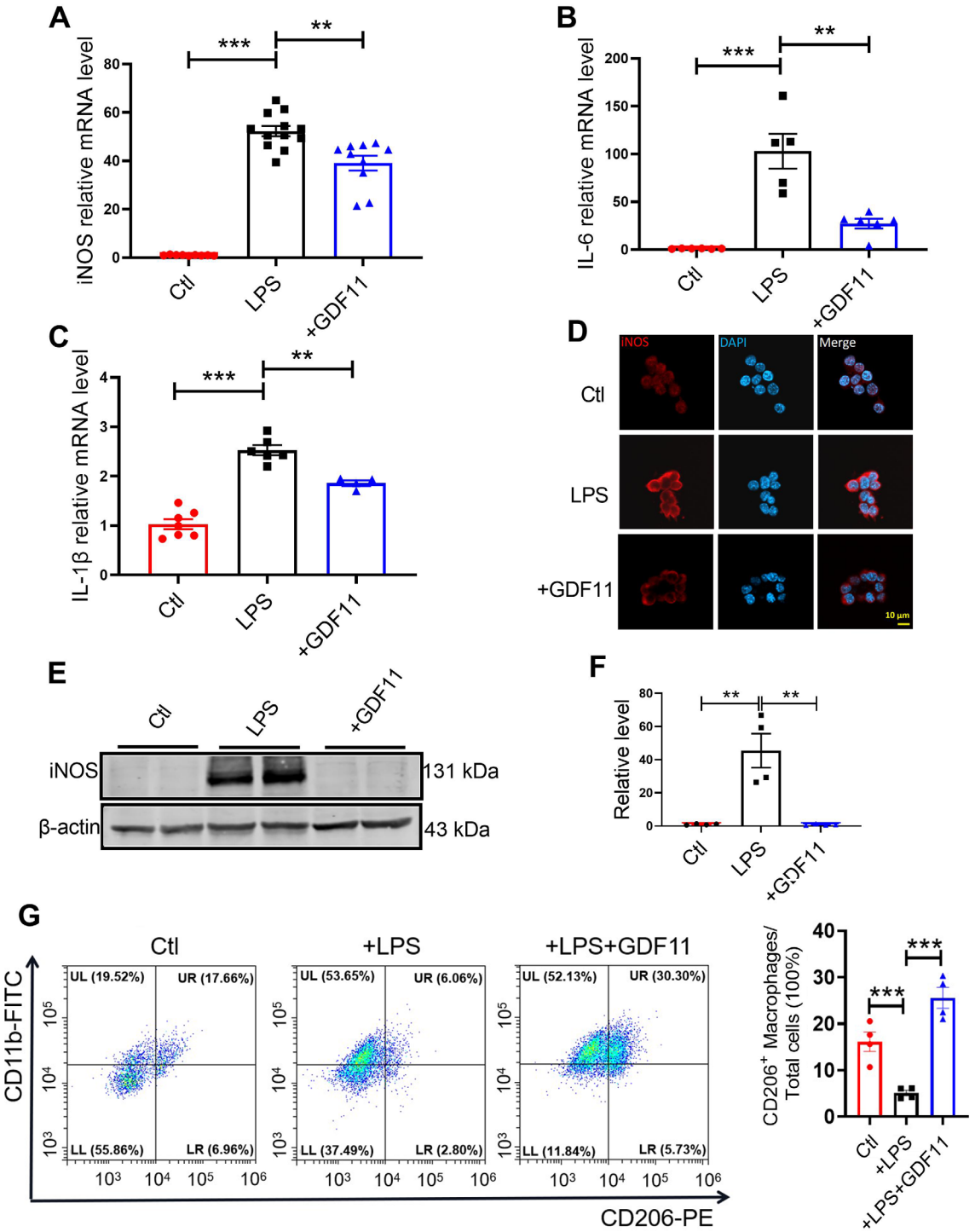

Fig. 4 GDF11 inhibits LPS-induced inflammatory response in RAW264.7

(A-C) The mRNA levels of iNOS, IL-1β and IL-6 in the LPS (100 ng/mL) treated macrophages at the presence or absence of GDF11 (50 ng/mL). *N* = 3-5 per group. (D) Levels of iNOS was tested by Immunofluorescence (iNOS was shown in red, nucleus was stained by DAPI and shown in blue). (E-F) iNOS protein levels were measured using western blotting analysis. *N* = 4 per group. (G) The number of CD206+ cells was measured byflow cytometry analysis. *N* = 4 per group. Data are described as means ± SEM. $^{**}P < 0.01$; $^{***}P < 0.001$; GDF11, growth differentiation factor 11; IL, interleukin.

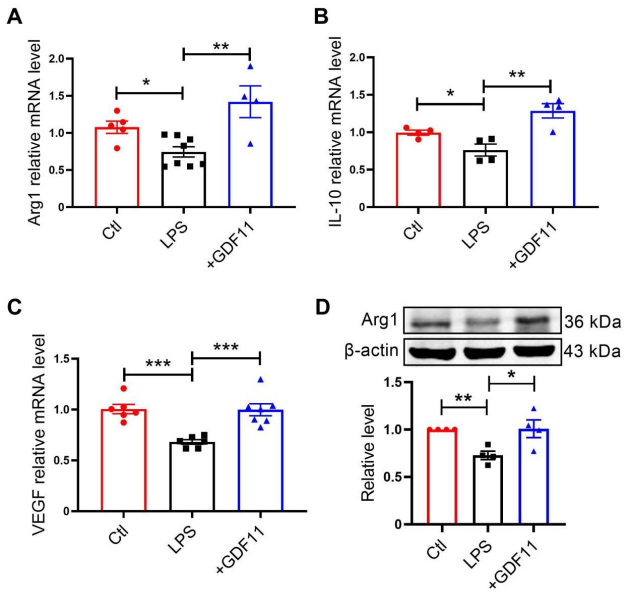

Fig. 5 GDF11 enhances anti-inflammatory reaction in LPS treated RAW264.7 cells (A-C) The mRNA levels of Arg1, VEGF and IL-10 in the LPS treated macrophages at the presence or absence of GDF11. *N* = 4-6 per group. (D) The protein level of Arg1 was verified by western blotting. *N* = 4 per group. Data are described as means ± SEM . $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; GDF11, growth differentiation factor 11; IL, interleukin; Arg1, arginase 1; VEGF, vascular endothelial growth factor.

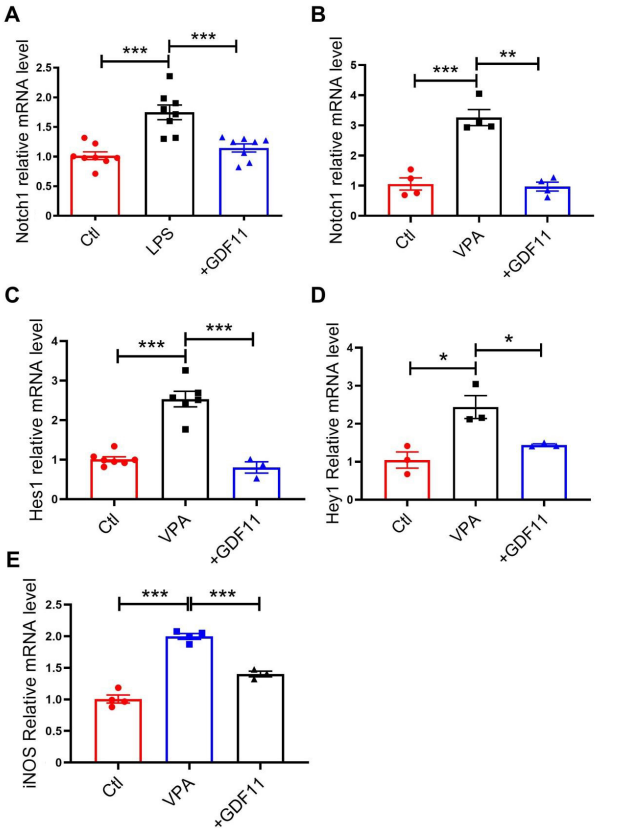

Fig. 6 GDF11 inhibits the activation of Notch1 signaling pathway

(A) The mRNA levels of Notch1 in the LPS treated macrophages at the presence or absence of GDF11. *N* = 8 per group. (B-E) The mRNA levels of Notch1, Hes1, Hey1 and iNOS in the VPA treated macrophages at the presence or absence of GDF11. *N* = 3-7 per group. Data are described as means ± SEM (*N* = 3/group). $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; GDF11, growth differentiation factor 11; IL, interleukin; Arg1, arginase 1; VEGF, vascular endothelial growth factor; VPA, valproic acid.

macrophages are of high value, so it is imperative to clarify their regulatory mechanisms and find safe and effective therapeutic drugs. Our findings uncovered a protective role of GDF11 in myocardial infarction by regulating macrophage polarization *via* inhibiting the Notch pathway (Fig. 7). Firstly, we found that GDF11 significantly improved cardiac function and reduced infarct size in mice with myocardial infarction. Next, we found that GDF11 could significantly decrease the expression of M1 macrophage markers iNOS, IL-1β, and IL-6 in a dose-dependent manner. In contrast, the expression of M2 macrophage markers Arg1, IL-10, and CD206 were increased with the application of GDF11. Considering that in the early stage of disease development, the tissue-resident macrophages and the recruited macrophages are mainly M1 type macrophages, which mediate inflammatory response and produce great damage. Therefore, we further explored the regulatory effect of GDF11 on M1-type macrophages. Our experimental results demonstrated that GDF11 could reverse the transformation of M1 macrophages to M2. We determined that the polarization of GDF11 macrophages could directly induce M0 macrophages and reverse the transformation of M1 macrophages into M2 macrophages. Our further mechanism study showed that GDF11 significantly inhibited the expression and the activation of the Notch1 signaling pathway, which suggests that GDF11 promotes M2 macrophage polarization by inhibiting the Notch1 signaling pathway.

To date, studies of the GDF11 have focused on aging, embryonic development, and bone formation and GDF11 plays a critical role in the occurrence and development of various diseases[26-30]. GDF11 has shown significant anti-inflammatory effects on inflammatory diseases such as rheumatoid arthritis[16] and ulcerative colitis[15]. Macrophages are an important part of innate immunity and play a central role in inflammation and host defense. In response to various environmental factors (microbial products, damaged cells, activated lymphocytes) or under different pathophysiological conditions, macrophages are transformed into different functional phenotypes[31-33]. The imbalance of M1/M2 polarization in macrophages is usually associated with various inflammatory diseases. M1

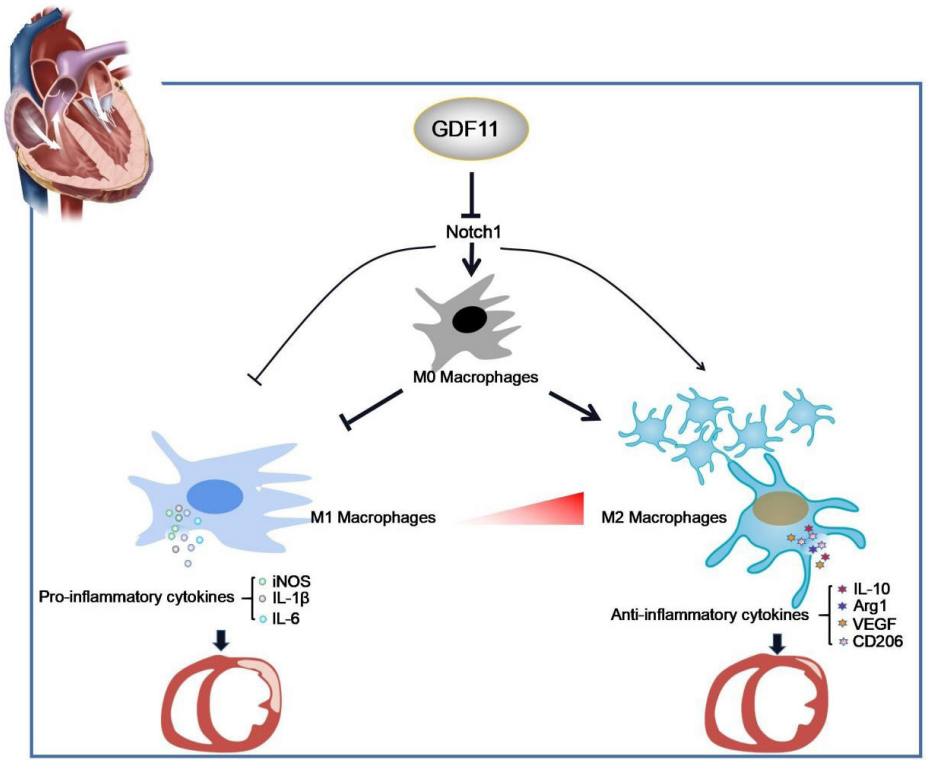

Fig. 7 Schematic diagram depicting the proposed signaling mechanisms.

GDF11, growth differentiation factor 11; IL, interleukin; Arg1, arginase 1; VEGF, vascular endothelial growth factor.

macrophages are mainly involved in initiating and maintaining an inflammatory response, and M2 macrophages are mainly involved in inflammation regression[10, 34-35]. Our previous study has identified that GDF11 could accelerate the healing of diabetic wounds[17]. In another study by our team, we found that GDF11 protected cardiomyocytes from hypoxia-mediated apoptosis by regulating autophagy[36]. As we have known that inflammation is involved in diseases such as aging, myocardial infarction, and diabetes. However, the effect of GDF11 on the polarization of macrophages in MI is unclear. In this study, we propose a novel function for the GDF11 that can direct the macrophage polarization state.

Taken together, this study explored the function of GDF11 to promote the polarization of macrophages to M2 phenotype, which has important reference significance for clinical inflammation-related diseases. This study also has shortcomings. Firstly, we did not clarify the specific downstream molecular mechanism of GDF11 in regulating the anti-inflammatory response of macrophages through the Notch1 signaling pathway. Secondly, we did not verify whether GDF11 administration could induce myocardial fibrosis in the MI mice. All these works will be finished in our next step study.

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Author contributions

All authors have read and approved the final manuscript.

Ethical approval

C57BL/6 mice ranging from 8 to 10 weeks in age and weighing between 22-25 g each were used for animal studies (Animal Experimental Ethical Inspection Protocol No. HMUIRB3021619). Use of animals was approved by the Ethic Committees of Harbin Medical University and conformed to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996).

Conflicts of interests

All authors declared that they have no conflict of interest.

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