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Immune-response gene 1 deficiency aggravates inflammation-triggered cardiac dysfunction by inducing M1 macrophage polarization and aggravating Ly6C^{high} monocyte recruitment

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Abstract
The immune response gene 1 (IRG1) and its metabolite itaconate are implicated in modulating inflammation and oxidative stress, with potential relevance to sepsis-induced myocardial dysfunction (SIMD). This study investigates their roles in SIMD using both in vivo and in vitro models. Mice were subjected to lipopolysaccharide (LPS)-induced sepsis, and cardiac function was assessed in IRG1 knockout (IRG1-/-) and wild-type mice. Exogenous 4-octyl itaconate (4-OI) supplementation was also examined for its protective effects. In vitro, bone marrow-derived macrophages and RAW264.7 cells were treated with 4-OI following Nuclear factor, erythroid 2 like 2 (NRF2)–small interfering RNA administration to elucidate the underlying mechanisms. Our results indicate that IRG1 deficiency exacerbates myocardial injury during sepsis, while 4-OI administration preserves cardiac function and reduces inflammation. Mechanistic insights reveal that 4-OI activates the NRF2/HO-1 pathway, promoting macrophage polarization and attenuating inflammation. These findings underscore the protective role of the IRG1/itaconate axis in SIMD and suggest a therapeutic potential for 4-OI in modulating macrophage responses.

Keywords Septic myocardial injury, Itaconate, Monocyte mobilization, Macrophage polarization, NRF2

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Introduction

Sepsis is a systemic inflammatory response to infection that results in septic shock and multiorgan failure [1, 2]. Statistical reports have suggested that almost 30 million patients suffer from sepsis worldwide [3], and 40–50% of patients with sepsis are accompanied by myocardial dysfunction. The mortality rate of patients with myocardial dysfunction has increased significantly [4, 5]. Sepsis-induced myocardial dysfunction (SIMD) is the leading cause of mortality in patients with sepsis.

Previous studies have reported that during sepsis, the hyperpolarization of macrophages towards M1

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accelerates the overproduction of nitric oxide (NO), interleukin 6 (IL-6), and interleukin-1β (IL-1β) [6], thereby resulting in myocardial cell damage and apoptosis, significantly increased brain natriuretic peptide and Troponin T, dilated cardiac ventricles, and decreased left ventricular ejection fraction [3, 7–9]. Macrophages located in the heart were rapidly activated after cardiac injury to protect the heart from damage. However, in the later stage of injury, monocytes in the blood circulation enter the heart and aggravate the inflammatory response, and further damage the heart tissue [10, 11]. When sepsis occurs, macrophages are converted to M2 and inhibit the hyperpolarization of macrophages to M1, thereby inhibiting the excessive inflammatory response [12–15]. However, the specific regulatory mechanism underlying the polarization changes in macrophages during SIMD is not completely known. Therefore, the effective regulation of macrophage activation, modulation of macrophage polarization, and inhibition of peripheral monocyte migration to the heart are potential targets for SIMD therapy.

Itaconic acid is a derivative of the tricarboxylic acid (TCA) cycle and is produced in the glucose metabolism pathway of macrophages [16, 17]. Endogenous and exogenous itaconic acid derivatives have the same modulating effect on macrophages [18]. They are an immune response product of enzymes encoded by immune response gene 1 (*IRG1*). Itaconic acid can reduce the production of pro-inflammatory mediators, such as IL-1β, IL-6, and NO induced by lipopolysaccharide (LPS)-treated macrophages, which results in anti-inflammatory effects [19–21]. There have been more studies on many inflammatory diseases. Previous studies have reported that itaconic acid had a cardiovascular protective effect. In the case of cardiac ischemia-reperfusion injury, intravenous injection of dimethyl itaconic acid (DI) can significantly reduce the myocardial infarction size after ligation of the anterior descending artery in mice [22], and intraperitoneal injection of DI can reduce doxorubicin-induced acute myocardial injury [23]. However, the studies on itaconic acid and SIMD are limited.

In this study, itaconic acid and IRG1 levels were measured by LC-MS and Western Blotting. IRG1 knockout ($IRG1^{-/-}$) and wild-type (WT) mice were treated with LPS and their cardiac functions were compared. Moreover, an itaconate derivative 4-octyl itaconate (4-OI) was administrated to investigate its pharmacological potential in SIMD. Here we report a therapeutic target of IRG1/itaconate for treatment of SIMD.

Methods

All the animal experiments were approved by the Ethics Committee of Nanjing Drum Tower Hospital (2020AE01065), followed the guidelines for the protection and use of laboratory animals, as specified by the NIH in the United States [24].

Animals and animal model

The $IRG1^{-/-}$ mouse model was generated through CRISPR/Cas9 system in C57B/6 background by Cyagen Biosciences Inc., Suzhou. The mice were bred at the animal center of Nanjing University affiliated Nanjing Drum Tower Hospital. C57/B6 wild-type (WT) mice were used as control. For acute sepsis-induced cardiac dysfunction model, the mice received 10 mg/kg LPS injected intraperitoneally in 0.2 mL saline. For the survival experiment, the mice received 15 mg/kg LPS injected intraperitoneally in 0.2 mL saline and then monitored every 4 h for 52 h.

Metabolomics study

Detection, identification and quantification of metabolites in cardiac tissue was as previously decribed [25]. Briefly, 100 mg of one sample was homogenized with methanol/water and further centrifuged at 12,000 rpm. The supernatant was anlayzed and quantified by a ultra-high-performance liquid chromatography system coupled with a Micromass system from Aptbiotech Inc.,Shanghai. The Raw data was extracted, followed by peak-identified, quality control and compound identified by comparison to the library. The data was further processed by bioinformatics using R 4.2.1.

Echocardiography

The echocardiography was performed using the Vevo 3100 Ultrasound System (Visualsonics, Toronto, Canada) as previous decribed [26]. The mice were anesthetized with isoflurane and cardiac function parameters including left ventricle internal end-systolic diameter (LVID; s) and left ventricle internal end-diastole diameter (LVID; d) was acquired. Left ventricular ejection fraction (EF) was calculated as following formula: LVEF (%) = 100 x (LVID; d [3] - LVID; s^3) / LVID; d [3].

Histological analyses

The heart samples were collected and fixed immediately in 4% paraformaldehyde overnight followed by embedded in paraffin. The heart sections were dissected, deparaffinized in xylene, and rehydrated with ethanol (100%, 95%, 80%, 50%) and water. The sections were subjected to antigen retrieval using a commercial Potent Antigen Retrieval Solution (Beyotime, #P0088). The slice was blocked with commercial blocking solution, followed by incubated with the primary antibody overnight. The slices were then incubated with the secondary antibodies for 2 h in room temperature. Finally, the slices was sealed with glycerin. All the images were captured with the Thunder Imaging Systems (Leica company).

Flow cytometry analysis
The proportion of macrophages/monocytes were detected and analyzed by flow cytometry as reported previously [25]. Briefly, the mice were sacrificed and the left ventricle, bone marrow, or spleen tissues were minced and digested with Collagenase II (1 mg/mL; Gibco, #17101015) and Dispase II (1U/mL, Sigma, #D4693), and then incubated at 37 °C for 30 min with gentle agitation every 5 min. The tissues were then homogenized with the gentleMACS dissociator (Miltenyi Biotec), passed through 70-μm cell strainer (BD Falcon #431751), and then centrifuged at 300 x*g* for 5 min. The pellets were resuspended in the flow stain buffer (BD) and blocked with the Fc-blocking solution (anti-CD16/32, eBioScience) for 10 min. After washing with PBS, the cells were stained with primary antibodies at 4 °C for 20 min in the dark. The cells were further washed, fixed, and permeabilized by the cytofix/perm solution (BD) for 25 min, followed by washing with perm/wash buffer and then subjected to intracellular cytokines staining. The cells were washed twice, and flow cytometry was performed with the BD FACSAria II and analyzed with the FlowJo 10.5.3.

Primary neonatal rat cardiomyocytes culture
Neonatal rat cardiomyocytes (NRCMs) were isolated from the ventricles of 1 day old neonatal SD rats as previously described [27]. Briefly, the neonatal rat heart was cut into pieces, followed by digested with 0.125% trypsin and 0.1% type I collagenase, cultured for 2 h for differential adherence, and fibroblasts and cardiomyocytes were separated. NRCM were then cultured in medium containing 5-BrdU. Through this method, the purity of cardiomyocytes can reach more than 90%. After 24 h of culture, 90% of cardiomyocytes beat spontaneously, showing good viability.

Statistical analysis
The data were reported as the mean ± SD. A one- or two-way ANOVA was performed to determine the difference among groups. Significance was reported at $p < 0.05$.

Results
Itaconate is highly increased in cardiac tissue of sepsis
Mice were given intraperitoneal injections of LPS to establish a sepsis model. The cardiac parameters including EF and FS were decreased after LPS injection, confirming the successful establishment of the SIMD mouse model (Fig. S1). We first evaluated the expression of cardiac metabolites in SIMD mice by LC-MS. KEGG enrichment analysis revealed that the metabolic changes associated with glucose metabolism were the most significant (Fig. S2). Among the metabolites increased in SIMD, itaconate was the second most significantly increased metabolites in the heart (Fig. 1A). The amount of acetyl-CoA was increased because it is the starting compound for the TCA cycle. However, the TCA cycle intermediate succinate decreased significantly, which indicated that the Krebs cycle was significantly blocked during SIMD.

As the enzyme cis-aconitate decarboxylase encoded by mouse immune responsive gene 1 (*IRG1*) promote the catalytic of cis-aconitic acid, an intermediate in the TCA cycle, to itaconate [28]. We further detected the

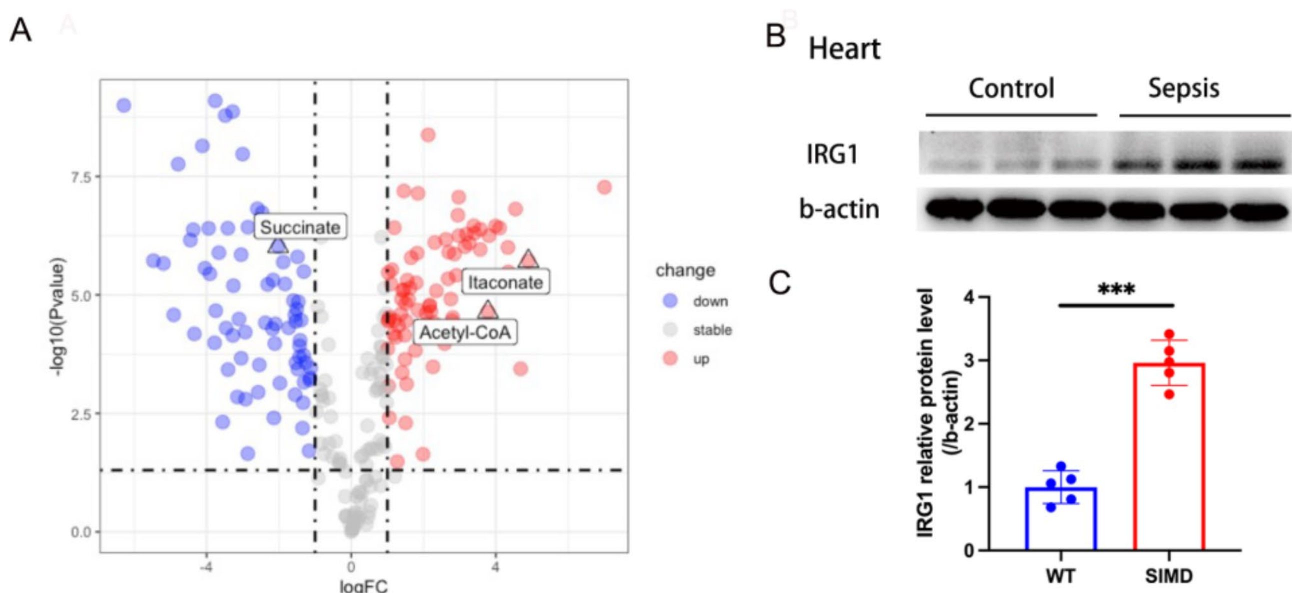

Fig. 1 Itaconate production is increased in cardiac tissue of SIMD mice. (**A**) Volcano plots for differential analysis of metabolites in mouse cardiometabolomics sequencing. (**B-C**) Representive western blots(WBs) of the protein levels of IRG1 in mice cardiac tissue. *** $p < 0.005$; $n = 5$ per group

alterations of IRG1 in cardiac during sepsis. Our results showed that protein levels of IRG1 in cardiac significantly increased during sepsis compared with those of the controls (Fig. 1B-C). These reults indicated that the level of itaconate was upregulated in SIMD.

IRG1 deficiency aggravates LPS-induced myocardial dysfunction and mortality

We next investigated whether IRG1 deficiency intensifies cardiac dysfunction in SIMD. We built IRG1$^{-/-}$ mice and the mice was validated by qPCR (Fig. S3).the Our results showed that under basal conditions (PBS injection), IRG1$^{-/-}$ mice showed normal cardiac functions similar to those in WT controls (Fig. 2A-D). However, cardiac functions of IRG1$^{-/-}$ mice deteriorated compared with WT control groups after LPS injection, as shown by worsened LVEF and LVFS compared with those of WT controls (Fig. 2A-B). The cardiac systolic functions altered more significantly than diastolic function as shown by significantly increased LVID; s rather than LVID; d (Fig. 2C-D). IRG1-KO mice are more prone to shock as shown by both lower systolic and diastolic blood pressure (Fig. 2E-F). Immunohistochemical results showed that LPS injection promoted cardiac apoptosis, whereas the knockdown of IRG1 promoted the increase of terminal deoxynucleotidyl transferase dUTP nick end

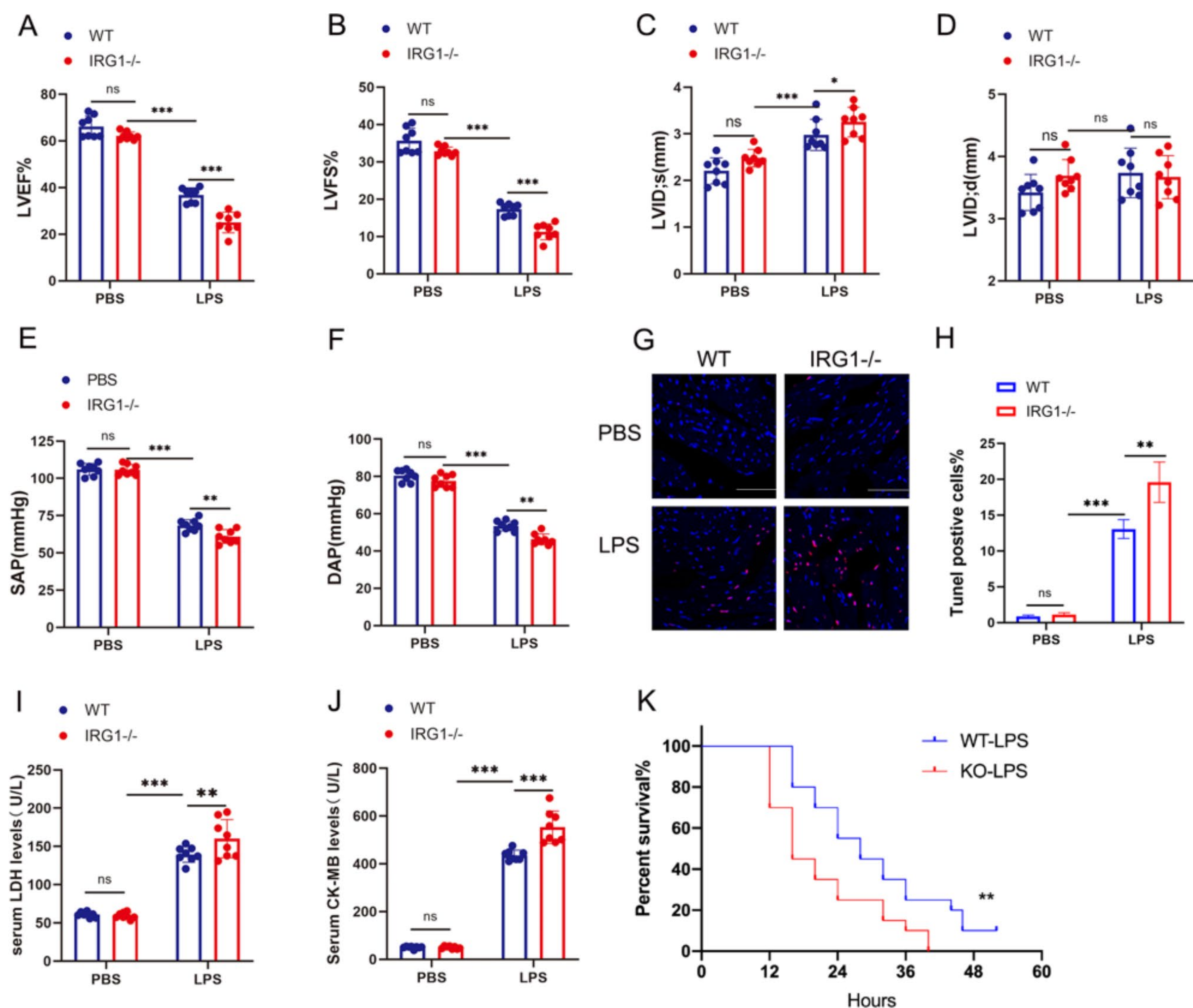

Fig. 2 IRG1 deficiency aggravates LPS-induced myocardial dysfunction and mortality in mice. (**A**–**J**) C57BL/6J WT and IGR1$^{-/-}$ mice were subjected to intraperitoneal injection of LPS (10 mg/kg) or saline. (**A**–**B**), Echocardiographic examination was used to evaluate cardiac function when LPS administrated for 12 h (n=8-10). A Left ventricular ejection fraction (LVEF). (**B**) Left ventricle fractional shortening (LVFS), (**C**) Left ventricular internal diameter at systole (LVIDs) and (**D**) Left ventricular internal diameter at dialation (LVIDd). (**E**–**F**) systolic and diastolic blood pressure 12 h after LPS injection. (**G**) Representative immunofluorescence micrographs showed that the Tunel-positive cells in each group (scale bar = 100 µm) and the (**H**) positive cells in each group were evaluate. (**I**–**J**) serum LDH and CK-MB 12 h after LPS injection (**K**) WT and IRG1 KO mice injected i.p. with LPS (15 mg/kg) were monitored for survival up to 52 h post-treatment. n=20. * $p<0.05$, ** $p<0.01$, *** $p<0.005$

labeling (TUNEL)-positive cells (Figure G-H). Moreover, mice from IRG1 deficiency group showed increased blood LDH (Lactate Dehydrogenase) and CK-MB (Creatine Kinase-Muscle/Brain Type) levels compared with WT control groups (Fig. 2I-J), which suggested that IRG1 deficiency exacerbated LPS-induced cardiac injury. Finally, we performed a survival experiment by injecting 15 mg/kg LPS. The results showed that the IRG1$^{-/-}$ mice showed higher mortality and shorter survival rate during the 52 h of LPS treatment (Fig. 2K). These data suggested that IRG1 plays an important role in cardiac function preservation on SIMD.

IRG1 deficiency aggravates cardiac inflammation and oxidative stress

We speculated that IRG1 protected cardiac from SIMD partly via suppressing oxidative stress. Therefore, we measured the levels of oxidative stress by DHE staining in the cardiac tissues of IRG1$^{-/-}$ and WT mice. We observed more DHE-positive cells in the cardiac tissue of IRG1$^{-/-}$ mice after LPS treatment than those in WT mice (Fig. 3A-B). Because macrophages are a major source of oxidative stress in the acute-infected host, we observed more F4/80 positive green cells in the cardiac tissues of IGR1$^{-/-}$ mice after LPS treatment (Fig. 3C-D). This was associated with markedly increased level serum levels of IL-1β and tumor necrotic factor- α (TNF-α) (Fig. 3E-F). Furthermore, the levels of M1 macrophage marker IL-1β and iNOS are increased in the cardiac tissues of IRG1$^{-/-}$ mice, whereas the M2 marker IL-10 and Arg1 decreased after IRG1 knockout (Fig. 3G-J). These findings suggested that IRG1 deficiency can promote cardiac inflammation and oxidative stress.

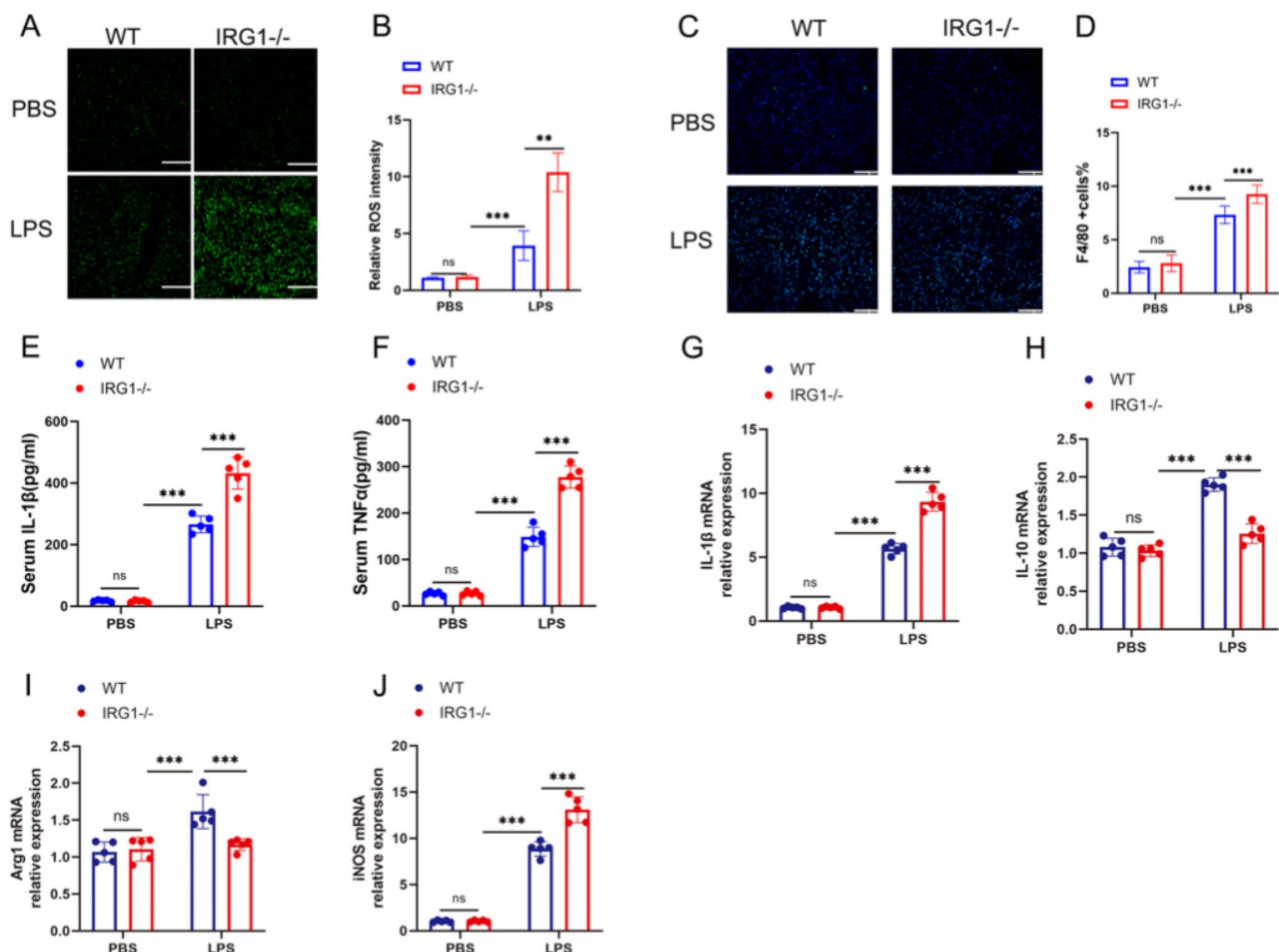

Fig. 3 IRG1 deficiency aggravates cardiac inflammation and oxidative stress. (**A**–**B**) Dihydroethidium staining was used to evaluate cardiac oxidative stress and the relative ROS intensity was calcuated. (**C**–**D**) Immunofluorescence staining for F4/80 in the hearts of each group ($n=4$; scale bar, 50 μm) (**E**–**F**) IL-1β and TNF-α serum levels were assessed in each group ($n=5$) . (**G**–**J**) RT-PCR analysis of IL-1β, IL-10,Arg1 and iNOS mRNA expression in each group ($n=5$). * $p<0.05$, ** $p<0.01$, *** $p<0.005$

IRG1 deficiency augments cardiac inflammation in SIMD via promoting macrophages towards a proinflammatory phenotype

Since macrophages play an important role in SIMD, we investigated the functions of macrophages present within the heart by FACS sorting. The gating strategy in shown in Fig. 4A. We found increased macrophage accumulation in cardiac from IRG1$^{-/-}$ mice after LPS injection, which was accompanied by a higher level of M1 macrophage markers, which included iNOS, IL-6, IL-1β, and TNF-α (Fig. 4B-E) and lower level of M2 macrophage markers, which including CD206, Arg1, IL-10 and TGF-β (Fig. 4F-I). Moreover, the percentage of MHC II+macrophages increased (Fig. 4J-K), whereas the percentage of CD163+macrophages decreased after IRG1 knockout (Fig. 4L-M), which suggested that IRG1 deficiency can skew macrophages towards a pro-inflammatory phenotype.

IRG1 deficiency aggravated Ly6Chigh monocyte recruitment from the spleen

Because the pathogenesis of SIMD is associated with immune infiltration of the myocardium by peripheral inflammatory cells, we detected the monocytes in the blood and heart. We found that both the percentage of CD11b+Ly6C^{high} monocyte and CD11b+Ly6C^{low} monocyte increased in the blood and heart of IRG1$^{-/-}$ mice (Fig. 5A-E). As monocyte-dominated immune cells migrate from the spleen and bone marrow to the heart in ischemic heart disease and myocarditis [29, 30], we measured the percentage of monocytes in SIMD and mainly focused on their inflammatory state and origins. Based on the results, in comparison to WT-LPS mice, neither the percentage of Ly6C^{high} monocytes nor Ly6C^{low} monocytes changed significantly in the bone marrow from IRG1$^{-/-}$ mice after LPS injection (Fig. 5F-H). We then assumed that the spleen may be involved in the inflammation of the cardiac. By performing flow cytometry, we found that IRG1 deficiency aggravated Ly6C^{high} monocyte recruitment from the spleen because the percentage of Ly6C^{high} monocyte decreased in spleen of IRG1$^{-/-}$ mice after LPS injection (Fig. 5I-J). However, the number of Ly6C^{low} monocytes was not significantly different among the groups (Fig. 5K). Taken together, these findings suggested that IRG1 deficiency aggravated cardiac inflammation partially by promoting Ly6C^{high} monocyte recruitment from the spleen to the heart.

Itaconate ameliorates cardiac injury and inflammation in sepsis-induced cardiac dysfunction

After establishing that IRG1 plays a protective role in SIMD, we investigated whether exogenous administration of 4-OI, a new synthesized cell-permeable itaconate derivative, can also protect cardiac in SIMD by regulating macrophage inflammation in our in vivo and in vitro models. Administration of 4-OI significantly improved

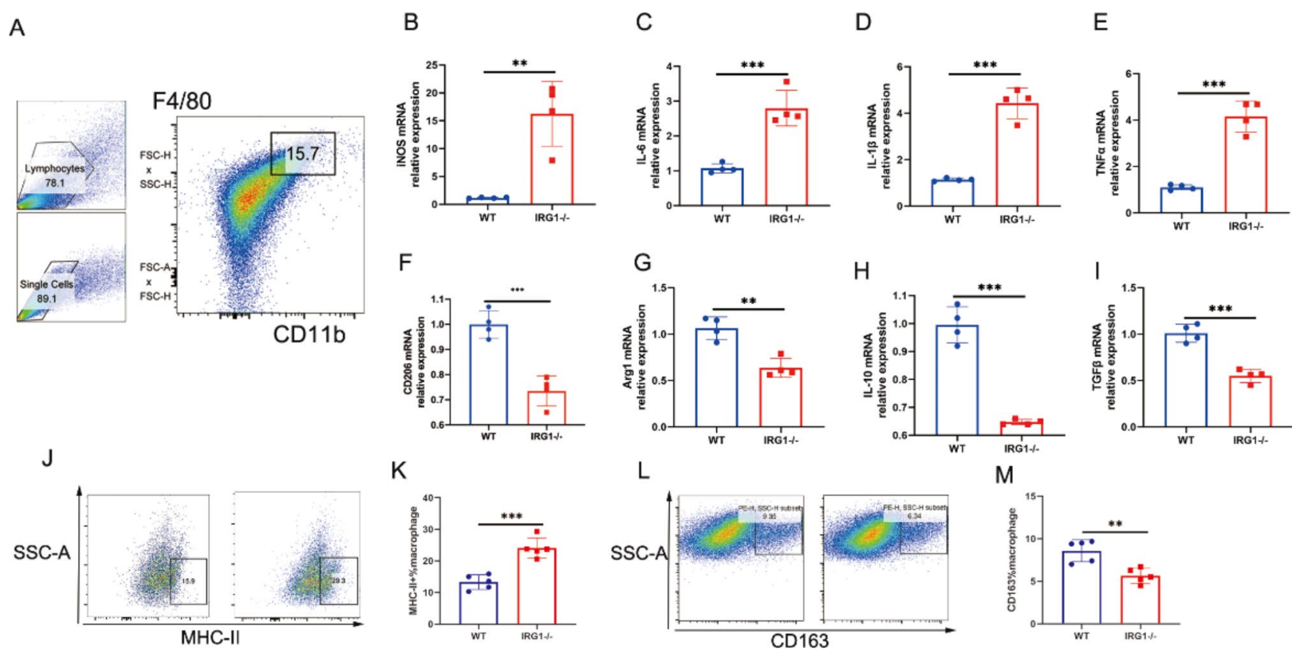

Fig. 4 IRG1 deficiency augments cardiac inflammation in SIMD via promoting macrophages towards a proinflammatory phenotype. (**A**) Gating strategy of macrophages. (**B**–**I**) RT-PCR analysis of M1 macrophage marker (iNOS, IL-6,IL-1β, TNF-α) and M2 macrophage marker (CD206, Arg1, IL-10 and iNOS) mRNA expression in each group ($n=4$). (**J**–**K**) cardiac cell was stained with CD11b and MHC-II for flow cytometry analysis and quantification results was shown ($n=5$ in each group) (**L**–**M**) cardiac cell was stained with CD11b and CD163 for flow cytometry analysis and quantification results was shown ($n=5$ in each group). * $p<0.05$, ** $p<0.01$, *** $p<0.005$

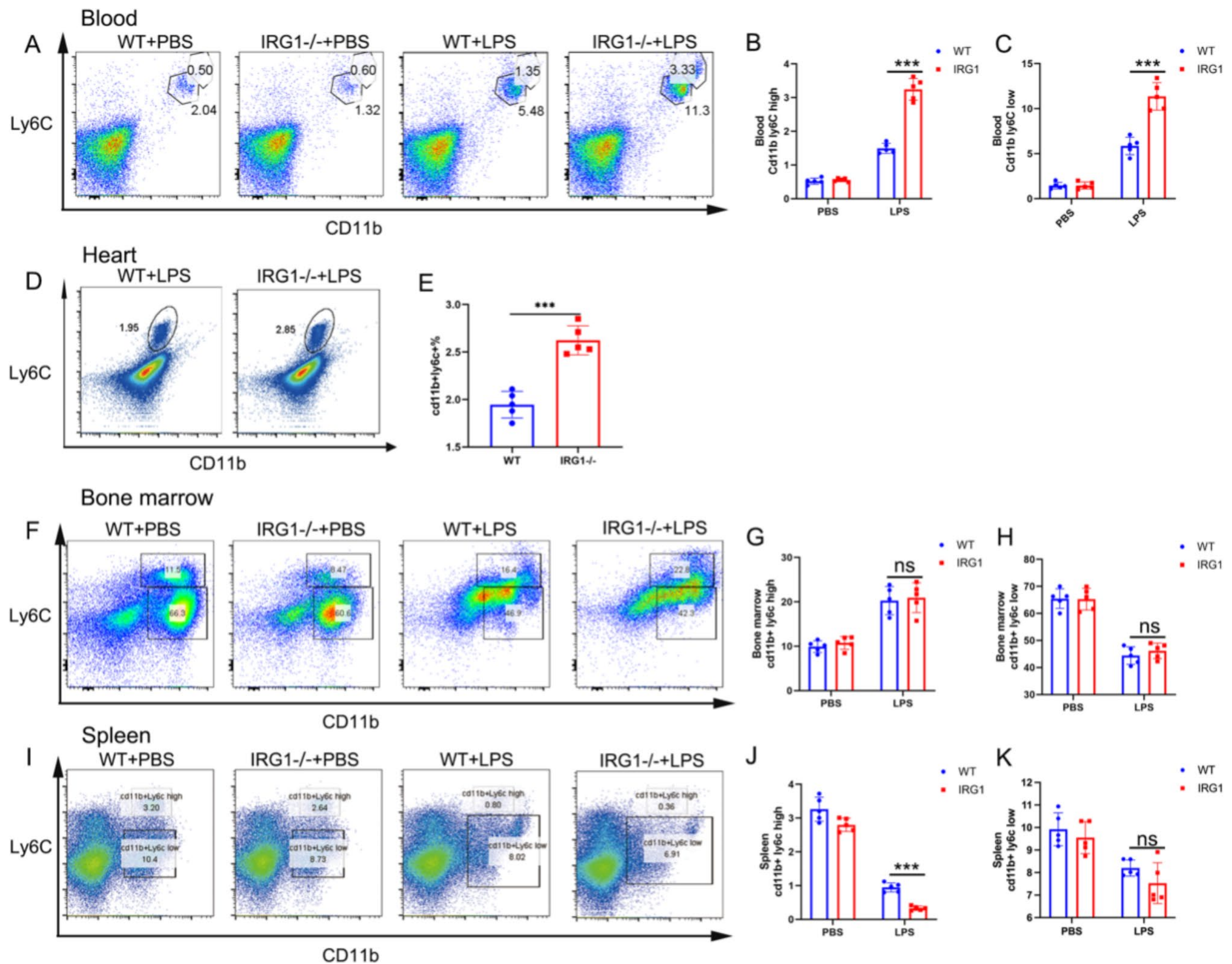

Fig. 5 IRG1 deficiency aggravated Ly6C^{high} monocyte recruitment from the spleen. (**A**) Representative flow cytometry plots showing the quantification of cells within (**B**–**C**) peripheral blood and (**D**–**E**) heart tissues. (**F**–**H**) Representative flow cytometry plots showing the quantification of CD11b+Ly6C^{high}and CD11b+Ly6C^{low} cells within bone marrow. (**I**–**K**) Representative flow cytometry plots showing the quantification of CD11b+Ly6C^{high}and CD11b+Ly6C^{low} cells within spleen. * $p<0.05$, ** $p<0.01$, *** $p<0.005$

the survival rate and cardiac function in SIMD mice as shown in longer survival time (Fig. 6I) and improved LVEF, LVFS and LVID; s (Fig. 6A-D). Similarly, 4-OI treatment reduced cardiac injury as determined by lower serum LDH and CK-MB levels (Fig. 6E-F). Furthermore, the systolic pressure and diastolic pressure was increased after 4-OI treatment in SIMD (Fig. 6G-H), indicating the improvement of cardiogenic shock. Consistent with the in vivo results, 4-OI significantly downregulated the expression of inflammatory genes in RAW264.7 cells and M1 macrophages in bone marrow-derived macrophages (BMDM) (Fig. 7A-D). Likewise, the levels of M1 macrophage markers were detected by flow cytometry, and the proportion of CD38+cells decreased significantly after 4-OI treatment, whereas the proportion of CD206+cells increased (Fig. 7E-G). Furthermore, 4-OI treatment attenuates macrophage ROS production in vitro as it shown by DCFH intensity (Fig. 7H-I).

Itaconate regulates the polarization of macrophages through NRF2

As itaconate was reported to promote nuclear factor (erythroid-derived 2)-like 2 (*NRF2*), which was reported to regulate macrophage polarization [18, 19], we determined whether 4-OI could regulate macrophage polariztion through NRF2 in vivo. We found that the 4-OI significantly augmented the expression level of NRF2-related genes and the protein level increased after 4-OI administration (Fig. 8A-C). Furthermore, the mRNA expression of M1marker (iNOS, IL-6, IL-1β, and TNF-α) increased after NRF2 silencing (Fig. 8D). As macrophage related inflammation promoted myocardial cell apoptosis, we co-cultured LPS-activated BMDM with neonatal

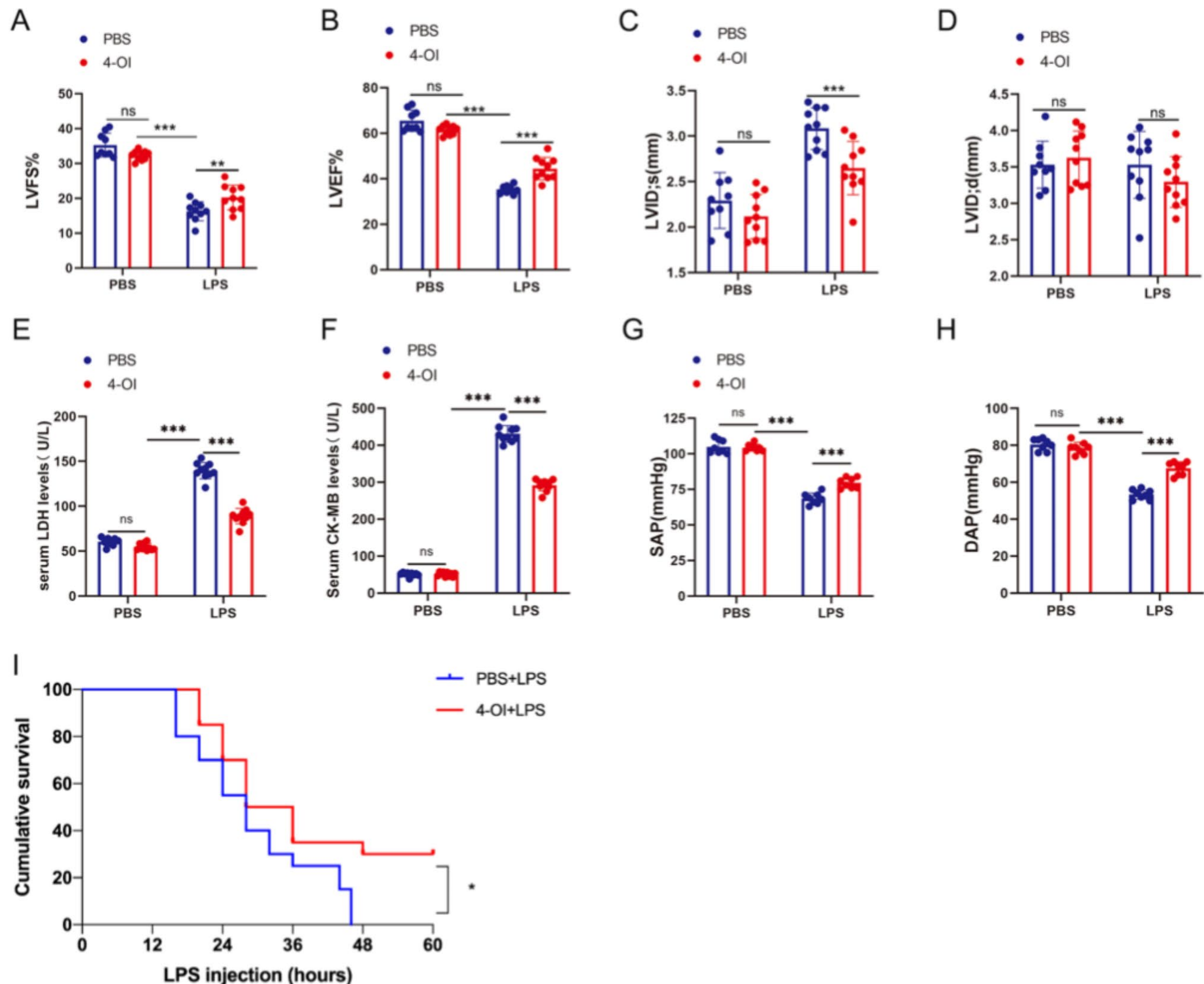

Fig. 6 Itaconate ameliorates cardiac injury and inflammation in sepsis-induced cardiac dysfunction. (**A**–**D**) Echocardiographic analysis of LVEF, LVFS, LVID; s and LVID; d in each group ($n = 10$). (**E**–**F**) The plasma levels of CK-MB and LDH were measured in each group ($n = 8$). (**G**, **H**) Systolic pressure and diastolic pressure in each group ($n = 10$) (**I**) Effect of itaconate adimisteration on the survival rate after LPS treatment ($n = 20$). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$

myocardial cells. Our results show that LPS-activated BMDM promoted neonatal myocardial cells apoptosis, while administration of 4-OI with BMDM protected myocardial cell from apoptosis. However, gene inhibition of NRF2 through NRF2 siRNA negated the protective effect of 4-OI/itaconate. Taken together, our findings suggested that itaconate regulated macrophage polarization through NRF2.

Discussion

Sepsis is a life-threatening organ dysfunction caused by a dysregulated host response to infection. It is often accompanied by septic cardiac dysfunction, which increases mortality and limits effective treatment. In this study, we showed that itaconate acid is increased in cardiac tissues during sepsis. We found that IRG1 knockout aggravated cardiac dysfunction during sepsis, whereas exogenous 4-OI supplementation can restore cardiac function and decrease systemic inflammatory response during sepsis. Moreover, we also found that IRG1 knockout promoted the polarization of cardiac macrophages towards the M1 phenotype along with promoting splenic monocyte recruitment. Our results suggested that 4-OI can be a potential therapeutic target for the treatment of SIMD.

Sepsis-induced myocardial dysfunction (SIMD) is characterized by impaired myocardial contractility and/or diastolic dysfunction caused by sepsis [31]. Currently, there is no clear definition or unified diagnostic criteria for SIMD in clinical practice. In the early stages of sepsis, the clinical manifestations are mainly those of a hyperdynamic, low-resistance “warm” shock, characterized by an increased left ventricular ejection fraction, increased cardiac output, and reduced peripheral capillary resistance, among other hyperdynamic signs; if not treated promptly and effectively,

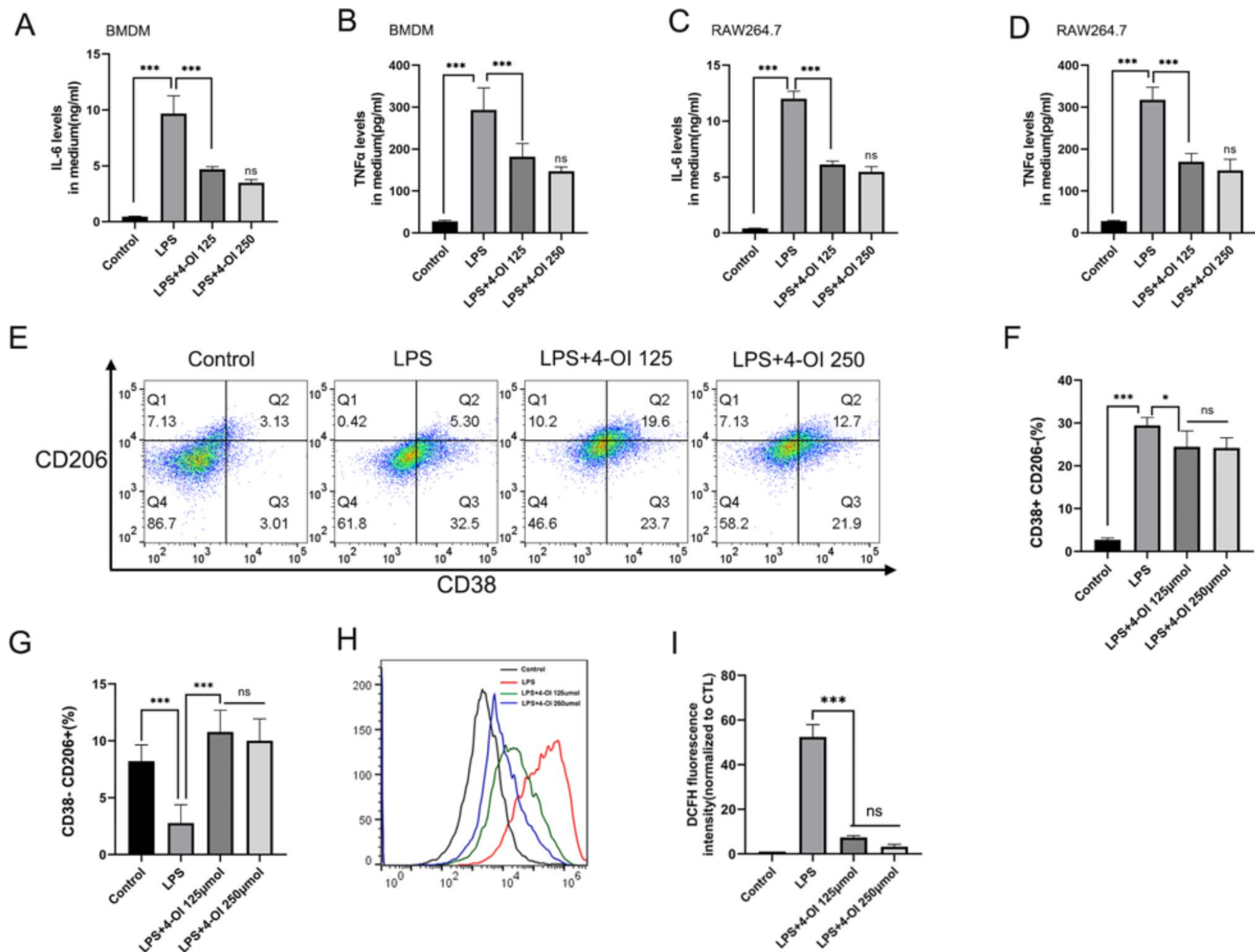

Fig. 7 Itaconate promote macrophage towards M2 phenotype in vitro. (**A**–**D**) RAW264.7 and BMDM was treated with 4-OI and LPS for 12 h. IL-6 and TNF-α level in the medium was assessed in each group. (**E**–**G**) The BMDM was stained with CD206 and CD38 for flow cytometry analysis. Quantification results for (**F**) CD38 positive macrophages and (G) CD206 positive macrophages. (**H**–**I**) BMDM was stained with DCFH for flow cytometry analysis. Quantification results for (**I**) relative DCFH intensity was shown
 * $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$

it will gradually progress to a low-output "cold" shock, characterized by a decreased left ventricular ejection fraction, decreased cardiac output, left ventricular dilation, and increased peripheral small vessel resistance, among other hypodynamic phenotypes. In addition, studies have also found that in patients with septic shock, a subset of patients may have right ventricular systolic or diastolic dysfunction [32]. Therefore, sepsis-induced myocardial dysfunction is a form of global cardiac dysfunction caused by sepsis. Our findings revealed a reduced cardiac ejection fraction in mice with sepsis-induced myocardial dysfunction which is similar to previous studies.

IRG1 links metabolism with inflammation by catalyzing endogenous itaconate production. Previous studies found that itaconate can be detected in the plasma of patients with inflammatory diseases, such as rheumatoid arthritis [33], and the rejection of allogeneic kidney transplantation [34]. However, itaconate was also decreased in some patients with autoimmune diseases such as systemic lupus erythematosus [35]. Over the last few decades, studies have shown that IRG1 can regulate inflammatory responses in various systems. Zhongjie Yi et al. reported that IRG1 deficiency can aggravate hepatic ischemia and reperfusion by suppressing the Nrf2 antioxidative response in hepatocytes [36]. Similarly, Alexander Hooftman reported that IRG1 can suppress macrophage inflammasome formation through modified NLRP3[21]. On the contrary, other reports showed that IRG1 can augment the bactericidal activity of macrophages and promote mitochondrial ROS production [37]. These results suggested that IRG1 can play dual or distinct roles in different inflammatory cells in different acute inflammatory settings.

Therefore, we investigated the role of IRG1 in LPS-induced acute cardiac inflammation. Interestingly, we found that IRG1 expression is remarkably increased in the cardiac of SIMD mice compared with that of the controls,

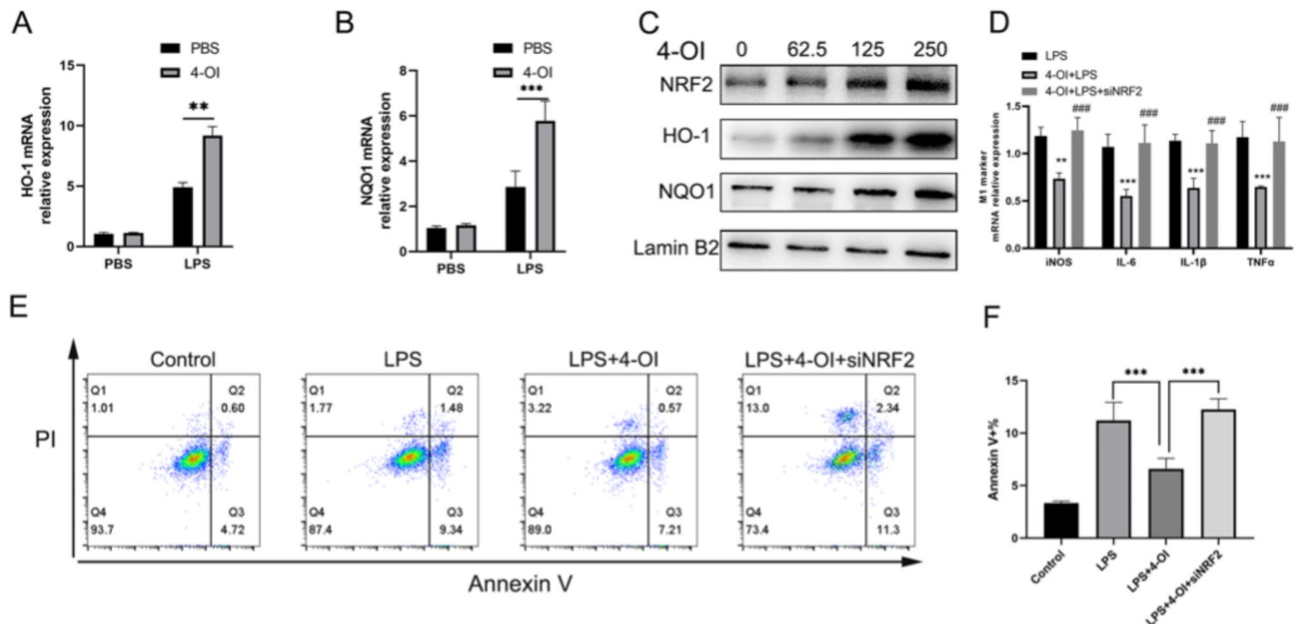

Fig. 8 Itaconate regulates the polarization of macrophages through NRF2. (**A**–**B**) BMDM was treated with LPS and itaconate, the relative expression of HO-1 and NQO1 was assessed by RT-PCR. (**C**) BMDM was treated with LPS and itaconate, the nuclear protein level of NRF2, HO-1 and NQO1 was assessed by western blotting. Lamin B2 was used as an internal control for nuclear protein; (**D**) BMDM was treated with siNRF2 and 4-OI, the relative expression of iNOS, IL-6, IL-1β, TNF-α was assessed by RT-PCR. (**E**) cardiac cell was co-culture with macrophages in each group, followed by stained with annexin V and PI for flow cytometry analysis. Quantification results for (**F**) Annexin V postive cell was shown.* $p<0.05$, ** $p<0.01$, *** $p<0.005$

which has not been reported in previous studies to the best of our knowledge. We also found that the metabolite product of IRG1, itaconate, is highly increased. This suggested that IRG1 gene expression can be increased during pathophysiological processes such as inflammation and oxidative stress. Furthermore, we found that IRG1 is highly increased in SIMD and acts as an innate immune suppressor which protects the body from hyperactive inflammatory responses and oxidative stress. In the $IGR1^{-/-}$ mice, the cardiac function and average survival time were significantly worsened. Meanwhile, the serum proinflammatory cytokines, such as IL-1β, TNF-α, and oxidative stress, increased significantly, which suggested increased inflammatory activity in the absence of IRG1. To conclude, the loss of IRG1 aggravates myocardial inflammation.

Macrophages are central mediators of cardiac inflammation and are involved in the initiation and resolution of cardiac inflammation [38, 39]. Studies have proven the infiltration of macrophages in the sepsis-induced cardiac dysfunction [40, 41]. The activated macrophages can secrete large amounts of inflammatory factors, such as NO, TNF-α and IL-1β, which participate in the inflammatory response and also aggravate tissue injury [10]. In the early stage of sepsis, macrophages can be hyperactivated and undergo M1 differentiation, leading to the overproduction of proinflammatory cytokines, which is considered to be the main cause of high mortality in sepsis [42, 43].Therefore, modulating the balance of M1 and M2 macrophages is an attractive approach to mitigate sepsis-induced cardiac damage [44–46].In this study, we evaluated the levels of inflammatory markers in the blood and cardiac in an LPS-induced sepsis model. We found that IRG1 deficiency promoted the polarization of cardiac macrophages to M1 phenotype because the F4/80 cd11b+macrophages separated from cardiac had higher expression of M1 markers, such as IL-6, iNOS, IL-1β and TNF-α. Meanwhile, flow cytometry showed an increased percentage of MHC+macrophages in the cardiac of $IRG1^{-/-}$ mice. Thus, IRG1 dampened the cardiac inflammation partially by suppressing regulating macrophage polarization.

As circulating $Ly6C^{high}$ monocytes are direct precursors of lesional macrophages and are recruited from the periphery when inflammation occurs in myocardial tissue, which further differentiates into cardiac proinflammatory macrophages [47, 48]. We determined the $Ly6C^{high}$ monocytes in the blood and heart. Based on our results, we found a higher ratio of $Ly6C^{high}$ monocytes in the blood and heart of $IRG1^{-/-}$ mice. Thus, we further investigated its organ origins. Bone marrow and spleen are the two main peripheral reserve pools of monocytes during inflammation [49, 50]. By detecting the ratio of bone marrow and spleen monocytes, we found that the proportion of spleen $Ly6C^{high}$ monocytes was significantly decreased after IGR1 knockout, whereas the proportion of $Ly6C^{high}$ monocytes in bone marrow was unchanged. Our results suggested that IRG1 deletion can regulate splenic $Ly6C^{high}$ monocyte mobilization during SIMD and accelerate the infiltration of peripheral monocytes into the myocardium. However, this can be

due to the mobilization of mature monocytes in the bone marrow and the differentiation and maturation of myeloid precursor cells during inflammation, which resulted in relatively insignificant changes after IRG1 knockout.

Itaconate, a metabolite of the TCA cycle, has recently showen protective properties in a number of inflammatory pathologies such as ischemia-reperfusion [51], abdominal aortic aneurysm [52], and doxorubicin-induced myocardial injury [23]. We determined whether an exogenous supplement of itaconate can protect the cardiac from SIMD. Consequently, cardiac function and mortality were significantly improved by itaconate in vivo. Furthermore, macrophage inflammation was significantly reduced in response to LPS stimulation in vivo. Thus, the decreased cardiac inflammation after the itaconate supplement was partly attributed to the decreased proinflammatory capability of macrophages. NRF2 was reported to regulate the progression of oxidative stress and macrophage polarization [53, 54]. Furthermore, Evanna L Mills et al. reported that itaconate can activate NRF2 via the alkylation of KEAP1[19]. Our findings suggest that itaconate can regulate macrophage inflammation by activating NRF2, which further promotes macrophage polarization.

The present study has certain limitations. First, we established an animal SIMD model by the intraperitoneal injection of LPS, which has been used widely [55, 56]. More models should be established in different ways to determine the IRG1 in SIMD such as caecal ligation and punctur surgery. Secondly, $IRG1^{-/-}$ mice were used to establish the SIMD model in our experiment. However, the macrophage-specific knockout of IRG1 can provide more definitive evidence on IRG1 and macrophage. Our model can cause limitations for further research. Besides, SIMD is an acute inflammation and some other inflammatory cells, such as neutrophils, T lymphocytes and cardiac fibroblasts, are also involved besides macrophages [57–59]. The regulation of IRG1 on different immune cells requires further investigation. Finally, by either gene editing or drug intervention, the change of itaconate levels in cardiac occurred before SIMD. Thus, the role of itaconate intervention after sepsis stills requires further investigation.

Conclusion

Itaconate regulates the secretion of inflammatory cytokines of monocytes/macrophages by regulating the NRF2-HO-1 signaling pathway, promoting the transformation of cardiac macrophages towards M2 type during SIMD, inhibiting the mobilization of peripheral monocytes, and inhibiting the myocardial inflammatory response. This reduces myocardial inflammation during SIMD. This study provides a new therapeutic approach for SIMD.

Abbreviations

IRG1	Immune response gene 1
SIMD	Sepsis-induced cardiac dysfunction
4-OI	4-octyl itaconate
NRF2	Nuclear Factor erythroid 2-Related Factor 2
qPCR	Quantitative real–time polymerase chain reaction
LPS	Lipopolysaccharide
TCA	Tricarboxylic acid
LVID;d	Left ventricle internal end-diastole diameter
LVID;s	Left ventricle internal end-systolic diameter
BMDM	Bone marrow-derived macrophages

Supplementary Information

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Supplementary Material 1

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Author contributions

SS and JL：Conceptualization, Methodology and Writing- Original draft preparation ; ZW: Software; YL: Formal analysis ; LK, RG: Supervision ; XS, YQ: Resources ; QL and BX：Writing- Reviewing，Editing and Funding.

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Data availability

The datasets used and/or analyzed during the present study are available from the corresponding author upon reasonable request.

Declarations

Ethics approval and consent to participate

This study was approved by the Ethics Committee of Nanjing Drum Tower Hospital, affiliated hospital of Nanjing university medical school(2020AE01065).

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

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