Cardiac fibroblasts pyroptosis mediated by NLRP3/Caspase-1/GSDMD-N pyroptotic pathway produces CXCL4 via Wnt/β-Catenin signaling and induces cardiac fibrosis

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Abstract

Severe myocarditis is often accompanied by cardiac fibrosis, but the underlying mechanism has not been fully elucidated. NOD-like receptor protein 3 (NLRP3) inflammation, closely related to apoptosis pyroptosis, participates in the development of myocarditis. Inhibiting pyroptosis mediated by NLRP3 inflammasome can reduce cardiac fibrosis, although its exact mechanism remains unknown. In this study, we induced Viral myocarditis (VMC) via infection of CVB3 to explore the relationship between pyroptosis and fibrosis. Our results showed that intraperitoneal injection of an NLRP3 inhibitor MCC950 or use of NLRP3−/− mice inhibited cardiac pyroptosis mediated by NLRP3 inflammasome in VMC. CXCL4 is a chemokine that has been reported to have pro-inflammatory and pro-fibrotic functions. In VMC, we further found that pyroptosis of Mouse myocardial fibroblasts (MCF) promoted the secretion of CXCL4 by activating Wnt/β-Catenin signaling. Subsequently, the transcriptome sequencing data showed that CXCL4 could promote cardiac fibrosis by activating PI3K/AKT pathway. In summary, infection of CVB3 induced host oxidative stress to further activate the NLRP3 inflammasome and ultimately lead to heart pyroptosis, in which MCF secreted CXCL4 by activating Wnt/β-Catenin signaling and CXCL4 participated in cardiac fibrosis by activating PI3K/AKT pathway. Therefore, our findings revealed the role of CXCL4 in VMC and unveiled its underlying mechanism. CXCL4 appears to be a potential target for the treatment of VMC.

1. Introduction

VMC is a heart disease mainly caused by CVB3 (Coxsackie B Type 3) virus infection. In the early stages of infection, inflammatory cells infiltrate the heart, and in the later stages of the disease, myocardial fibrosis can occur. VMC is the main cause of sudden cardiac death in children and adolescents, which poses a major threat to human health[1, 2]. Although the prognosis of VMC patients is generally good, the disease is likely to develop into dilated cardiomyopathy, leading to heart failure and even sudden death. A large number of articles have been published on VMC, but its specific pathogenesis and effective treatment methods are still unclear. Studies have shown that maladaptive immune responses are an extremely critical participant in the occurrence of VMC, so the immune mechanism of VMC still needs further research.

When pyroptosis occurs, inflammasomes are formed, Gasdermin and Caspase are activated, and inflammatory cytokines are released. NLR protein-3 (NLRP3) inflammasome is one of the most extensively studied inflammasomes. Damage-associated molecular patterns (DAMPs) or pathogen-associated molecular patterns (PAMPs) can activate NLRP3 inflammasome composed of NLRP3, ASC and pro-caspase-1. When pyroptosis occurs, pores form in the plasma membrane and the volume of the cell expands until the membrane ruptures, eventually leading to a variety of leakage of cytoplasmic contents. Pyroptosis usually has two main ways, namely the classical ways and the non-classical ways[3]. In the classical pathway, DAMPs and PAMPs trigger aggregation of inflammasomes and activation of Caspase-1[4]. In addition, Caspase-1 also can cleave and activate inflammatory cytokines such as IL-18 and IL-1β, thereby enhancing the inflammatory response. [5]. However, the exact mechanism by which pyroptosis regulates cardiac fibrosis is still unknown.

CXCL4 is a multifunctional chemokine, also known as platelet factor 4, was first discovered by Deutsch et al in 1955. As research continues to deepen, researchers have found that CXCL4 is secreted by a variety of immune cells, such as mast cell, activated T cells and monocyte, and has the function of regulating immune response, angiogenesis, endothelial cell activation, proliferation and migration[6–8]. CXCL4 has a strong pro-inflammatory and pro-fibrotic effect, which has been proved to be up-regulated in inflammatory diseases including Rheumatoid arthritis, Atherosclerosis and myocardial infarction. In addition, CXCL4 is upregulated in fibrotic diseases including chronic liver fibrosis, primary bone marrow fibrosis, and systemic sclerosis[9, 10]. Several studies have shown that CXCL4 is closely related to the occurrence and development of cardiovascular diseases, which is a serious threat to the health of the heart. Therefore, we hypothesize that VMC induced pyroptosis promotes cardiac fibrosis by inducing the production of CXCL4.

In conclusion, the purpose of this study was to investigate the effect of pyroptosis on VMC and to further study the mechanism of pyroptosis regulating cardiac fibrosis, providing a new direction for the treatment of myocarditis.

2. Material and Methods

2.1 Mice

Male BALB/c (6–8 weeks) mice were obtained from Kavins Laboratory Animal Company (Changzhou, China). All mice housed in the Animal Center of Nanjing Medical University in compliance with the guidelines outlined in the Guide for the Care and Use of Laboratory Animals.

2.2 CVB3 infection and drug treatment

CVB3 virus (Nancy strain) was maintained through Hela cells (ATCC number: CCL-2). Mice were infected with CVB3(100TCID50, 200 µL) via intraperitoneal (i.p.) injections. MCC950 (MCE, China) were given to VMC mice by intraperitoneal (i.p.) injection every day after the infection, 10mg/kg/d[11]. LY294002 (MCE, China) were given to VMC mice by intraperitoneal (i.p.) injection every day after the infection, 0.3mg/kg/d.[12] Seven days later, the hearts and serum were collected for the experiment.

2.3 Cell culture
MCF were purchased from BNCC (Beijing, China) and were cultured with DMEM medium (Gibco) containing 10% FBS in a 5% CO₂ incubator. The 3–6 passages of cells were used for experiments. Cells were stimulated with AngII (10µM, R&D Systems) for 24 h. The 10µM MCC950 were added for 2 hours prior to the stimulation with AngII.

2.4 Quantitative RT-PCR

A RNA extraction kit (Invitrogen) was used to extract total RNA from cells and tissues. Purified RNA was reverse transcribed into cDNA, then amplified by SYBR-Green master mix kit. The relative expression of genes was calculated by the $2^{-\Delta\Delta Ct}$ method. Primer sequences were showed in Table 1.

<table>
<thead>
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<th>Target DNA</th>
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<td>β-actin</td>
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2.5 Western blot

RIPA buffer was used for preparing whole cell lysates. Protein was separated by SDS-PAGE and transferred to PVDF membranes (Millipore). 1% BSA was added in order to block the membranes. Then, the membranes were washed three times with TBS-0.1% Tween 20 (TBST). The membranes were incubated primary antibodies at 4°C overnight. The primary antibodies as followed anti-NLRP3 (1:1000, Abcam), anti-Gasdermin D-N-terminal (1:1000, Invitrogen), anti-caspase-1 (1:1000, Invitrogen), anti-cleaved caspase-1 (1:1000, Invitrogen), anti-GAPDH, Collagen-I (1:1000, Immunoway), Collagen-III (1:500, Proteintech), α-SMA (1:3000, Proteintech), anti-actin (1:5000, Bioworld), anti-β-catenin (1:5000, Proteintech), anti-CXCL4 (1:3000, Proteintech). And then, incubated with secondary antibodies (1:3000; Proteintech, America) at room temperature for 1–2 h. Finally, the antigen-antibody reactions were visualized by chemiluminescence (ECL) kit, and the intensity of protein bands was quantified by using ImageJ software.

2.6 ELISA

AngII, IL-1β, CXCL4 levels were quantified in serum or culture media according to the manufacturer's instructions. The results were normalized to the control condition. Data were expressed as a fold change relative to the control conditions.

2.7 ROS detection

DCFH-DA was diluted in serum-free medium at 1:1000 to a final concentration of 1 mmol/L. The cells were placed in diluted DCFH-DA and incubated in a cell incubator at 37°C for 25 min. When the time was up, cells were washed three times with serum-free cell culture solution to fully remove DCFH-DA that had not entered cells.

2.8 Immunofluorescence staining

The mice were sacrificed by neck breaking, and the middle heart tissue was selected to make paraffin sections. Then, the prepared paraffin sections were placed in oven at 60°C and waited for 1h. The slices were then soaked with xylene for 2 times, about 15min each time. After that, the slices were immersed in gradient anhydrous ethanol (100%, 95%, 85%, 70%) for one time. 6min each time. Subsequently, put the hydrated slices into 10mM citrate buffer in advance. boil for 10min, take out and cool for about 30min naturally; Weigh 0.05g BSA and add it into 1mL PBS solution to prepare 5%BSA, then drip it into the tissue on the section and wait for 1h. Incubation of primary antibody, and incubated overnight at 4°C. The primary antibody was discarded and washed with PBS for 3 times, for about 10 minutes each time. Secondary antibody (1:200) was added and incubated for 1h at room temperature and away from light. The secondary antibody was recovered and cleaned with PBS for 3 times, 10 minutes each time. Diluted DAPI was added and incubated in the dark for about 15 minutes.

2.9 Histopathological examination of the heart

Mice heart tissues were fixed in 4% polyformaldehyde and embedded in paraffin. And then, stained with hematoxylin-eosin (H&E). H&E staining was used to analyze the level of inflammation under a microscope in random order.

2.10 Masson staining

Mouse heart tissues were embedded in paraffin, and 7 µm sections were obtained for staining. The standard procedures for the Masson's staining were performed.
2.11 Immunohistochemistry (IHC)

Heart tissues were fixed and embedded in paraffin, and analyzed by immunohistochemical analysis. Heart tissues were incubated with anti-CXCL4 and c-Myc (1:500; Proteintech) at 4°C overnight. Then, heart tissue was incubated with biotin-labeled secondary antibodies. The immunoreaction signal was developed with DAB staining. Finally, sections were viewed under a light microscope.

2.12 Examination of myocardial markers

The levels of lactate dehydrogenase (LDH), creatine kinase (CK-MB) and glutamic oxalacetic transaminase (AST) in serum were measured using detection kit.

2.13 Transfections

INTERFERin® (Polyplus) were performed according to the manufacturer’s instructions within the recommended reagent/siRNA ratio range. MCF cells were plated at a density 5×10^4 cells/well in 24 well plates one day prior to transfection with siRNA against CXCL4 at a final concentration of 50 nM/well.

2.14 PCR Array

Gene expression profiles were analyzed using the Mice Targets of Pyroptosis Signaling Related Gene qPCR Array (Wgene Biotech, Shanghai, China) according to the manufacturer’s protocol. Data were analyzed using Wgene Biotech software (http://www.wgenre.com). Genes with fold-changes greater than 2.0 or less than −2.0 were considered to be of biological significance. The genes are listed in Table 2.

Table 2

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2.15 Statistical Analysis

All statistical analysis were performed using GraphPad Prism 8.0 software. Data are presented as mean ± SD. A t test was used to compare the data between two groups and the differences between multiple groups were analyzed via a one-way analysis of variance. *P < 0.05 was considered as statistically significant.

3. Results

3.1 Cardiac pyroptosis occurs in VMC by activating NLRP3 inflammasome

VMC is a common heart disease, which can cause the infiltration of inflammatory cells and the damage of the heart tissue. Pyroptosis is a kind of inflammatory cell death, which can lead to a strong inflammatory response and participate in the occurrence and development of a variety of cardiovascular diseases[13]. Despite research have shown, pyroptosis of the heart occurs in CVB3-induced myocarditis, the involvement of NLRP3-mediated classical pyroptosis pathway in myocarditis has not been fully established[14, 15]. Our data showed that intraperitoneal injection of MCC950, an NLRP3 inhibitor, resulted in decreasing protein expression of NLRP3, GSDMD, GSDMD-N, Caspase-1, Cleaved Caspase-1, pro-IL-1β and IL-1β compared to the VMC group (Figure.1A). Results of qRT-PCR also showed that genes expression of NLRP3 and IL-18 was significantly inhibited by MCC950 treatment (Figure.1B-C). In addition, the condition of pyroptosis was assessed using ELISA assay, and showed that levels of serum IL-1β was increased in the CVB3 group, but this effect was reversed by MCC950 treatment (Figure.1D). These results showed that the heart occurs pyroptosis through the NLRP3 signaling pathway in VMC (Figure.1A-D). Further research found that the infiltration of inflammatory cells in the heart tissue of VMC + MCC950 group decreased significantly by observing results of HE staining (Figure.1E). Moreover, the MCC950-treated group decreased the levels of serum CK, AST, LDH, suggesting that pyroptosis is involved in cardiac injury caused by VMC and depends on...
activation of NLRP3 inflammasome (Figure.1F-H). In addition, the levels of ROS also decreased in VMC + MCC950 group. The relationship between pyroptosis and myocarditis was further investigated using NLRP3\(^{-/-}\) mice, and the results were consistent with MCC950 treatment (Figure.S1).

3.2 Increased AngII in VMC can cause oxidative damage and pyroptosis of MCF.

Although cardiac pyroptosis is involved in the development of VMC, its mechanism is still not fully understood. The heart is composed of a variety of cells, mainly including myocardial cells, MCFs, and macrophages[16, 17]. The most obvious pyroptosis phenomenon was found in MCFs by infecting cardiomyocytes, macrophages, and MCFs with CVB3 virus in vitro (Figure.S2). In addition, Immunofluorescence detection showed that the expressions of Caspase-1, TUNEL and Vimentin were significantly increased in the heart tissue of the VMC group, while these changes were reversed by MCC950 treatment. This result indicated that pyroptosis of MCF occurred in VMC (Figure.2A). Angiotensin II (AngII) is a regulator of heart function, of which content is elevated in various models of heart disease[18, 19]. Studies have shown that AngII can induce pyroptosis, which can be speculated to be involved in cardiac pyroptosis mediated by MCF. The level of serum AngII in mice was detected by ELISA, and the result showed that the level of AngII in VMC group was significantly increased (Figure.2B). In vitro, the data showed that the release of AngII in the supernatant of MCFs infected with CVB3 increased (Figure.S2). Next, we will investigate the effect of AngII on cardiac cell pyroptosis. AngII treatment of MCF cause significant pyroptosis in MCFs rather than macrophages and cardiomyocytes (Figure.2G, Figure.S2). In addition, further study showed that compared with control group, AngII treatment led to the increase of MDA content, ROS and LDH, the decrease of SOD activity (Fig. 2.C-F). In addition, AngII treatment can promote gene expression of NLRP3 and IL-18 and secretion of IL-1β in MCFs (Fig. 2.H-J). Together, these results suggest that increased AngII in VMC can cause oxidative damage and pyroptosis of MCF.

3.3 The pyroptosis of MCF promotes the secretion of CXCL4

CXCL4 is a multifunctional chemokine that regulates immune response and participates in angiogenesis[20–22]. CXCL4 has been reported to be increased in a variety of inflammatory diseases[23, 24]. Therefore, Multiple experiments were conducted to study the relationship between pyroptosis and CXCL4 in VMC mice. Results of Western Blot showed that the protein expression of CXCL4 was significantly increased in the heart tissues of VMC mice (Figure.3A). Results of immunohistochemistry showed that the expression of CXCL4 was significantly increased in the heart of VMC mice (Figure.3B). In addition, serum CXCL4 was also significantly increased in VMC mice by ELISA (Figure.3C). However, these effects were reversed by MCC950 treatment. In vitro, the data showed that AngII could up-regulate the protein expression of CXCL4 (Figure.3D and E). Furthermore, MCC950 treatment inhibited pyroptosis of MCF and secretion of CXCL4 in vivo and in vitro (Figure.3D-H) and deficiency of NLRP3 had less pyroptosis of MCF and production of CXCL4 in vivo and in vitro (Figure.S3). Together, these evidences suggest that pyroptosis of MCF promotes production of CXCL4.

3.4 Pyroptosis of MCF induces CXCL4 production by activating the Wnt/β-Catenin pathway

In order to investigate the mechanism of CXCL4 production by pyroptosis of MCF, PCR array was used to detect the differences of MCF gene expression in Control group, AngII group, AngII + MCC950 group, and the results showed that the gene expressions of Wnt pathway related factors were significantly different as shown in the heatmap and volcano plot. The mRNA levels of Axin2 were significantly decreased, whereas c-Myc was significantly upregulated in MCF treated by AngII (Figure.4A-B). This suggests that the Wnt pathway is activated in the progress of VMC. Further analysis showed that the Wnt pathway changed significantly, so we focused on the Wnt pathway (Figure.4C). Immunohistochemical data showed that the expression of protein c-Myc in heart of VMC + MCC950 mice decreased compared to VMC mice (Figure.4E). Immunofluorescence data showed that the expression of β-Catenin was reduced in the heart sections of MCC950 + VMC mice compared to VMC mice but the expression of Axin2 was reversed (Figure.4F,G), confirming that pyroptosis of MCF may induce CXCL4 production by activating the Wnt/β-Catenin pathway in vivo (Figure.4F). Western blot analysis showed that the treatment of MCC950 can increase the expression of protein β-Catenin, c-Myc in MCF and decrease the expression of protein Axin2 in MCFs. In addition, inhibitor of Wnt pathway can decrease expression of protein CXCL4, which further verified that the pyroptosis of MCF promoted CXCL4 generation by activating the Wnt/β-Catenin pathway (Figure.4G). In a word, these results suggest that pyroptosis of MCF induces CXCL4 production by activating the Wnt/β-Catenin pathway.

3.5 Pyroptosis of MCF promotes cardiac fibrosis by producing CXCL4

Cardiac fibrosis is a common pathological phenomenon in the occurrence of myocarditis, and persistent or excessive fibrosis will cause serious adverse reactions[25–27]. Masson staining showed collagen deposition in the hearts of mice in the VMC group (Figure.5A). In addition, the fibrosis-related proteins Collagen-I, Collagen-III, α-SMA were expressed in the hearts (Figure.5B). Not only did MCC950 treatment significantly improve cardiac fibrosis, but NLRP3 knockout mouse also showed significant improvement in cardiac fibrosis (Figure.S4). CXCL4 has been reported to be involved in the development of fibrosis. Results of western blot and q-RT PCR show that CXCL4 treatment can promote the gene and protein expression of Collagen-I, Collagen-III and α-SMA in MCFs (Figure.5C-D). Therefore, we verified in vitro, whether the secretion of CXCL4 by pyroptosis promotes cardiac fibrosis. Firstly, the gene of CXCL4 was knocked down by small interfering RNA(Figure.5E). Then, pyroptosis of MCF was induced by AngII. Finally, the supernatants were used to treat MCF and CXCL4-treated group as positive control. The results showed that knockdown of CXCL4 significantly inhibited the activation of MCF (Figure.5F). This evidence suggests that pyroptosis of MCF promotes cardiac fibrosis by producing CXCL4.
3.6 CXCL4 promotes cardiac fibrosis through activating the PI3K/AKT pathway.

The PI3K/Akt signaling pathway is related to cell growth, proliferation, survival, apoptosis, reverse transcription, migration, protein synthesis, etc. The inhibition of the PI3K/Akt pathway can improve fibrosis. In order to determine the role and mechanism of CXCL4 on MCF, RNA sequencing was performed to detect gene expression differences. The sequencing results showed that CXCL4 induced a significant increase in the expression of fibrosis-related genes (Figure 6A). In addition, the expression of genes related to the PI3K-AKT signaling pathway was significantly increased (Fig. 6B). In order to verify whether CXCL4 mediates MCF activation through the PI3K-AKT signaling pathway, we treated MCF with CXCL4, which results in a transient increase in the phosphorylation on PI3K and AKT within the MCF, peaking at 30 min and 45 min, respectively (Fig. 6C). In addition, pretreatment of MCF with PI3K inhibitor LY294002 (10µM) significantly reduced the expression of fibrosis-associated proteins (Figure 6D). To further confirm in vivo that CXCL4 promotes fibrosis by activating the PI3K-AKT signaling pathway, mice were intraperitoneally injected with LY294002. The data showed that PI3K inhibitor decreased the protein expression of Collagen-I, Collagen-III, α-SMA in the heart tissue (Figure 6E-F). HE staining indicated that administration of LY294002 reduced the number of inflammatory cells in the heart (Figure 6G) and Masson staining were consistent with those phenomenon (Figure 6H). Taken together, these results suggest that CXCL4 promotes cardiac fibrosis through activating the PI3K/AKT pathway.

4. Discussion

In this study, we demonstrated that pyroptosis of MCF is involved in the development of VMC and depends on activation of the NLRP3 signaling pathway. Furthermore, We found that pyroptosis promotes the production of CXCL4 by activating the Wnt/β-Catenin pathway, which in turn promotes myocardial fibrosis by activating the PI3K/AKT pathway.

VMC is an acute inflammatory disease of the heart caused by viral infection, which is the main cause of sudden cardiac death in children and adolescents[28–30]. About 21% of patients with acute VMC will progress to dilated cardiomyopathy, leading to heart failure and even sudden death[25]. During this period, the heart occurred pyroptosis and is infiltrated by immune cells, which subsequently causes the activation of myocardial fibroblasts, leading to cardiac fibrosis[31]. However, the precise molecular mechanisms underlying the association between pyroptosis and VMC development need to be investigated further. Numerous studies have shown that the occurrence of cardiovascular diseases is often accompanied by the increase of AngII, and the level of AngII is positively correlated with cardiac fibrosis. AngII is a polypeptide produced by the hydrolysis of angiotensin by angiotensin converting enzyme. It effects cardiovascular function and is specifically involved in the course of disease. Studies have shown that AngII can cause pyroptosis of cells[32, 33]. In this study we demonstrated that during the development of VMC, elevated AngII induces pyroptosis of MCF resulting in the production of CXCL4, which subsequently activates MCF and promotes cardiac fibrosis.

Pyroptosis is a programmed necrosis of inflammatory cells mediated by inflammasome, accompanied by the release of several inflammatory cytokines including IL-1β and IL-18 from the cell[34, 35]. The inflammasome is a multiprotein complex composed of pattern recognition receptors, adaptor ASC, and Cysteine aspartic protease cysteine proteinogen-1(Caspase-1) that recognizes pathogen-associated molecular patterns (PAMPs) or host-derived danger signaling molecules (DAMPs) and activates Caspase-1[36, 37]. Activated Caspase-1 enables the precursors of IL-1β and IL-18 to transform into mature IL-1β and IL-18, and eventually these inflammatory cytokines are released into the extracellular membrane through pores formed by GSDMD-N to induce inflammatory responses. NLRP3 inflammasome is a critical component of pyroptosis induction[38]. In our study, we demonstrated that VMC can induce pyroptosis of myocardial fibroblasts by activating the NLRP3 inflammasome. Using the NLRP3 inhibitor MCC950 and NLRP3−/− mice, we further found that pyroptosis of myocardial fibroblasts is induced by activation of NLRP3 inflammasome and that inhibition of pyroptosis is beneficial for VMC. Furthermore, we also found that AngII treatment of myocardial fibroblasts can cause pyroptosis through in vitro experiment but MCC950 treatment or transfection of NLRP3 siRNA can inhibit pyroptosis. Therefore, inhibiting NLRP3 inflammasome activation may be beneficial in viral myocarditis. Our study provides new perspectives on the regulation of fibrosis in myocarditis and suggests that pyroptosis of MCF promotes myocardial fibrosis. Therefore, targeting the NLRP3 inflammasome may be a novel treatment for VMC.

In the development of myocarditis, cardiac fibrosis occurs and myocardial fibroblasts are the main cells that cause cardiac fibrosis[16, 39]. When cardiac fibroblasts are activated, they produce large amounts of collagens, and as collagen is deposited, the heart eventually develops fibrosis. Cardiac fibrosis, which causes arrhythmias and abnormal wound healing, eventually leads to heart failure, is a common pathological phenomenon of heart disease[40, 41]. Myocardial fibroblast is an important part of the heart and plays an indispensable role in the physiological and pathological conditions of the heart. When the body is affected by acute myocardial injury, various inflammatory cytokines and pro-fibrotic factors stimulate the activation of myocardial fibroblasts and produce extracellular matrix (ECM), matrix metalloproteinases (MMP) and tissue inhibitors of metalloproteinases (TIMPs). Poor cardiac fibrosis can lead to the disorder of myocardial cell arrangement, myocardial hypertrophy, cytoskeletal rearrangement, collagen deposition, which poses a major threat to heart health[42]. In this study, we found that the occurrence of VMC is accompanied by cardiac fibrosis, which can be reduced by inhibiting pyroptosis of MCF.

CXCL4 is a multifunctional chemokine involved in a variety of biological responses, including the regulation of immune response and angiogenesis. CXCL4 not only has proinflammatory function but also promotes fibrosis[43–45]. In myeloproliferative tumors, hematopoietic deficiency of CXCL4 inhibits fibrosis. CXCL4 knockout alleviates cardiac fibrosis in a model of fibrosis caused by transverse aortic contraction. Our study shows that
pyroptosis activates the Wnt pathway of MCF to produce CXCL4, which has a profibrotic effect. Transcriptome sequencing analysis showed that CXCL4 treatment activated the PI3K/AKT signaling pathway of MCF. In order to verify whether the PI3K/AKT signaling pathway is related to the profibrotic function of CXCL4, and then pretreatment of MCF with PI3K inhibitors, it was found that PI3K inhibitors inhibited the profibrotic effect of CXCL4. Together, our study shows that pyroptosis of MCF produces CXCL4 by activating the Wnt/β-Catenin pathway. In addition, further studies have shown that CXCL4 promotes fibrosis through PI3K/AKT pathway.

In conclusion, our results suggest that the vital role of regulation of the NLRP3 inflammasome in the development of viral myocarditis. Inhibition of MCF pyroptosis caused by activation of NLRP3 can reduce heart damage. In addition, pyroptosis induced MCF to secrete CXCL4 by activating the Wnt/β-Catenin pathway, which promote cardiac fibrosis through activating the PI3K/AKT signaling pathway. NLRP3 may be considered a potential therapeutic target for myocarditis treatment via inhibition of CXCL4 secretion through the Wnt/β-Catenin pathway and inhibition of fibrosis through the PI3K/AKT pathway.

Declarations

Acknowledgements

Not applicable.

Author contributions

J W, M Y P, S N W. performed and analyzed experiments and participated in drafting the manuscript. T.J.J. analyzed data and participated in drafting of the revised manuscript. L HX. conceived the idea, oversaw the project, analyzed results and wrote the final manuscript.

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Availability of data and materials

The data that supports the findings of this study are available in the supplementary material of this article.

DECLARATION OF INTERESTS

The authors declare no commercial or financial conflict of interest.

References


NLRP3 inflammasome inhibitor reduces pyroptosis and ameliorates cardiac injury. Mice were intraperitoneally injected with CVB3 to construct a VMC model. MCC950 was then injected intraperitoneally daily at a dose of 10 mg/kg to inhibit NLRP3 inflammasome activation. Heart tissues and serum from Control, VMC and VMC+MCC950 mice were collected at the indicated time points. The VMC model was established successfully after seven days. A Representative image of western blot results of NLRP3, GSDMD, GSDMD-N, Caspase 1, Cleaved-Caspase-1, IL-1β, pro-IL-1β in heart tissues. B-C Representative image of the quantitative analysis of NLRP3, IL-18 in heart tissues. D Levels of serum IL-1β in mice. E HE staining of mouse heart tissue. F-H Levels of serum CK, AST, LDH in mice. I Levels of ROS in heart. Representative data from one of three independent experiments are shown. *P<0.05, **P<0.01, ***P<0.001. Comparisons between groups were performed using paired t-test or one-way ANOVA with Bonferroni correction.
Figure 2

Increased AngII in VMC can cause oxidative damage and pyroptosis of MCF. In vivo, Mice were intraperitoneally injected with CVB3 to construct a VMC model. MCC950 was then injected intraperitoneally daily at a dose of 10 mg/kg to inhibit NLRP3 inflammasome activation. In vitro, After AngII (1μM) treated MCF 24h, cell supernatants and cell lysates were collected respectively, and then analyzed as follows. A The pyroptosis of MCF was analyzed by immunofluorescence staining. Cardiac sections were taken from Control, VMC and VMC+MCC950 on day 7 and stained using with anti-Vimentin, anti-Caspase-1, and anti-TUNEL antibodies (40×) B Levels of serum AngII in mice. N=8 mice in each group. C-F Levels of ROS, SOD, MDA, LDH in cell supernatants and cell lysates. G Representative image of western blotting results of NLRP3, GSDMD, GSDMD-N, Caspase 1, Cleaved-Caspase-1, IL-1β, pro-IL-1β. H-I qRT-PCR analyses of the mRNA expression of NLRP3, IL-18. J Levels of IL-1β in culture medium. Representative data from one of three independent experiments are shown. *P<0.05, **P<0.01, ***P<0.001. Comparisons between groups were performed using paired t-test or one-way ANOVA with Bonferroni correction.
Figure 3

The pyroptosis of MCF promotes the secretion of CXCL4. In vivo, Different treatment groups mice hearts were collected, and then analyzed as follows. In vitro, MCC950-pretreated MCF 2h, AngII was added for 24h, and the following were assessed. A The protein levels of CXCL4 were detected in heart tissues. N=5 mice in each group. B Immunohistochemistry staining showing the expression of CXCL4 in heart sections (20× and 40×). C Levels of serum CXCL4 in mice. N=5 mice in each group. D Representative image of western blotting results of CXCL4 in MCF. E Levels of CXCL4 in different group cell supernatants. F-G qRT-PCR analyses of the mRNA expression of NLRP3, IL-18 in MCF. H Levels of IL-1β in cell supernatants. Representative data from one of three independent experiments are shown. *P<0.05, **P<0.01, ***P<0.001. Comparisons between groups were performed using paired t-test or one-way ANOVA with Bonferroni correction.
Pyroptosis of MCF induces CXCL4 production by activating the Wnt/β-Catenin pathway. A MCF were divided into control groups, MCC950+AngII group and AngII group and subjected to the pyroptosis qPCR array. Heatmap showed gene expression level detected by the pyroptosis qPCR array. B The volcano plot showed differentially expressed genes in the AngII group versus control group. C The bubble diagram showed pathway enrichment analyses of differentially expressed mRNAs. D Representative image of western blotting results of β-Catenin, c-Myc and Axin2 in heart tissues. E Representative images of immunohistochemistry staining showing the expression of c-Myc in heart sections. F Immunofluorescence staining showing expression of Axin2 in heart sections. G Immunofluorescence staining showing expression of β-Catenin in heart sections. H Representative image of western blotting results of β-Catenin, c-Myc and Axin2 in MCF. I Representative image of western blotting results of CXCL4 in MCF. MCC950-pretreated MCF 2h, AngII was added for 24h, and the following were assessed. Representative data from one of three independent experiments are shown. *P<0.05, **P<0.01, ***P<0.001. Comparisons between groups were performed using paired t-test or one-way ANOVA with Bonferroni correction.
Figure 5

Pyroptosis of MCF promotes cardiac fibrosis by producing CXCL4. Mice were intraperitoneally injected with CVB3 to construct a VMC model. MCC950 was then injected intraperitoneally daily at a dose of 10 mg/kg to inhibit NLRP3 inflammasome activation. Heart tissues from Control, VMC and VMC+MCC950 mice were collected at the indicated time points. A Masson staining for one of the 6 mice were shown. B Representative image of western blotting results of Collagen-I, Collagen-III, α-SMA in heart. Representative data from one of three independent experiments are shown. C The protein levels of Collagen-I, Collagen-III, α-SMA in MCF. MCF treated with CXCL4 for 24h, and then collected for analysis. D qRT-PCR analyses of the mRNA expression of Collagen-I, Collagen-III, α-SMA in MCF. E The protein levels of CXCL4 were detected in MCF. F The protein levels of Collagen-I, Collagen-III, α-SMA were detected in MCF. Representative data from one of three independent experiments are shown. *P<0.05, **P<0.01, ***P<0.001. Comparisons between groups were performed using paired t-test or one-way ANOVA with Bonferroni correction.
CXCL4 promotes cardiac fibrosis through activating the PI3K/AKT pathway. **A** The volcano plot showed differentially expressed genes in the CXCL4 group versus control group. **B** The bubble diagram showed pathway enrichment analyses of differentially expressed mRNAs. **C** Activation of PI3K and AKT in MCF was assessed after treatment with 5μM CXCL4. **D** The protein levels of Collagen-I, Collagen-III, α-SMA was detected in CXCL4-treated MCF with/without LY294002. **E** Before inducing VMC, LY294002 was intraperitoneally injected into the mice. After inducing VMC, LY294002 was injected every 2 days. Heart tissues from Control, VMC and VMC+LY294002 mice were collected at the indicated time points. Representative image of western blotting results of Collagen-I, Collagen-III, α-SMA in mouse heart tissue. N=5 mice in each group. Representative data from one of three independent experiments are shown. **F** Immunofluorescence staining showing expression of Collagen-III and α-SMA in heart sections. **G** HE staining of mouse heart tissue. **H** Masson staining of mouse heart tissue. Representative data from one of three independent experiments are shown. *P<0.05, **P<0.01, ***P<0.001. Comparisons between groups were performed using paired t-test or one-way ANOVA with Bonferroni correction.
In the mouse VMC model, activation of NLRP3 inflammasome promotes the occurrence of pyroptosis in MCF. The MCF of pyroptosis promotes the production of CXCL4 by activating the wnt pathway. CXCL4 activates the PI3K/AKT signaling pathway, leading to MCF activation and ultimately leading to myocardial fibrosis.

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