

Original Article

Macrophage NLRP3-dependent IL-1 β production contributes to aortic fibrosis in heart failure with preserved ejection fraction

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Abstract

Fibrosis is the main pathological feature of aortic stiffness, which is a common extracardiac comorbidity of heart failure with preserved ejection fraction (HFpEF) and a contributor to left ventricular (LV) diastolic dysfunction. Systemic low-grade inflammation plays a crucial role in the pathogenesis of HFpEF and the development of vascular fibrosis. In this study, we investigate the inflammatory mechanism of aortic fibrosis in HFpEF using a novel mouse model. LV diastolic dysfunction with preserved ejection fraction and aortic fibrosis induced by a high-fat diet (HFD) combined with subcutaneous aldosterone infusion are utilized. The constructed model exhibits augmented macrophage recruitment and NLR family pyrin domain containing 3 (NLRP3)-dependent interleukin (IL)-1 β production in fibrotic aortas. In addition, a bone marrow transplant is employed to induce macrophage-specific NLRP3 deficiency in the HFpEF mouse model. These mice show almost completely suppressed cleaved-caspase-1 and mature IL-1 β protein expression in the aortas, indicating that macrophage NLRP3 inflammasome activation enhances the IL-1 β overproduction in fibrotic aortas. Furthermore, we show that macrophage NLRP3 inflammasome inhibition improves aortic fibrosis and LV diastolic dysfunction. In conclusion, this study demonstrates the pivotal effect of macrophage NLRP3-dependent IL-1 β production on aortic fibrosis and cardiac function in HFpEF, suggesting a potential target for HFpEF therapy.

Key words macrophage, NLRP3 inflammasome, aortic fibrosis, left ventricular diastolic dysfunction, heart failure with preserved ejection fraction

Introduction

Heart failure with preserved ejection fraction (HFpEF) accounts for approximately half of all cases of heart failure. In recent years, the prevalence of HFpEF has been increasing, attracting considerable attention worldwide [1,2]. In addition to cardiac phenotypes such as left ventricular (LV) diastolic dysfunction, HFpEF is characterized by multiple extracardiac comorbidities, such as non-alcoholic fatty liver disease and vascular diseases. Among vascular diseases, vascular stiffness, especially aortic stiffness, deserves particular attention, as patients with HFpEF are more likely to develop large artery stiffness. Aortic stiffness, which is usually caused by excessive fibrosis manifesting as degradation of elastic fibers and increased collagen deposition, can lead to adverse ventricular-arterial interaction and subsequently diastolic dysfunction [3–6]. A correlation between aortic stiffness and LV diastolic dysfunction has been demonstrated in patients with HFpEF [7]. Nevertheless, only a

handful of studies on aortic fibrosis and its underlying mechanisms have been conducted in HFpEF mouse models.

Systemic low-grade inflammation plays a pivotal role in the pathogenesis of HFpEF and is associated with deleterious vascular changes [8,9]. Inflammatory biomarkers are also associated with vascular fibrosis [10,11]. Immune cell infiltration, especially macrophages, is the main manifestation of vascular inflammation. Macrophages and macrophage-derived inflammatory cytokines increase matrix metalloproteinase (MMP) expression, leading to matrix collagen deposition and driving myofibroblast activation [12,13]. Interleukin (IL)-1 β is one of the most abundant inflammatory cytokines secreted by macrophages [14]. NLR family pyrin domain containing 3 (NLRP3) inflammasome activation induces IL-1 β production [15]. Previous research has demonstrated that increased macrophage IL-1 β in the heart promotes cardiac dysfunction during the development of HFpEF [16]. Moreover,

NLRP3 inflammasome activation in the arterial smooth muscle layer is associated with vascular calcification in diabetic mice [17]. However, whether macrophages and NLRP3-dependent IL-1 β production contribute to aortic fibrosis in HFpEF remains unknown.

In this study, we developed a mouse model of HFpEF induced by a high-fat diet (HFD) combined with subcutaneous aldosterone (ALD) infusion. Subsequently, pathological fibrosis in the aortas and the underlying mechanism were explored. We found that inhibiting NLRP3-dependent IL-1 β overproduction in macrophages mitigates aortic fibrosis and LV diastolic dysfunction in HFpEF.

Materials and Methods

Animals

All animal experiments were approved by the Animal Care and Use Committee of the School of Basic Medical Sciences of Fudan University, and the investigation complied with the Guide for the Care and Use of Laboratory Animals by the US National Institutes of Health. C57BL/6 wild-type (WT) mice were obtained from Charles River Laboratories (Beijing, China). *NLRP3* knock-out mice (*NLRP3*^{-/-}) on a C57BL/6 background were generated by Cyagen Company (Suzhou, China). Spontaneous green fluorescent mice H11-G/R were generated by GemPharmatech Company (Nanjing, China). All the mice were kept in cages under a controlled temperature (22°C–24°C) and humidity (50%–60%) in a 12 h light/dark cycle. The animal experiments were performed using male C57BL/6 mice aged 8–10 weeks. To induce HFpEF with aortic fibrosis, the mice were fed with a 60% high-fat diet (HFD) for three months. In the last month of HFD feeding, the mice received 1% NaCl drinking water and were continuously infused with aldosterone (600 μ g/(kd*d), dissolved in 10% dimethyl sulfoxide and polyethyleneglycol 300) by using subcutaneously implanted osmotic mini-pumps (Model 2004; Alzet Durect Corp, Cupertino, USA).

Mouse vascular ultrasound and echocardiography

Mouse vascular ultrasound and echocardiography were performed using the Vevo100 high-resolution imaging system. The mice were anesthetized with 1% isoflurane and placed on a heating pad at 37°C in the supine position. Depilatory cream was used to remove hair from the chest and abdomen of the mice. Motion (M)-mode images of the abdominal aorta were recorded to determine the circumferential cycle strain. The systolic diameter (Ds) and diastolic diameter (Dd) were quantified from M-mode images. The circumferential cycle strain was calculated as (Ds–Dd)/Dd. Long-axis LV imaging was obtained, and M-mode tracings were recorded for analysis of the LVEF. Apical four-chamber views were obtained in mice for diastolic function measurements using pulsed-wave and tissue Doppler imaging at the level of the mitral valve (E/e', the ratio of the early transmitral flow velocity to the early diastolic mitral annular velocity).

Western blot analysis

Tissues and cells were homogenized in RIPA buffer containing protease and phosphatase inhibitors. The protein concentration was determined using the BCA assay (Beyotime Biotechnology, Shanghai, China). Proteins were loaded on an SDS-PAGE gel for separation and transferred to a PVDF membrane. The membrane was blocked with 5% fat-free dry milk for one hour at room temperature and then incubated with the primary antibody overnight at 4°C. The membranes were subsequently incubated with a

horseradish peroxidase-conjugated secondary antibody for an additional hour. The protein bands were visualized through enhanced chemiluminescence. Blots were quantified using Image J software. The antibodies used for immunoblots were as follows: α -Tubulin (Proteintech, Chicago, USA); β -actin (Proteintech); ICAM-1 (ABclonal, Wuhan, China); MMP2 (Cohesion Biosciences, London, UK); MMP9 (Cohesion Biosciences); NLRP3 (CST, Danvers, USA); Caspase1 (Santa Cruz, Dallas, USA); IL-1 β (Santa Cruz); CD68 (CST); CCL2 (Proteintech); and HRP-conjugated anti-rabbit IgG (Proteintech) and HRP-conjugated anti-mouse IgG (Proteintech).

RNA isolation and quantitative real-time PCR

For RNA isolation, the tissues were snap-frozen with liquid nitrogen, and the aortas were homogenized in a Tissue Lyser (EZBioscience, Roseville, USA) with metal beads. Then, the RNA was transcribed into complementary DNA via a reverse transcriptase kit (EZBioscience). Quantitative real-time PCR was performed using SYBR Green master mix (EZBioscience). Gene expression was quantified using comparative threshold cycle (Δ Ct) method with normalization to the reference gene β -actin. The primer sequences are listed in [Supplementary Table S1](#).

Histology and immunofluorescence staining

For histological analysis, the tissues were quickly removed, fixed with 4% formaldehyde for paraffin embedding and sectioned at 5- μ m intervals. The sections were deparaffinized and rehydrated by immersion in xylene and alcohol. The sections were subsequently stained with hematoxylin and eosin (HE) in accordance with the manufacturer's protocol for a commercial kit (Servicebio, Wuhan, China). Sirius Red staining and Masson staining were performed in accordance with the manufacturer's protocol of commercial kits (GC307014-5 g, and G1006-20ML; Servicebio). For immunofluorescence staining, the sections were heat retrieved in sodium citrate for 20 min in 95°C water bath and were permeated and blocked in 0.1% Triton X-100 with 5% goat serum for 1 h. The sections were subsequently incubated with the following primary antibodies: mouse anti-CD68 (CST), mouse anti-CD20 (Proteintech), rabbit anti-CD4 (Abcam, Cambridge, UK), rabbit anti-ICAM-1 (ABclonal), and goat anti-CD31 (R&D SYSTEMS, Minneapolis, USA) overnight at 4°C and then incubated with secondary Alexa FluorTM594 donkey anti-rabbit IgG (H + L) (Invitrogen, Carlsbad, USA), rabbit antilexa FluorTM594 donkey anti-mouse IgG (Invitrogen), Alexa FluorTM488 donkey anti-rabbit IgG (H + L) (Invitrogen) or Alexa FluorTM594 donkey anti-goat IgG (H + L) (Invitrogen) for 1 h and DAPI for 10 min at room temperature. Images were captured via a fluorescence microscope.

Bone marrow transplantation (BMT)

Briefly, donor bone marrow (BM) was isolated from H11-G/R and *NLRP3*^{-/-} mice. The recipient mice were C57BL/6 WT mice subjected to 10-Gy lethal-dose irradiation. Within 4–6 h after irradiation, 1×10^7 donor BM cells were reinfused into each recipient mouse via tail vein injection. Recipient mice were allowed a 2-week recovery period for bone marrow regeneration and then fed with an HFD and infused with aldosterone for the subsequent experiments.

Flow cytometry

The mice were anesthetized, and then, blood was taken by eyeball

extirpation and placed into 1.5-mL EP tubes treated with heparin sodium. Erythrocyte lysate was added to the blood and incubated for 10 min at room temperature, followed by centrifugation to remove red blood cells. Blood cells were suspended in PBS and analyzed by flow cytometry.

Peritoneal macrophage isolation

WT mice were injected with 1 mL of 3% brewer thioglycollate broth into the peritoneal cavity. After three days of injection, the macrophages were collected via injection, and 10 mL of RPMI 1640 medium (HyClone, Logan, USA) was aspirated from the peritoneum. The collected peritoneal lavage fluid was centrifuged, and the supernatant was discarded. The cells were resuspended in fresh RPMI 1640 medium supplemented with 10% fetal bovine serum and cultured for subsequent experiments.

Tail-cuff blood pressure recordings

Systolic blood pressure (SBP) was measured via the noninvasive tail-cuff technique (Visitech Systems, Apex, USA). The mice were placed individually on a temperature-controlled platform (37°C). Before testing, all the mice were trained to become accustomed to short-term restraint. Blood pressure measurements were averaged from at least ten readings per session.

Cell culture and stimulation

Human umbilical vein endothelial cells (HUVECs; Cell Bank of Chinese Academy of Sciences, Shanghai, China) were cultured with high-glucose DMEM (HyClone, Logan, USA) supplemented with 10% fetal bovine serum. Peritoneal macrophages were pretreated with or without MCC950 (1 μ M; MCE, Monmouth Junction, USA) (designated as MCC950 and CON groups) or lipopolysaccharide (LPS, 1 μ g/mL; Sigma-Aldrich, Burlington, USA) for 4 h and then stimulated with palmitate (500 μ M; Sigma-Aldrich) or ALD (100 nM; MCE) for 30 h, followed by the addition of adenosine triphosphate (ATP, 2 mM; Sigma-Aldrich) for 1 h. The cell lysate was collected for western blot analysis. The supernatants collected from the two groups were mixed with equal volumes of complete DMEM and used to culture HUVECs. HUVECs were incubated for 36 h and collected for western blot analysis.

Statistical analysis

Statistical analyses were performed via GraphPad Prism 9.0 software (GraphPad Software, San Diego, USA). Flow cytometry analysis was performed via FlowJo software (BD Bioscience, Franklin Lakes, USA). Data are presented as the mean $\pm$ SEM. The number of experimental mice per group was 4 or 5. Unpaired Student's *t* tests were used for 2-group comparisons to determine statistical significance. The normality test was conducted before the unpaired Student's *t* test. If the data did not conform to a normal distribution, a non-parametric test was performed to determine statistical significance. *P* < 0.05 was considered statistically significant.

Results

HFDs and ALDs induce aortic fibrosis in HFpEF

To investigate the mechanism of aortic fibrosis in HFpEF, a mouse model of HFpEF was constructed by feeding mice with a HFD for 12 weeks, followed by ALD infusion during the last 4 weeks of HFD treatment (Figure 1A). The echocardiographic evaluation showed a preserved ejection fraction (Figure 1B) and increased E/e', indicat-

ing impaired cardiac diastolic function (EF) (Figure 1C,D) in the model group (HFD + ALD). HE staining of liver tissue revealed marked hepatic steatosis, which was consistent with the clinical features of HFpEF (Supplementary Figure S1). Vascular ultrasound showed decreased aorta circumferential cycle strain (aorta strain) in the model group mice (Figure 1E,F). Additionally, tail-cut blood pressure measurement showed that SBP was increased in model group mice subjected to HFD and ALD (Figure 1G). Masson and Red Sirius staining showed that the aortas contained distinct collagen accumulation and interstitial fibrosis in HFpEF (Figure 1H-J). MMPs facilitate the accumulation of stiffer, uncoiled collagen by disrupting the cross-linking of collagen and elastin in the pathogenesis of arterial stiffness [18]. Our data showed that the protein and gene expression levels of MMP2 and MMP9 were upregulated in the model group (Figure 1K-N). Collectively, these findings indicated that HFD and ALD induced the typical pathological features of aortic fibrosis in HFpEF.

HFD and ALD treatment increases macrophage recruitment in the aortas

Western blot and real-time PCR analyses showed that C-C motif chemokine ligand 2 (CCL2) and intercellular adhesion molecule-1 (ICAM-1) were overexpressed in the aortas from the HFD and ALD treatment group (Figure 2A-F). Furthermore, immunofluorescence staining results verified the increased expression of ICAM-1 in aortic endothelial cells (ECs) (Figure 2G). Cluster of differentiation 68 (CD68) is an antigen marker expressed on the surface of macrophages. The infiltration of CD68⁺ cells in the aortas was examined to determine whether enhanced CCL2 and ICAM-1 levels increase the number of macrophages. The results revealed increased CD68⁺ cells in the aortas, and western blot analysis confirmed enhanced CD68 protein expression in the fibrotic aortas. However, no differences in the number of CD4⁺ and CD20⁺ cells in the aortas were found between the two groups (Figure 2H-M). Taken together, these data indicate that macrophages, rather than T or B-cell infiltration, may contribute to aortic fibrosis in HFpEF patients.

Macrophage NLRP3 inflammasome activation results in IL-1 β overproduction in aortas after HFD and ALD treatment

It is believed that the inflammatory cytokines secreted by macrophages promote the progression of aortic diseases [19]. Therefore, we explored whether HFD and ALD treatment affects the expressions of inflammatory cytokines, including IL-1 β , IL-6, and TNF- α , which are mainly secreted by macrophages. The results showed that HFD and ALD treatment elevated IL-1 β , IL-6, and TNF- α levels in the aortas, with IL-1 β being the most highly expressed (Supplementary Figure S2 and Figure 3A). Further analyses revealed that mRNA and protein expression levels of NLRP3 were significantly upregulated in the model group compared with those in the sham group (Figure 3B-D). Consistently, western blot analysis also showed enhanced caspase1 cleavage and downstream pro-IL-1 β cleavage (Figures 3 and 4). Wen *et al.* [20] reported that palmitate, which is used to stimulate the effects of HFD, induced NLRP3 inflammasome activation and IL-1 β maturation in bone marrow-derived macrophages *in vitro*. On the basis of these findings, we hypothesized that NLRP3 inflammasome activation in macrophages is mainly responsible for IL-1 β overproduction in

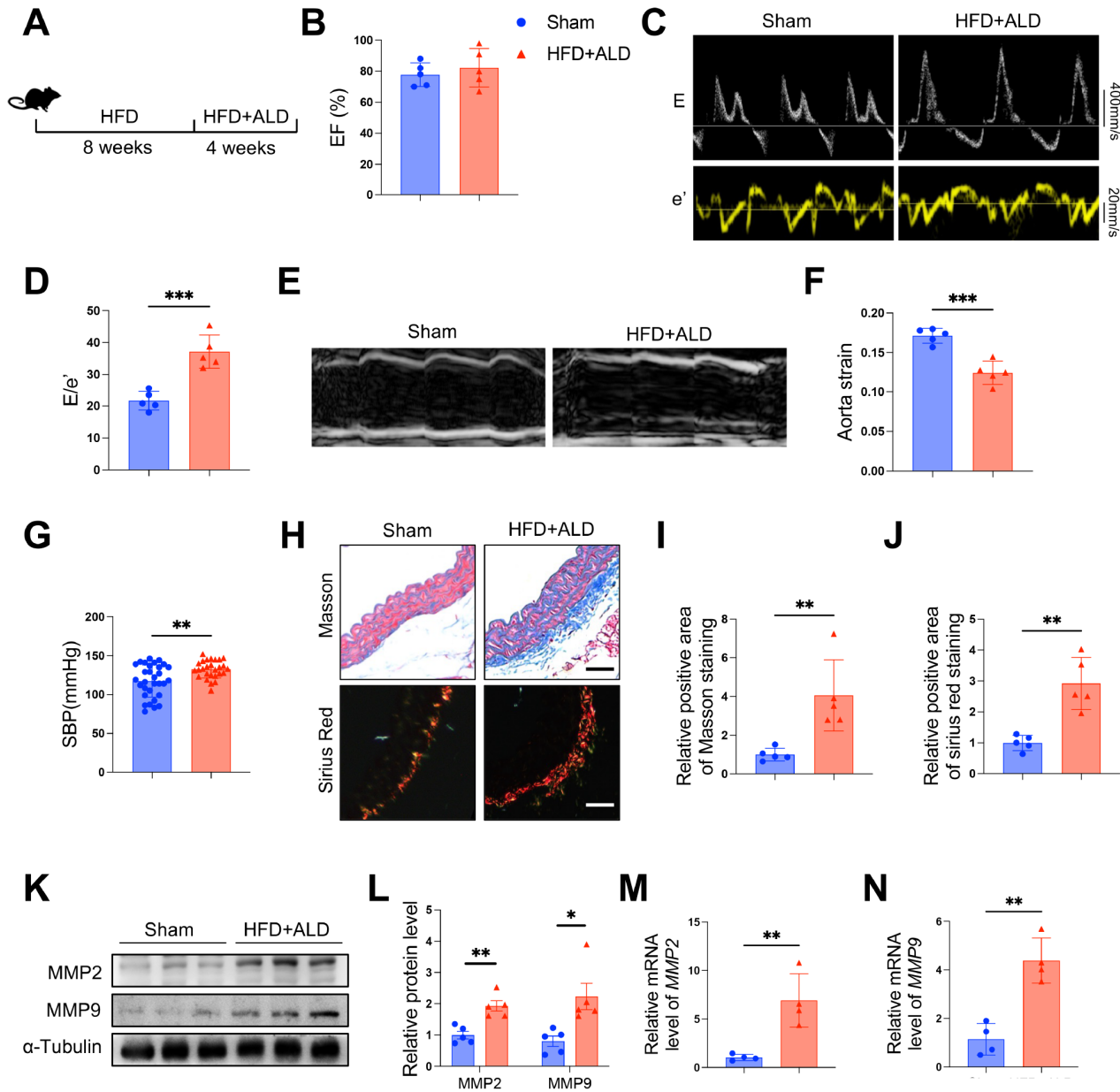


Figure 1. HFpEF mouse model generated by HFD and ALD treatment exhibits aortic fibrosis (A) Schematic representation of the HFpEF mouse model. (B) Percentage of the left ventricular ejection fraction. $n = 5$ mice per group. (C,D) Representative pulsed-wave Doppler (top) and tissue Doppler (bottom) tracings and the ratio between the mitral E wave and e' wave (E/e'). $n = 5$ mice per group. (E,F) Representative images of the M mode for the abdominal aorta monitored by ultrasound and analysis of circumferential cyclic strain. $n = 5$ mice per group (G) SBP. $n = 5$ mice per group (31 recordings in the sham group and 27 recordings in the HFD + ALD group). (H–J) Representative images of Masson and Sirius Red staining of the aortas and quantification of the collagen content. Scale bar: 25 μm . $n = 5$ mice per group (K,L) Protein levels of MMP2 and MMP9 in aortas detected by western blot analysis. $n = 5$ mice per group. (M,N) mRNA levels of MMP2 (M) and MMP9 (N) in aortas detected by real-time PCR. $n = 5$ mice per group. Data are expressed as the mean $\pm$ SEM. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

the aortas of HFpEF mice.

To verify this hypothesis, we established a macrophage-specific NLRP3-deficient mouse model via bone marrow transplantation (BMT) from H11-G/R mice or NLRP3-knockout mice (NLRP3 $^{-/-}$) into wild-type (WT) mice, which were subsequently fed with a HFD and subjected to ALD (Figure 3E). Since H11-G/R mice exhibited green fluorescence, the percentage of green fluorescence in blood cells from BMT recipient mice was detected to evaluate BMT efficiency. The flow cytometry results showed that the average percentage of

green fluorescence was 97.01% in the H11-G/R BM group and only 0.012% in the NLRP3 $^{-/-}$ BM group (Figure 3F,G), suggesting high BMT efficiency and successful NLRP3 knockout in macrophages. The protein expression levels of NLRP3, cleaved caspase-1 pro-IL-1 β , and mature IL-1 β were almost completely suppressed in the aortas of NLRP3 $^{-/-}$ BM mice (Figure 3H,I), suggesting that NLRP3 inflammasome activation in macrophages is the primary pathway for mature IL-1 β production in the aortas of mice treated with HFD and ALD.

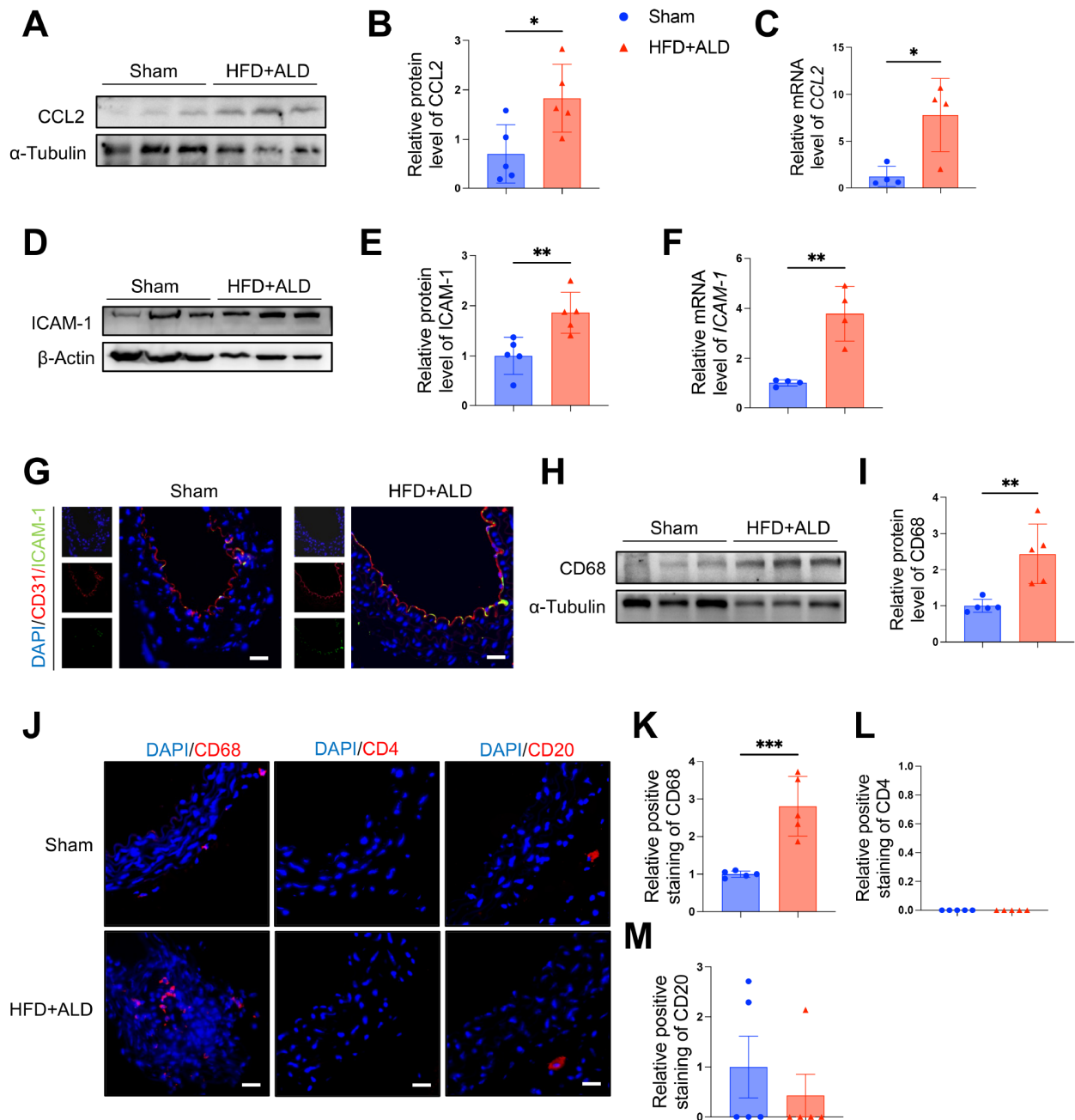


Figure 2. Macrophage markers are upregulated in the aortas from the HFD and ALD treatment groups (A,B) Protein levels of CCL2 in the aortas were detected by western blot analysis. $n = 5$ mice per group. (C) mRNA levels of *CCL2* in aortas detected by RT-PCR. $n = 4$ mice per group. (D,E) Protein levels of ICAM-1 in aortas were detected by western blot analysis. $n = 5$ mice per group. (F) mRNA levels of *ICAM-1* in aortas detected by RT-PCR. $n = 4$ mice per group. (G) Representative immunofluorescent staining images of ICAM-1-positive aortic endothelial cells. Scale bar: 20 μ m. $n = 4$ mice per group. (H,I) Protein levels of CD68 in the aortas were detected by western blot analysis. $n = 5$ mice per group. (J–M) Representative images of immunofluorescence staining and quantification of CD68, CD4, and CD20 in the aortas. Scale bar: 20 μ m. $n = 5$ mice per group. Data are expressed as the mean $\pm$ SEM. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Inhibiting the macrophage NLRP3 inflammasome activation attenuate aortic fibrosis and LV diastolic dysfunction in HFpEF

To investigate whether NLRP3-dependent IL-1 β production in macrophages facilitates aortic fibrosis in HFpEF, we detected aortic compliance and extracellular matrix remodeling in the H11-G/R BM and NLRP3 $^{-/-}$ BM groups. The vascular ultrasound results showed

that macrophage NLRP3 deficiency increased aortic strain (Figure 4A,B). Consistently, SBP was also reduced in the NLRP3 $^{-/-}$ BM group (Figure 4C). Mason and Sirius Red staining revealed less collagen accumulation in the aortas of the NLRP3 $^{-/-}$ BM group than in those of the H11-G/R BM group (Figure 4D–F). Intriguingly, MMP2 and ICAM-1 protein expression levels were reduced in the aortas of the NLRP3 $^{-/-}$ BM group compared to those in the aortas of

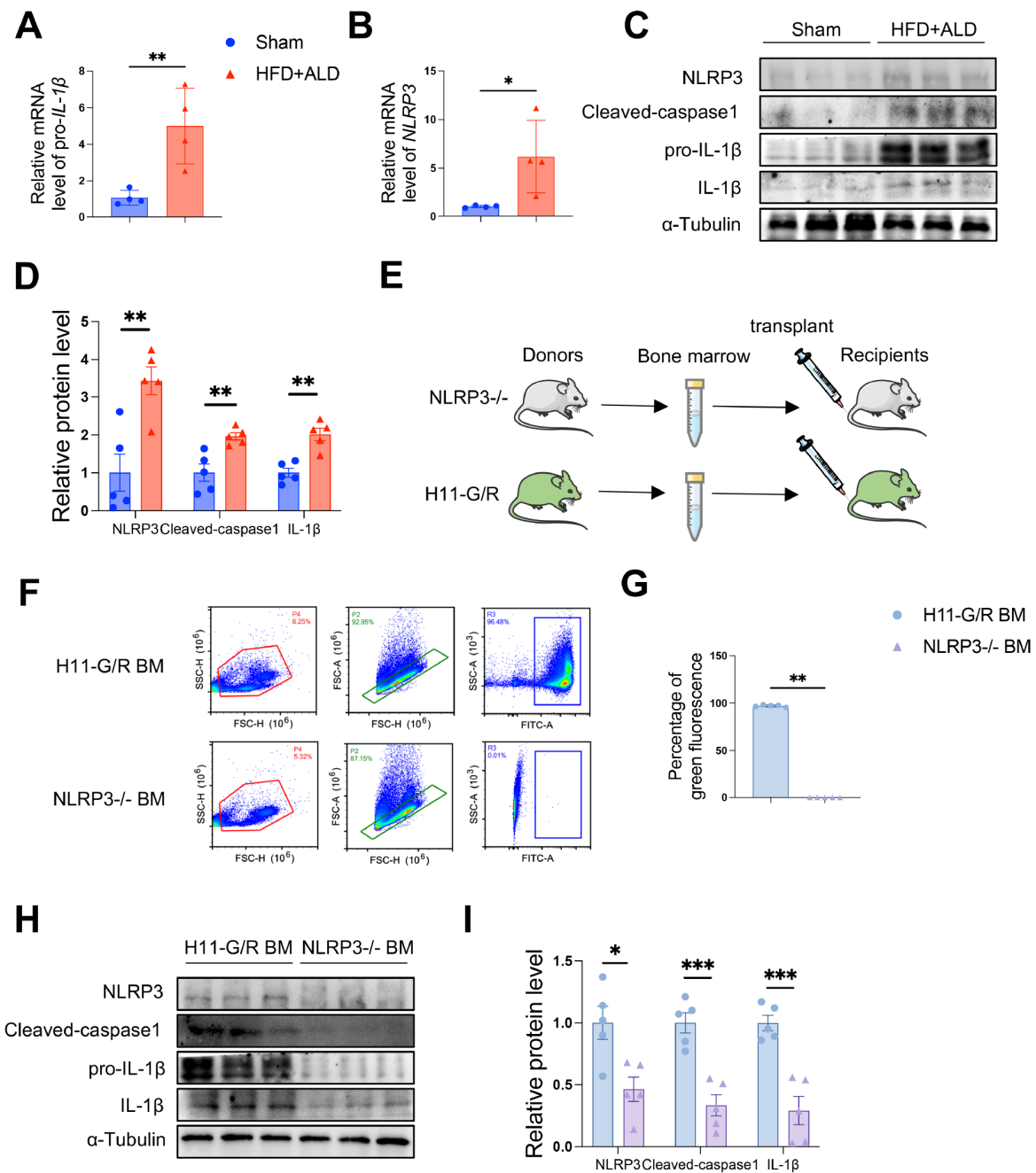


Figure 3. Macrophage NLRP3 inflammasome activation results in IL-1 β overproduction in the aortas of mice treated with HFD and ALD (A,B) mRNA levels of pro-IL-1 β (A) and NLRP3 (B) in the aortas detected by RT-PCR. $n = 4$ mice per group. (C,D) Protein levels of NLRP3, cleaved caspase-1, pro-IL-1 β , and IL-1 β in the aortas were detected by western blot analysis. $n = 5$ mice per group. (E) Bone marrow transplant (BMT) strategy. (F,G) Representative images and analysis of the percentage of green fluorescence in blood cells detected by flow cytometry for evaluating BMT efficiency. $n = 5$ mice per group. (H,I) Protein levels of NLRP3, cleaved caspase-1, and IL-1 β in the aortas were detected by western blot analysis. $n = 5$ mice per group. Data are expressed as the mean $\pm$ SEM. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

the H11-G/R BM group (Figure 4G–J). To explore whether the IL-1 β secreted by macrophages could affect the ICAM-1 expression in ECs, we conducted *in vitro* experiments. The results showed that MCC950 significantly prevented the increase in the protein expression levels of NLRP3, cleaved caspase-1 and mature IL-1 β in macrophages treated with palmitate, ALD, LPS and ATP (Supplementary Figure S3A,B). ICAM-1 protein expression in

HUVECs cultured with the supernatant from MCC950-pretreated macrophages (MCC950) was significantly decreased compared to that in HUVECs cultured with the supernatant from non-MCC950-pretreated macrophages (CON) (Supplementary Figure S3C,D). Moreover, NLRP3 inflammasome blockade in macrophages improved LV diastolic dysfunction in mice with HFpEF (Figure 4K–M). Taken together, these data demonstrated that inhibition of NLRP3

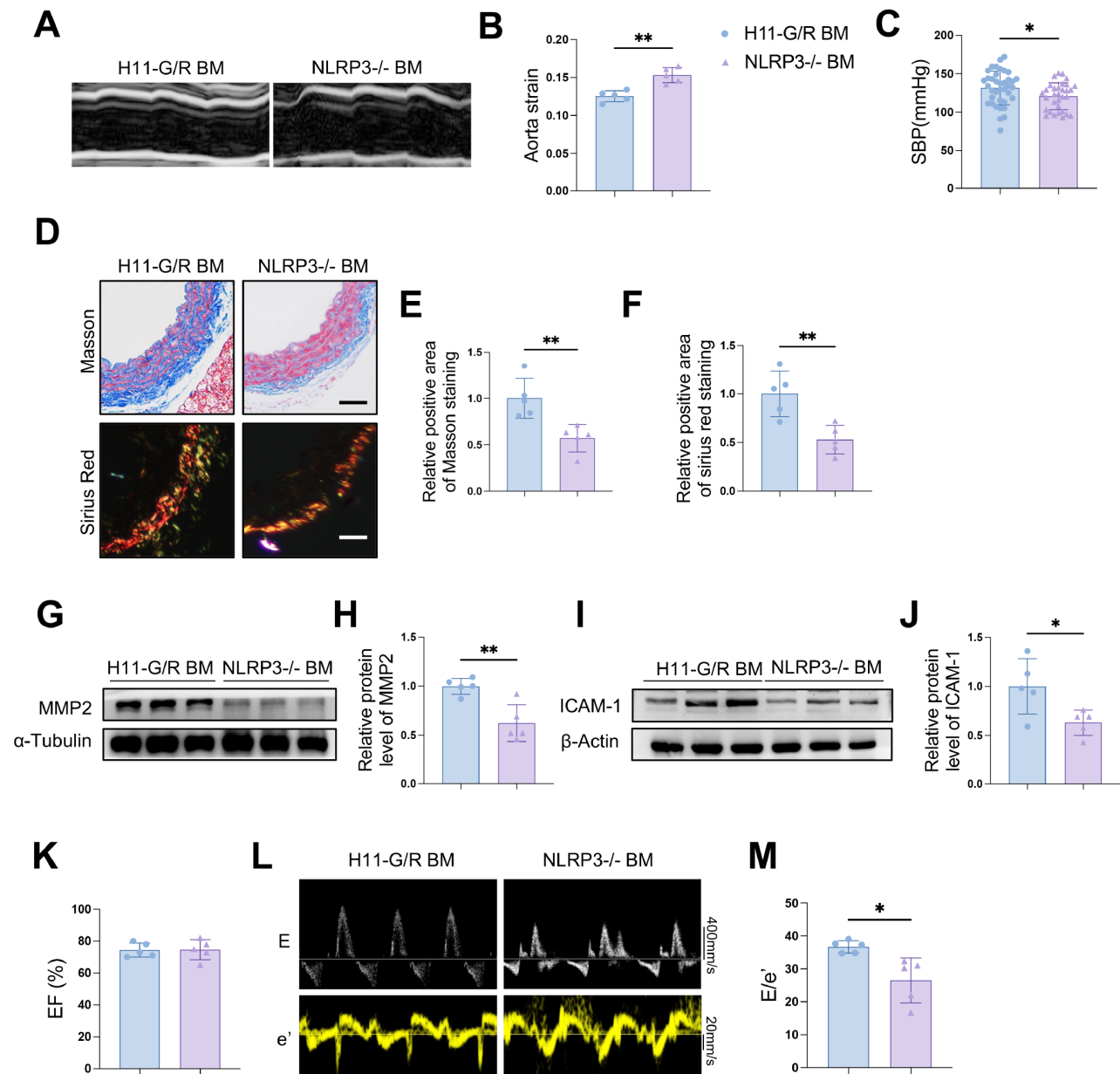


Figure 4. NLRP3 deficiency in macrophages attenuates aortic fibrosis and improves cardiac diastolic dysfunction (A,B) Representative images of the M mode for the abdominal aorta monitored by ultrasound and analysis of circumferential cyclic strain. $n = 5$ mice per group. (C) SBP. $n = 5$ mice per group (38 recordings in the H11-G/R BM group and 32 recordings in the NLRP3 $^{-/-}$ BM group). (D–F) Representative images of Masson and Sirius Red staining of the aorta and quantification of the collagen content. Scale bar: 25 μ m. $n = 5$ mice per group. (G–J) Protein levels of MMP2 (G–H) and ICAM-1 (I,J) in the aortas detected by western blot analysis. $n = 5$ mice per group. (K) Percentage of the left ventricular ejection fraction. $n = 5$ mice per group. (L,M) Representative pulsed-wave Doppler (top) and tissue Doppler (bottom) tracings and ratios between the mitral E wave and e' wave (E/e'). $n = 5$ mice per group. Data are expressed as the mean $\pm$ SEM. * $P < 0.05$, ** $P < 0.01$.

inflammasome-dependent IL-1 β production in macrophages attenuated aortic fibrosis and cardiac diastolic dysfunction in HFpEF mice.

Discussion

Due to its multiple manifestations and high heterogeneity, there is no standard for establishing mouse models of HFpEF. In recent years, a two-hit mouse model combining metabolic dysfunction and haemodynamic stresses, using HFD and N ω -nitro-L-arginine methyl ester, has emerged as a suitable model for HFpEF. However, female

sex has been found to be a protective factor against HFpEF, which is inconsistent with clinical observations [21,22]. Excessive ALD is a key feature of HFpEF which is known to induce arterial fibrosis. ALD infusion has been shown to independently induce HFpEF [23–25]. Although the overall therapeutic effect of spironolactone in patients with HFpEF is unsatisfactory, Jordana *et al.* [26] found that patients with HFpEF in the phenogroup with a high prevalence of obesity and diabetes exhibited a preferential response to spironolactone. Therefore, to recapitulate the characteristics of metabolic

dysfunction, excessive ALD and cardiac dysfunction, we developed a two-hit mouse model of HFpEF by administering HFD and ALD infusion. The results indicated that HFD and ALD treatment adequately induced aortic fibrosis and reduced cardiac diastolic function with preserved EF. Moreover, aortic fibrosis was accompanied by an increase in macrophage infiltration in the aortas and macrophage NLRP3-dependent IL-1 β overproduction. Conversely, inhibiting NLRP3 inflammasome activation in macrophages using BMT significantly ameliorated aortic fibrosis and diastolic dysfunction in HFpEF.

Macrophage recruitment in the heart during HFpEF has been verified in mouse models and patients [27,28]. Macrophage infiltration was implicated in aortic fibrosis in a pressure overload mouse model [29]. However, whether macrophages are involved in the process of aortic fibrosis in HFpEF remains elusive. Histological examination, molecular biological detection, and ultrasonography confirmed the presence of aortic fibrosis in our HFpEF mouse model, which was consistent with the clinical features of patients with HFpEF. CCL2 and ICAM-1 are important regulators of macrophage recruitment [30,31]. Macrophage infiltration is accompanied by CCL-2 and ICAM-1 upregulation in the aortic valve of aging mice [32]. The present study revealed that CCL2 and ICAM-1 levels were elevated in the aortas of HFpEF mice. Thus, it is reasonable to examine whether macrophages are recruited to fibrotic aortas. Consistent with previous studies, our findings showed that only macrophages, not lymphocytes, were recruited to the aortas and might contribute to aortic fibrosis in HFpEF.

Recent studies have suggested that macrophages contribute to the inflammatory environment by secreting cytokines, potentially leading to arterial fibrosis and stiffness. Deuell *et al.* [33] reported that increased IL-6 and TNF- α in macrophages mediated receptor activator of nuclear factor kappa-B ligand (RANKL)-induced smooth muscle cell calcification. IL-1 β is a critical cytokine in the inflammatory response. NLRP3 inflammasome activation triggers IL-1 β maturation and release. There are two steps in NLRP3 inflammasome activation. The first step, usually called the priming step, is the transcriptional upregulation of NLRP3 and pro-IL-1 β . Thereafter, the inflammasome is fully assembled and activated, ultimately inducing IL-1 β maturation [34]. Low-density lipoprotein in the aortic wall activates NLRP3 inflammasome-dependent IL-1 β release in macrophages, promoting the development of atherosclerosis in patients and mouse models [35]. Moreover, blockage of NLRP3 inflammasome in macrophages alleviated hyperhomocysteinemia-aggravated abdominal aortic aneurysm [36]. Inspired by the above findings, we first assessed the activation of the NLRP3 inflammasome in the aorta. The results showed that both mRNA and protein expressions of NLRP3 inflammasome pathway were increased, as expected. IL-1 β is an inflammatory cytokine that can be produced not only by macrophages but also by vascular smooth muscle cells (VSMCs) and ECs. However, the latter two generally secrete lower levels than do macrophages [19]. To explore whether activation of the NLRP3 inflammasome occurs in the development of aortic fibrosis, we generated macrophage-specific NLRP3-deficient mice via BMT. The results showed that NLRP3 depletion in macrophages significantly inhibited inflammasome activation and IL-1 β production, indicating that IL-1 β in the aortas of mice treated with HFD and ALD model mice was derived mainly from macrophages but not from VSMCs or ECs, which is consistent with the findings of previous studies [19]. As a secretory protein, IL-1 β

can mediate the interaction between macrophages and other organs or cells [13]. Agrawal *et al.* [37] reported that macrophage IL-1 β promoted pulmonary hypertension in HFpEF. Our results confirmed that NLRP3 inflammasome inhibition in macrophages mitigated aortic fibrosis, accompanied by reduced MMP2 and ICAM-1 expressions in the aorta. MMP2 and ICAM-1 are expressed primarily in VSMCs and ECs, respectively. Given that IL-1 β is an inducer of these two factors [31,38], we postulated that macrophages might modulate aortic fibrosis by interacting with VSMCs and ECs. The *in vitro* experiments provided supporting evidence for the effect of IL-1 β secreted by macrophages on the expression of ICAM-1 in HUVECs. Decreased ICAM-1 expression in ECs may further inhibit inflammation in aortas, thereby preventing aortic fibrosis. Although the downstream signaling pathways mediating the effects of IL-1 β are unknown, our results suggested that NLRP3 inflammasome-dependent IL-1 β production in macrophages may contribute to the development of aortic fibrosis in HFpEF.

Numerous studies have identified aortic stiffness as a contributing factor to HFpEF, as it increases cardiac afterload, further exacerbating LV diastolic dysfunction [3,39]. In our study, we found that the reduction in aortic fibrosis following NLRP3 inhibition was accompanied by improvement in LV diastolic dysfunction. Considering the contribution of aortic stiffness to HFpEF and the role of fibrosis in promoting aortic stiffness, the improvement in LV diastolic dysfunction observed in this study may be partially due to the amelioration of aortic fibrosis. Therefore, targeting macrophages may be a potential approach for alleviating HFpEF progression by attenuating aortic fibrosis. However, because many comorbidities in HFpEF such as diabetes and hypertension, may have common additive effects on the heart and vessels, causal sequences between aortic fibrosis and cardiac dysfunction during the progression of HFpEF are not entirely clear [7]. Therefore, the causal relationship between decreased aortic fibrosis and diastolic function improvement in our study deserves further investigation.

This study has several limitations. First, the effects of neutrophil-derived IL-1 β and other inflammatory cytokines secreted by macrophages, including IL-6 and TNF- α , on aortic fibrosis in HFpEF cannot be ruled out in our study. Additionally, BMT inhibited NLRP3-dependent IL-1 β production not only in the aortas but also in other tissues, suggesting that the extravascular effects of reduced IL-1 β regulate aortic fibrosis and cardiac function through crosstalk between organs. Finally, although our findings identified IL-1 β as a potential target for regulating aortic fibrosis in HFpEF, further *in vivo* and *in vitro* studies are required to unravel the mechanism by which macrophage-derived IL-1 β contributes to aortic fibrosis in HFpEF.

In conclusion, this study demonstrated increased macrophage infiltration in fibrotic aortas in HFpEF and identified the adverse effect of macrophage NLRP3-dependent IL-1 β overproduction on aortic fibrosis in HFpEF. Our findings provide important insights into the pathogenesis of HFpEF and a potential therapeutic strategy for aortic stiffness in HFpEF.

Supplementary Data

Supplementary data is available at *Acta Biochimica et Biophysica Sinica* online.

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Conflict of Interest

The authors declare that they have no conflict of interest.

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