Original Article
CXCR6 deficiency attenuates pressure overload-induced monocytes migration and cardiac fibrosis through downregulating TNF-α-dependent MMP9 pathway

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Abstract: An immerging role of TNF-α in collagen synthesis and cardiac fibrosis implies the significance of TNF-α production in the development of myocardial remodeling. Our previous study showed a reduction of TNF-α and attenuated cardiac remodeling in CXCR6 knockout (KO) mice after ischemia/reperfusion injury. However, the potential mechanism of TNF-α-mediated cardiac fibrosis with pressure overload has not been well elucidated. In the present study, we aim to investigate the role of CXCR6 in TNF-α release and myocardial remodeling in response to pressure overload. Pressure overload was performed by constriction of transverse aorta (TAC) surgery on CXCR6 KO mice and C57 wild-type (WT) counterparts. At 6 weeks after TAC, cardiac remodeling was assessed by echocardiography, cardiac TNF-α release and its type I receptor (TNFRI), were detected by ELISA and western blot, collagen genes Col1a1 (type I) and Col3a1 (type III) were examined by real-time PCR. Compared with CXCR6 WT mice, CXCR6 KO mice exhibited less cardiac dysfunction, reduced expression of TNFRI, Col1a1 and Col3a. In vitro, we confirmed that CXCR6 deficiency led to reduced homing and infiltration of CD11b+ monocytes, which contributed to attenuated TNF-α release in myocardium. Furthermore, TNFRI antagonist pretreatment blocked AT1 receptor signaling and NOX4 expression, reduced collagen synthesis, and blunted the activity of MMP9 in CXCR6 WT mice after TAC, but these were not observed in CXCR6 KO mice. In the present work, we propose a mechanism that CXCR6 is essential for pressure overload-mediated myocardial recruitment of monocytes, which contributes to cardiac fibrosis through TNF-α-dependent MMP9 activation and collagen synthesis.

Keywords: CXCR6, pressure overload, cardiac fibrosis, TNF-α, MMP9

Introduction

Biomedical stress and pressure overload cause cardiac hypertrophy, as well as inflammation, and the development of cardiac fibrosis is usually associated with inflammation [1-3]. Recent studies indicated that inflammation is critically involved in the pathogenesis of pressure overload-mediated cardiac fibrosis [2, 4, 5]. For example, bone marrow (BM)-derived monocytes and mast cells are rapidly homing and accumulated in myocardium during pressure overload, and these cells are the major sources of pre-inflammatory cytokines production [1, 6, 7]. In contrast, genetic deletion of interleu-kin-10 (IL-10), a potent anti-inflammatory cytokine, exacerbated pressure overload-induced cardiac remodeling [8].

Accumulative studies revealed that potent chemoattractants, such as the chemokine receptors, played a critically role in orchestrating neutrophils and monocytes infiltration into inflammatory sites, such as the injury vascular endothelium and ischemic myocardium [9, 10]. These chemokine receptors belonged to a big family, including CCRs, CXCRs and CX_3CRs, and played different roles in regulating the maturation, adhesion and immigration of inflammatory cells [11, 12]. Liehn EA. reported that neutro-

phil were unaltered, but monocyte infiltration was blunted in CXCR2$^{-/-}$ mice [13]. Previous studies indicated that CXCL16 was a marker of inflammation in cardiovascular disease, in vitro CXCL16 promoted proliferation of myocardial fibroblasts and collagen synthesis [14], which indicated its important role in cardiac matrix remodeling and heart failure. CXCR6, the only unique receptor to CXCL16 [14, 15], could be activated by shear stress and promoted monocytes infiltration [16]. Inflammation-mediated hepatic and myocardial fibrosis was significantly reduced in CXCR6$^{-/-}$ mice [15].

Although the mechanism underlying mechanical stress-mediated cardiac fibrosis is yet not to be fully defined, evidences suggest that TNF-α signaling was critically involved in pressure overload induced cardiac fibrosis [17, 18]. Upregulation of TNF-α promoted fibrogenic response, and inhibition of TNF-α using neutralizing antibody improved cardiac remodeling and dysfunction [19, 20]. AngII infusion-induced cardiac fibrosis was suppressed in TNF-α receptor1 (TNFR1) KO mice [18]. Interestingly, CXCR6 promoted atherosclerosis via recruitment of leukocytes and secretion of pre-inflammatory cytokines including TNF-α [21], and TNF-α was significantly decreased in fibrotic liver of CXCR6 KO mice [15]. We previously also proved that CXCR6 deficiency led to reduced secretion of TNF-α in ischemic hearts. However, little was known about the precise role of CXCR6-mediated TNF-α signaling in pressure overload-induced cardiac fibrosis.

In the present study, our data implicated that pressure overload-mediated myocardial fibrosis, infiltrating CD11b monocytes and TNF-α production were significantly attenuated in CXCR6 KO mice. Particularly, we provided evidences for the involvement of CXCR6 in the pathogenesis of cardiac remodeling through TNF-α-dependent MMP9 activation and fibrogenic response.

Materials and methods

Pressure overload model and echocardiography analyses

8~10 weeks old male CXCR6 KO mice and their wild-type (WT) counterparts were used in these experiments. Pressure overload was preformed by TAC as previously described [22]. In brief, mice were anesthetized by intraperitoneal injection of a cocktail of ketamine (100 mg/kg)

and xylazine (5 mg/kg), respiration was controlled with a tidal volume of 0.2 ml and a respiratory rate of 110 breaths/min. After the heart was exposed, the transverse aorta was constricted by ligating with a 7-0 nylon string, and together with a 27-gauge blunt-ended needle, which pulled out 30 seconds later. 6 weeks post-TAC surgery was chosen as the experimental endpoint, transthoracic echocardiography was performed using animal specific instrument (Visual Sonics® Vevo770®, VisualSonics Inc. Canada). Mice were anesthetized and M-mode images of the left ventricle were record. We measured the left ventricular (LV) anterior and posterior wall at diastole (LVAWd, LVPWd) in M-mode. Percentage fractional shortening (FS%) and percentage ejection fraction (EF%) were calculated as described previously [22]. All animal experimental protocols were approved by Tongji University Committee on Laboratory Animals, in accordance with "Guidelines for the Care and Use of Laboratory Animals" published by the National Academy Press (NIH Publication No. 85-23, revised 1996).

Cell culture

Spleen-derived monocytes were isolated from CXCR6 KO mice and WT counterparts, and screened by CD11b Microbeads (Miltenyi Biotec, Germany). Resuspended cells were seeded into 6-well cell culture plates and cultured for 48 h in Gibco® RPMI 1640 medium containing 10% fetal bovine serum (FBS). Cells were stimulated by CXCL16 (100 ng/ml, R&D Systems, Inc.) for 24 h, TNF-α release was determined by ELISA

Collagen Masson's trichromic staining

Sliced paraffin sections of left ventricular tissues from CXCR6 KO mice and WT counterparts were stained with Masson's trichrome technique. Collagen stained in blue and images were captured with a Nikon DXM1200 camera. Stained sections were quantified by Image Pro Plus 6 software (Media Cybernetics, Inc.).

IHC analysis of myocardial infiltration of monocytes

Cardiac dissected tissues were fixed in 4% (w/v) paraformaldehyde for about 20 hours, embedded in paraffin, and sectioned into microscope slides (Leica Biosystems) for immunohistochemistry. The slides were incubated

with anti-mouse CD11b antibody (R&D Systems, Inc.) for 24 h. CD11b+ monocytes were detected and visualized by ABC-POD substrate kit for Mouse (Vector Labs).

Cell isolation and FACS analysis

Single-cell suspension from mice heart tissue was produced as previously described [23]. Heart was rapidly removed and placed into 37°C water bath. The aorta was cannulated with a 20-gauge blunt-ended needle connected to a Langendorff preparation system. The heart was perfused for 2 min with a constant flow of 1.1 ml/minute, and then enzymatic digestion was started by adding collagenase D (0.895 mg/ml, Worthington Biochemical) within the above solution. The heart was placed into a Petri dish with chilled staining buffer (1% FBS, 0.05% sodium azide in PBS) and dispersed into Single-cell suspension, passed sequentially through 70 μm and 40 μm cell strainers (Falcon; BD Biosciences). The cells were washed by 200 μl PBS containing 2% BSA and incubated with anti-CD11b-FITC (BD Biosciences) or anti-TCR-PE antibodies on ice for 1 h. Finally, each sample was washed twice and loaded onto a flow cytometer instrument (Beckman, Germany), raw data were analyzed.

RNA isolation and quantitative RT-PCR

Total RNA from left ventricular was extracted using One Step PrimeScript® RT-PCR Kit II (TaKaRa). Quantitative RT-PCR was performed on Rotor-Gene 3000 (Corbett Research, Australia) by a program of 42°C for 5 min and 30 cycles of 94°C for 5 s, 60°C for 30 s. The primers used as follows: Col1a1, 5'-CTTTGCTTCCC-AGATGTCCTAT-3', 3'-GACCTTACTTCCCTGTGGC-5'; Col3a1, 5'-CCCTGGCTCAAATGGCTCA-3', 3'-TTCTTGCCACGCTTCCCTC-5'. All the gene expressions were normalized to house-keeping gene GAPDH, and data were analyzed by ChemiDoc™ XRS system (Bio-Rad).

Western blot analysis

Myocardial proteins were isolated from left ventricular tissue and lysed in a buffer containing 50 mM Tris (pH 7.4), 20 mM HEPES, 150 mM NaCl, 12.5 mM β-glycerophosphate, 1% Triton X-100, 2 mM EGTA, 5 mM $MgCl_2$ 10 mM NaF, 2 mM DTT and 1 mM phenylmethylsulfonyl fluoride. Equal amount of total proteins were separated on 10% SDS-PAGE and transferred to PVDF membranes (Millipore Corp.). The membrane with blotted proteins were blocked for 1 h in 5% bovine serum albumin (BSA), followed by probing with anti-TNFRI (ab19139, Abcam), anti-AT1 receptor (ab9391, Abcam), anti-NOX4 (Santa Cruz sc-21860).

ELISA analysis of TNF-α release

TNF-α level from blood serum and cytoplasm of spleen-derived CD11b+ monocytes were measured using a commercially available ELISA kit (R&D Systems) according to manufacturer's instructions.

MMPs activities determined by gelatin zymography

Myocardium tissues were pulverized and homogenized in Tris buffer (10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 10 mM $CaCl_2$) containing 0.1% Triton-X100. Homogenates were centrifuged at 10,000×g for 30 min at 4°C. Supernatants were collected and protein concentration in was determined using a Bradford protein assay kit (Bio-Rad). Samples were electrophoresed in 10% SDS-polyacrylamide under non-reducing conditions, containing gelatin (1 mg/ml; Sigma-Aldrich) as MMPs substrate. After separation, gels were placed in 2.5% Triton X-100 in water for 1 hour, then gelatinases were activated by overnight incubation in 50 mM Tris-HCl, pH 8.0, and 5 mM $CaCl_2$ at 37°C. Gels were stained with 0.5% Coomassie blue and then de-stained in 10% acetic acid with 30% methanol solution until the gelatinolytic bands were visible.

Statistical analysis

Data were expressed as mean±s.e.m. Multiple-group statistical analysis was performed by 1-way ANOVA analysis of variance followed by the Tukey-Kramer post hoc test. Comparison between two groups under identical conditions was performed by the 2-tailed student's t-test. A value of *P* < 0.05 was considered statistically significant.

Results

Myocardial remodeling was attenuated in CXCR6 KO mice after pressure overload

To investigate the role of CXCR6 in pressure overload induced cardiac remodeling, we performed TAC on CXCR6 KO mice and C57-WT littermates, and evaluated the cardiac hypertro-
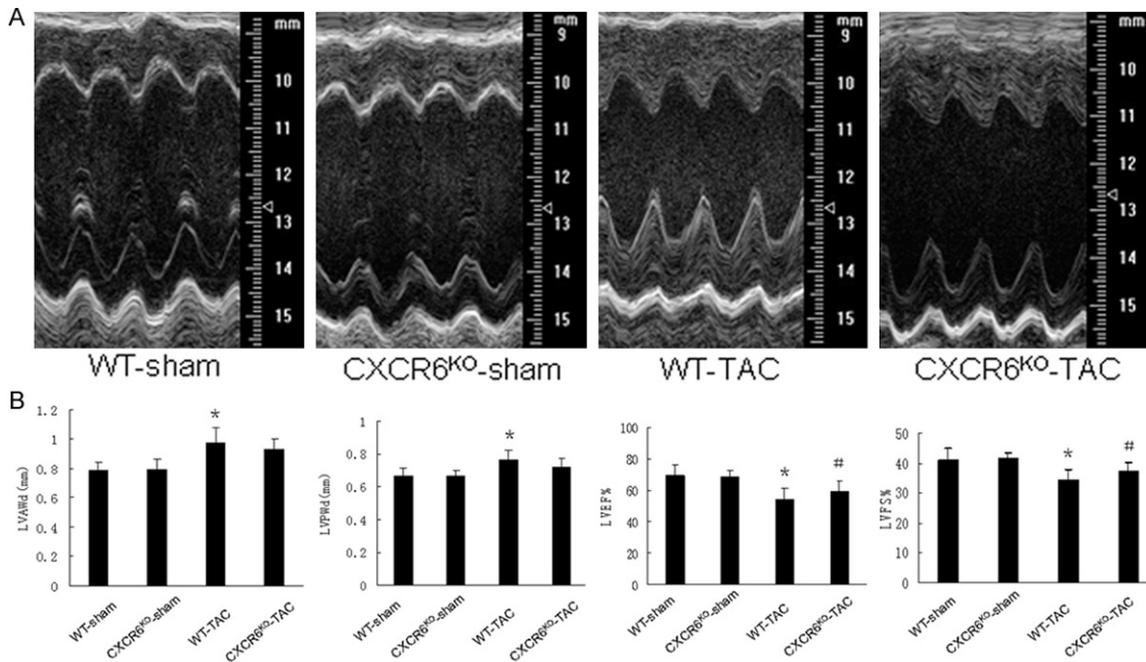
Figure 1. Echocardiographic analyses of the cardiac function. A. Representative echocardiographic M-mode of left ventricular images from CXCR6 WT and KO mice. B. Quantifications of LVAWd, LVPWd, LVEF% and LVFS%. Values are expressed as mean±SEM (n=6), where $^*P < 0.05$ vs. sham-operated mice of the same genotype; $^#P < 0.05$ vs. TAC-operated WT counterparts.

phic response and function by echocardiography at 6 weeks after TAC. Both groups of mice showed increased wall thickness of LV in response to TAC, but it was attenuated in CXCR6 KO mice when compared with WT littermates, the differences were not statistically significant. However, both LVEF% and LVFS% were significantly increased in CXCR6 KO mice after TAC when compared with WT littermates (**Figure 1**). These data indicated cardiac remodeling and dysfunction were largely prevented in CXCR6 KO mice after long-term pressure overload.

TNF-α signaling-induced cardiac fibrosis after pressure overload was attenuated in CXCR6 KO mice

Next, we examined the cardiac fibrogenic response after TAC for 6 weeks, the data showed that the transcriptional levels of both Col1a1 (type I collagen) and Col3a1 (type III collagen) were significantly reduced in myocardia of CXCR6 KO mice, when compared with their WT counterparts (**Figure 2A**). Masson's staining also confirmed the reduced collagen deposition in myocardium of CXCR6 KO mice after pressure overload (**Figure 2B**). Previous studies indicated that TNF-α signaling was centrally

involved in pressure overload mediated fibrogenisis and cardiac remodeling [17]. Therefore, we tested the role of TNF-α in the pressure overload model with CXCR6 KO mice. As shown in **Figure 1**, TNF-α serum level was lower in CXCR6 KO mice than that in WT mice (**Figure 2C**), and importantly that TNF-α type I receptor (TNFRI) was significantly decreased in CXCR6 deficient heart when compared with that in WT counterparts (**Figure 2D**). These data suggested that TNF-α activation contributed to enhanced cardiac fibrosis under pressure overload, which might be prevented by loss of CXCR6 expression.

Loss of CXCR6 impaired cardiac infiltration of monocytes and TNF-α release

Previous study suggested that monocyte and natural killer cells is one of the major sources for TNF-α production during chronic inflammation [24]. Interestingly, CXCR6 activation is essential for maturation and migration of CD11b^{+} monocytes and NKT cells. Thus, we further investigated whether these subpopulations of cells were infiltrated in pressure overloaded hearts. As shown in **Figure 3A**, a significant increase of leukocytes and monocytes infiltration was observed in CXCR6 WT
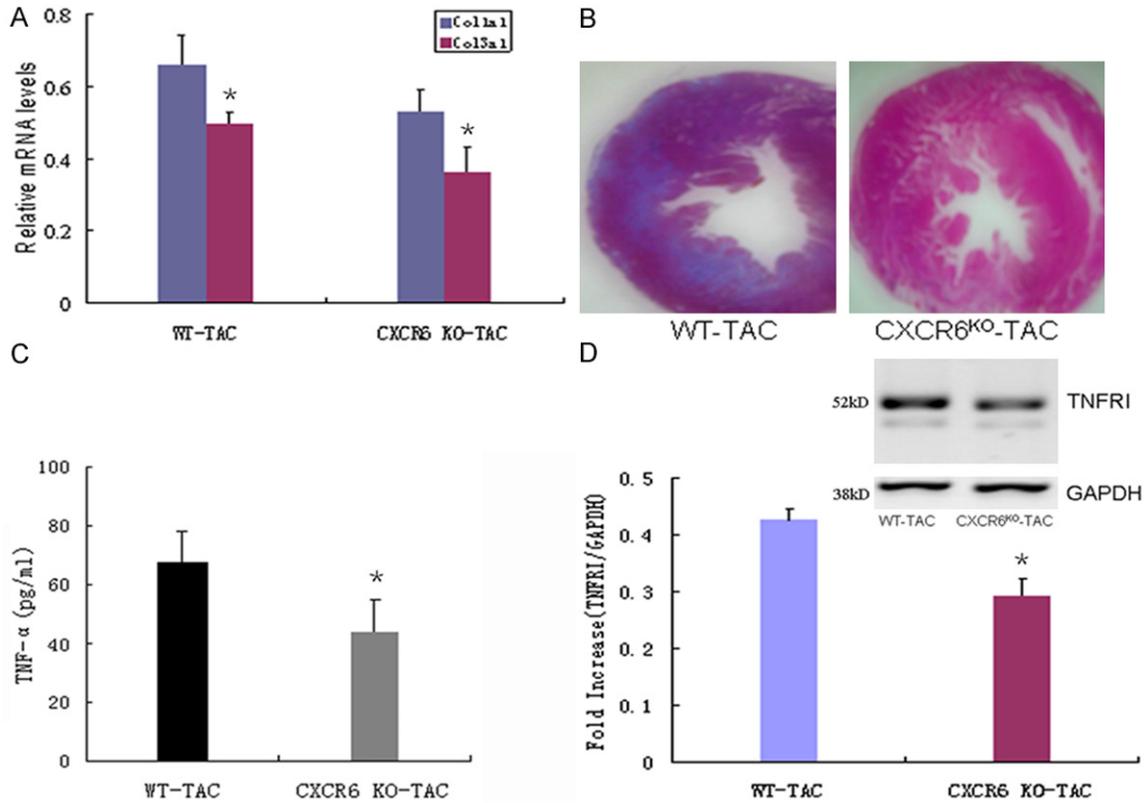
Figure 2. CXCR6 KO mice showed reduced cardiac fibrosis and TNF-α activation at 6 weeks after TAC. A. mRNA levels of collagen type I (Col1a1) and type III (Col3a1) in CXCR6 WT and KO mice. B. Masson's trichromic staining showed the collagen deposition (stained in blue) in myocardium in CXCR6 WT and KO mice. C. Serum levels of TNF-α were detected by ELISA. D. Western blot analysis of myocardial expression of TNF-α type I receptor (TNFRI). Values are expressed as mean±SEM (n=6), where $^{*}P < 0.05$ vs. TAC-operated WT mice.
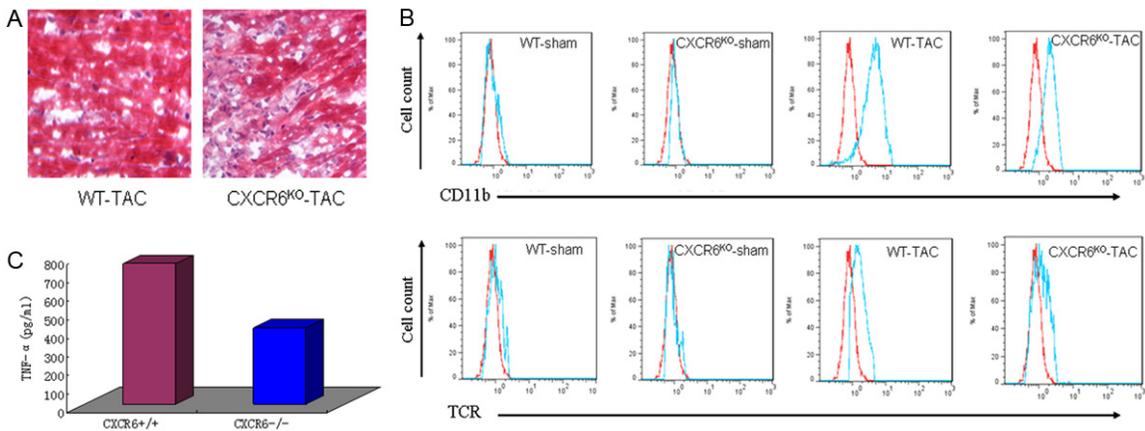
Figure 3. CXCR6 was essential for pressure overload-induced monocyte infiltration and TNF-α release. A. Representative photomicrographs of left ventricular sections labeled with H&E stain. B. Flow cytometry detection of CD11b (or TCR) positive cells isolated from myocardium. C. In vitro spleen-derived cells from CXCR6 WT and KO mice were isolated by anti-CD11b^{+} and cultured for 48 h, then stimulated by CXCL16 for another 24 h, TNF-α release in cytoplasm was detected by ELISA.

myocardium after TAC for 6 weeks, while it was significantly reduced in CXCR6 KO mice (**Figure 3A**). According to FACS analysis, we confirmed that CD11b positive cells and T cells were
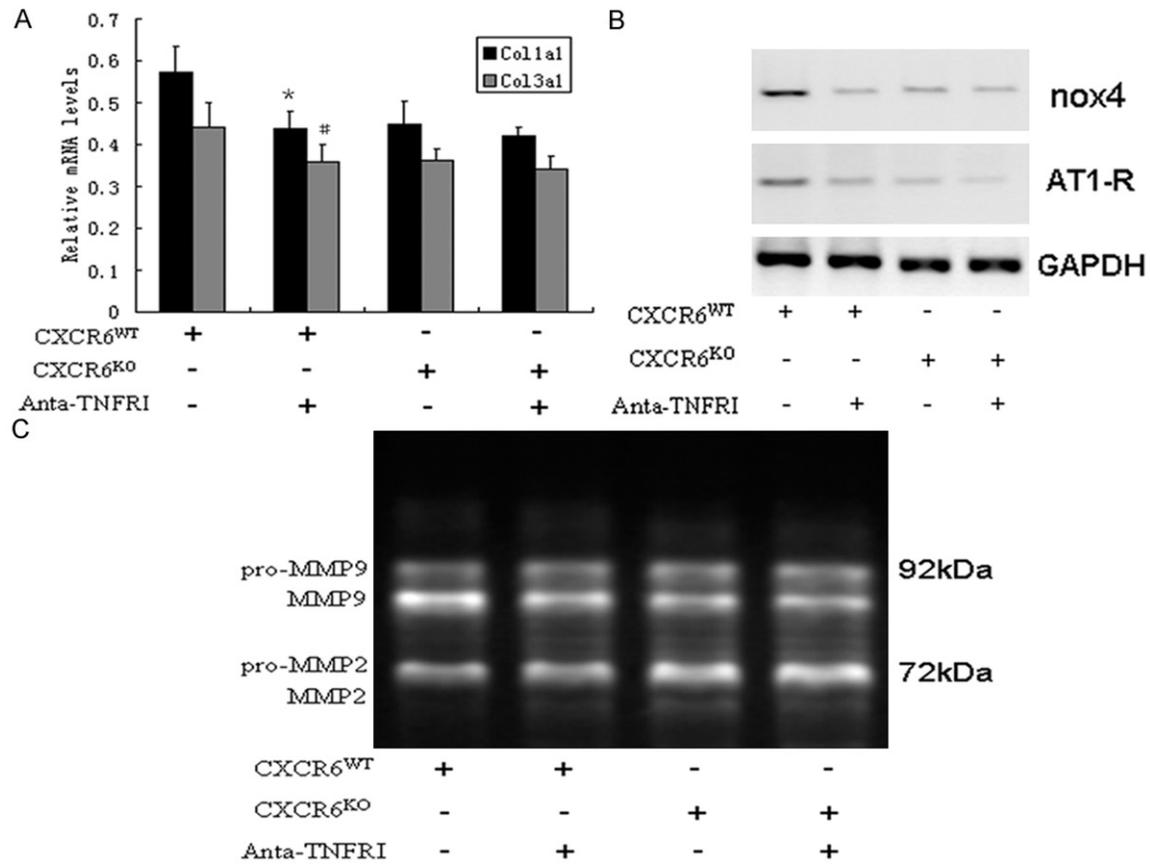
Figure 4. TNF-α receptor signaling was essential for CXCR6-mediated cardiac fibrosis. CXCR6 WT and KO mice were infused with TNFRI antagonist (sc-358755, Santa Cruz Biotech.) using Alzet osmotic minipumps (DURECT corporation, CA, USA), which implanted subcutaneously into the back of mice from 1 w to 6 w after TAC. A. Real-time PCR detection of the mRNA levels of collagen type I (Col1a1) and type III (Col3a1). B. Western blot showed the protein levels of NOX4 and AT1 receptor. C. Zymographic analysis showed the pro- and active-form of MMP2 and MMP9 in myocardium tissue. *,#$P < 0.05$ vs. TAC-operated WT mice.

increased in hearts response to pressure-overload when compared with in sham-operated hearts, but only CD11b+ subpopulations was markedly reduced by CXCR6 KO (**Figure 3B**), indicating a critical role of CXCR6 in mediating monocyte infiltration in hearts with pressure overload.

To further confirm whether CD11b+ subpopulations was the major source contributes to TNF-α release, we stimulated in vitro cultured spleen-isolated CD11b+ monocytes by CXCL16, and examined TNF-α release in cytoplasm after stimulation for 24 h. The results showed that TNF-α production was significantly reduced in spleen-derived CD11b+ monocytes from CXCR6 KO mice compared with that in WT control (**Figure 3C**), suggesting a critical role of CXCR6 in maintenance of monocytes function and TNF-α release.

TNF-α receptor inhibition attenuated MMP9 activity and cardiac fibrosis during pressure overload

We asked whether CXCR6-mediated myocardial fibrogenic response and remodeling in vivo was TNF-α signaling dependent. The TNF-α type I receptor (TNFRI) was blocked by pretreatment with TNFRI antagonist (WP9QY). At 6 weeks after TAC, pressure overload mediated enhancement of Col1a1 and Col3a1 gene expressions in CXCR6 WT mice were significantly reduced by pretreatment with TNFRI antagonist, which was not affected in CXCR6 KO mice (**Figure 4A**). Because TNFRI signaling was centrally involved in AT1 receptor-dependent cardiac fibrosis, we further examined the expressions of AT1 and NOX4 at 6 weeks after TAC in mice pretreated with TNFRI antagonist.
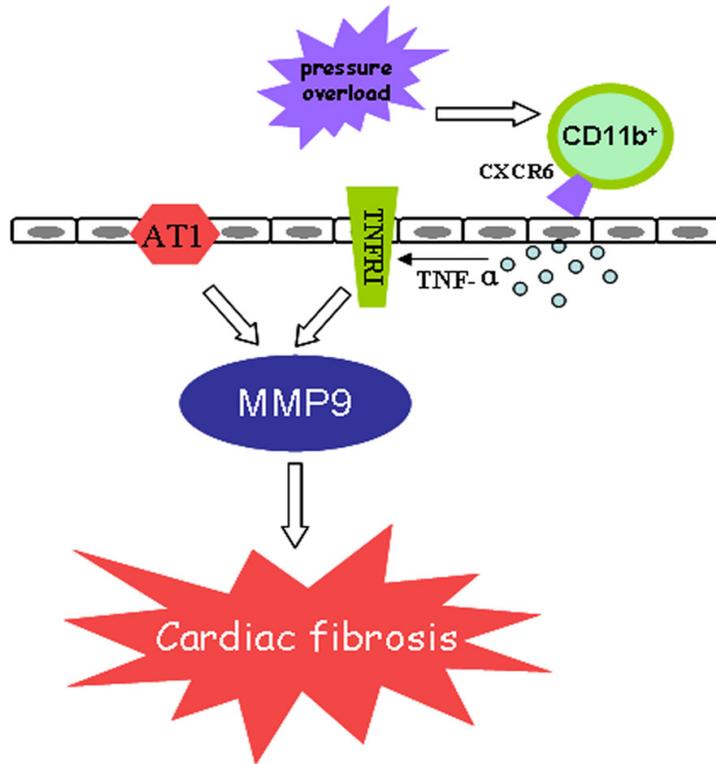
Figure 5. Schematic illustration depicting the major molecular mechanisms of CXCR6-mediated cardiac fibrosis in response to pressure overload.

Western blot analysis showed that both AT1 and NOX4 were decreased by TNFRI antagonist in CXCR6 WT mice after pressure overload, but not in CXCR6 KO mice (**Figure 4B**), indicating a critical role of TNF-α in cross-activation AT1 receptor mediated NOX4 signaling during pressure overload. Although TNFRI antagonist did not significantly alter the mRNA levels of MMP2 and MMP9 in CXCR6 WT and KO mice (data not shown), using gelatin zymography analysis, we found that MMPs activities induced by pressure overload, especially MMP9, were significantly inhibited by pretreatment of TNFRI antagonist in CXCR6 WT mice. Similar result was not observed in CXCR6 KO mice (**Figure 4C**). Collectively, these data demonstrated that TNF-α was required for pressure overload mediated cardiac remodeling, which was in part through AT1 receptor-dependent fibrogenic signaling pathway.

Discussion

In the present study, we revealed that CXCR6 played a critical role in promoting monocytes

adhesion and migration, which was greatly enhanced in myocardium after pressure overload. Accumulation of monocytes accelerated myocardial fibrogenic response and dysfunction through TNF-α release and TNF-α-dependent activation of MMP9 signaling as depicted in **Figure 5**.

CXCRs are one of the major members of chemokines family that preferentially bind CXC-chemokines. Increasing evidence indicated that CXCRs were linked to the inflammatory processes and myocardial remodeling. CCR2 and CXCR4 expressions were enhanced in patients with end-stage heart failure [25]. Monocytes infiltration was blunted in CXCR2 KO mice, the benefit of MIF for cardiac regeneration and scar formation was counteracted by CXCR2-dependent monocytes recruitment [13]. In our study, pressure overload induced upregulation of CXCR6 led to accumulation of CD11b+ cells in myocardia, which contributed to myocardial inflammation through releasing of TNF-α, IL-4 and IFN-γ. Zhao G et al. reported that CXCR6 deficiency significantly ameliorated IFN-γ secretion and autophagy during cardiac reperfusion injury [26]. Interestingly, our findings here suggested that monocytes infiltration caused activation of TNF-α instead of IFN-γ in a CXCR6-dependent way. Ischemia reperfusion induced CXCR6 positive monocytes recruitment to endothelium, and caused endothelial cells injury majorly depending on IFN-γ release, while CXCR6 might also contribute to TNF-α-dominant tissue injury, previous evidences indicated that TNF-α was critically involved in pressure overload-induced cardiac fibrosis [18, 26]. Our data confirmed that TNF-α and its receptor were significantly increased at 6w after TAC, thus we assumed CXCR6 deficiency ultimately led to attenuation of TNF-α and cardiac remodeling during pressure overload.

TNF-α was once considered as one of the important inflammatory cytokines to induce cell apoptosis via NF-κB activation. However, recent studies also indicated a critical role of

TNF-α in promoting collagen synthesis and fibrosis [17, 18, 27]. Serum TNF-α level was increased in experimental chronic renal failure patients with progressive cardiac fibrosis, and soluble TNF-α was regarded to mediate the transition from pulmonary inflammation to fibrosis [18]. TNF-α antibody or inhibitor was proved to be effective in the treatment of hepatic fibrosis through reducing inflammation and cell necrosis [19, 20]. Plenty of studies revealed that multiple resident innate immune cells producing TNF-α were mobilized during pressure overload [8, 28]. Mobilization of dendritic cells (DCs) controlled liver inflammation and fibrosis through activating TNF-α signaling [29]. We here proved that pressure overload caused accumulation of monocytes in myocardia, which was the major source of TNF-α production and might lead to cardiac fibrosis. Meanwhile, cardiac TNFRI was correspondently activated at 6 weeks after TAC. TNFRI activation was essential to AngII synthesis and AngII-mediated MCP-1 expression [30]. The later resulted in the uptake of monocytes into the heart. Notably, TNF-α/TNFRI signaling axis also contributed to cross-activation of TGF-β and connective tissue growth factor (CTGF) [31]. Both TGF-β and CTGF were profibrogenic cytokines that critically involved in pressure overload induced myocardial fibrosis [17, 27]. Our data implicated that blocking of TNF-α/TNFRI signaling could attenuate AT1 receptor and NOX4 expression, Nox4 NADPH oxidase was the major source of mitochondrial reactive oxygen species (ROS), which played a central role in AT1 receptor mediated cardiac remodeling via activating MMPs system and fibronectin production [21, 32]. We also revealed that CXCR6 deficiency significantly reduced MMP9 activity and fibrogenic response, this could also be achieved by inhibiting TNFRI. Thus we assumed that CD11b monocytes infiltration and TNF-α release resulted in TNF-α/AT1 receptor cross-activation, which led to MMPs activation and cardiac fibrogensis. In addition, CXCR6 also contributed to cell migration during hypoxia [33], especially for the recruitment of bone marrow derived fibroblasts and cell differentiation [34, 35]. CXCR6-depenent TNF-α signaling was required for MMP9 induction, whether it was also required for fibroblasts migration and differentiation would need further investigation.

CXCR6 has been speculated highly expressed on T cells subpopulations, and functioned in recruitment of CD8^{+} T cells and NKT cells [15, 36]. Interestingly, recruitment of CD8^{+} T cells was not found after pressure overload for 6 weeks. NKT cells were also not as significant as the infiltration of CD11b^{+} monocytes. One possible reason is that the acute phase response of immune reaction might not be monitored at a long-term pressure overload stress, and instead, chronic inflammatory response took place in the injured myocardia [37, 38]. Thereby, large amount of monocytes were observed at 6 weeks after TAC. Induction of CXCL16, the specific CXCR6 ligand, caused activation of in vitro cultured spleen-derived monocytes and TNF-α release, at least partly confirmed the critical role of CXCR6 in maturation and migration of monocytes. However, one recent study revealed that accumulation of CD4^{+} T cells resulted in adverse myocardial remodeling and promoted the transition from compensated cardiac hypertrophy to heart failure [28]. Therefore, additional researches will be required to ascertain the pathologic role of T cells in heart remodeling and their function associated with monocytes during myocardial injury during pressure overload.

In summary, our data emphasized the importance of CXCR6 in myocardial remodeling via regulating monocytes infiltration and TNF-α-dependent MMP9 signaling, and open up the possibility that targeting this chemokine might be a therapeutic option for hypertensive heart and vascular diseases.

Disclosure of conflict of interest

None.

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