Original Article
A critical role of CXCR6 in murine myocardial ischemia-reperfusion injury through activating IL-17a producing iNKT cells

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Abstract: The pathologic role of invariant natural killer T (iNKT) cells in reperfusion injury after myocardial infarction remains elusive although they induce detrimental inflammatory response in hepatic and renal ischemia/reperfusion (I/R) injury. Here we suppress the activation of iNKT cells by knocking out the chemokine receptor, CXCR6, and investigate the precise role of iNKT cells in cardiac I/R injury. Our data indicate that cardiac infiltrated iNKT cells promote IL-17a secretion and IL-17a-dependent cell apoptosis. IL-17a mRNA and serum level are significantly lower in hearts from CXCR6$^{-/-}$ mice than in those from wild-type (WT) mice, and IL-17a producing iNKT cells are also less activated in CXCR6$^{-/-}$ mice. Further results confirm that CXCR6 deficiency-mediated cardiac protection against I/R injury is abolished by recombined IL-17a treatment. Left ventricular (LV) infarct size was significantly increased and LV ejection fraction (LVEF) reduced by rIL-17a in CXCR6$^{-/-}$ mice, as were decreased expression of Beclin-1 and increased expression of Bax. We therefore conclude that CXCR6 activation is required for myocardial infiltration of IL-17a producing iNKT cells after I/R injury. CXCR6 inhibition before reperfusion period may reduce infarct size and preserve cardiac function partly through attenuating the activity of IL-17a producing iNKT cells.

Keywords: CXCR6, IL-17a, iNKT cells, ischemia/reperfusion

Introduction

Invariant natural killer T (iNKT) cells are considered to bridge the innate and adaptive immune system via their abilities to produce amounts of cytokines and modulate Th1 or Th2 response [1, 2]. Recent studies indicate that iNKT cells probably play a protective role in ischemic/reperfusion (I/R)-induced myocardial injury [3, 4]. iNKT cells activated by α-GalCer (α-GC) can enhance the level of IL-10, which ameliorates inflammatory response, reduces infarct size and improves cardiac function. However, the precise role of iNKT cells in I/R injury remains controversial, because inhibition of iNKT cells may reduce hepatic and renal I/R injury [5], and iNKT cells promotes kidney injury through increasing cell apoptosis [6]. In addition, iNKT cells also produce multiple inflammatory cytokines, such as TNF-α, IL-17a and IFN-γ, many of which have detrimental effects on cardiac I/R

injury [7-9]. Herein, we investigate the pathological role of iNKT cells in I/R-mediated cardiac injury in a model of CXCR6 knockout (KO) mice.

Both human and murine iNKT cells express high level of the chemokine receptor, CXCR6 [10, 11]. CXCR6 activation with ligands CXCL16 binding is essential for iNKT cells recruitment and homing [10-12]. A body of evidence indicates that CXCL16 is a hallmark of cardiovascular diseases [13-15]. It has been shown that CXCL16 promotes the distribution of iNKT cells in cardiac allograft [12, 16], and pulmonary and renal I/R injury are greatly attenuated in Jα18$^{-/-}$ mice lacking IL-17a-producing iNKT cells [8, 17]. We have also reported that cardiac ischemia injury is reduced and cardiac function is improved in CXCR6$^{-/-}$ mice [18]. Therefore, in this study we define iNKT cells as a key regulator of inflammatory cytokines that contribute to cardiac I/R injury.
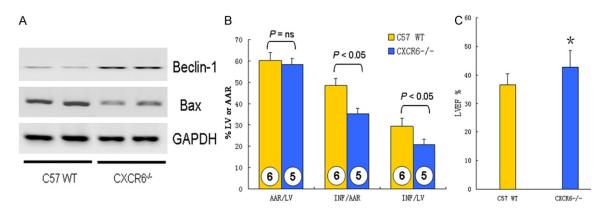
Figure 1. CXCR6 deficiency decreased the extent of I/R-induced cardiac injury and improved LV function in mice. A. Representative Western blots of Beclin-1 and Bax using LV tissue lysates after I/R injury. B. LV, AAR and INF were measured by image analyzer after TTC staining, cumulative data of AAR as a percentage of LV and INF as a percentage of AAR or LV for wild type (WT, n=6) and CXCR6$^{-/-}$ (n=5) mice. C. Echocardiographic acquisition and analysis of left ventricular ejection fraction (LVEF) of WT and CXCR6$^{-/-}$ mice. *$P < 0.05$, LV, left ventricle; AAR, area at risk; INF, infarct area.

Materials and methods

Experimental mice and model of myocardial I/R injury

CXCR6$^{-/-}$ mice were kindly provided by professor Wang Jianhua (Shanghai Jiao-tong University) and backcrossed into a pure C57BL/6 background for eight generations as previously described [18]. 8~10 weeks old male CXCR6$^{-/-}$ mice and their wild-type (WT) counterparts were performed with cardiac I/R injury models. In brief, Mice were anesthetized with isoflurane, and ventilated with room air supplemented with oxygen, at a rate of 130 strokes per minute and a tidal volume of 0.2 ml. Mice body temperature was maintained within 35~37°C using a heating pad. After exposure of the hearts, the left anterior descending coronary artery (LAD) was ligated with a 7.0 nylon suture from its origin with a slipknot. Followed by 45 min of ischemia, the LAD was reperfused for another 48 h. The experimental protocols were approved by Fudan University Committee on Laboratory Animals, in accordance with the guidelines of the China Council on Animal Care.

Measurement of area at risk (AAR) and infarct (INF) size

AAR and INF size were accessed by triphenyl-tetrazolium chloride (TTC) staining. In brief, mice were euthanized and hearts were removed and perfused with saline on a Langendorff system after 48 hours of reperfusion, then Evans blue dye (Sigma-Aldrich, Inc. USA) was injected into the right ventricular cavity to distinguish between ischemic (AAR) and nonischemic myocardium. Each heart was then sliced horizontally and incubated with 1.5% triphenyltetrazolium chloride (TTC) (Sigma-Aldrich, Inc. USA), For each slice, the AAR and MI areas were digitally recorded with a microscope attached camera, and AAR and INF size were measured from enlarged digital microscopic images by computer-assisted planimetry software (Qwin V3; Leica, Inc. Germany).

Echocardiography analyses of cardiac function

Transthoracic echocardiography was performed using a VisualSonics system (Vevo770®, VisualSonics Inc. Toronto, Canada) equipped with a linear 30-MHz probe, as previously described [18]. Mice were anesthetized and M-mode images of the left ventricle were recorded. All the measurements were performed with double-blind methods.

Cell culture

Mice spleens were removed and pooled, Single-cell suspensions were generated by grinding tissue with a sterile nylon mesh. Erythrocytes were suspended and removed in the lysis buffer (0.15 mol/L NH$_4$Cl, 1 mol/L KHCO$_3$, 0.1 mmol/L EDTA-2Na, pH 7.2) and rinsing the cells twice with Gibco® RPMI 1640 medium. Resuspended cells were seeded into 6-well cell culture plates, cultured in RPMI 1640 medium containing 10% fetal bovine serum (FBS). Cells were stimulated by α-GalCer (100 ng/ml, Enzo Life Sciences, Inc.) for 48 h, and then single-cell suspensions were generated. Cells were
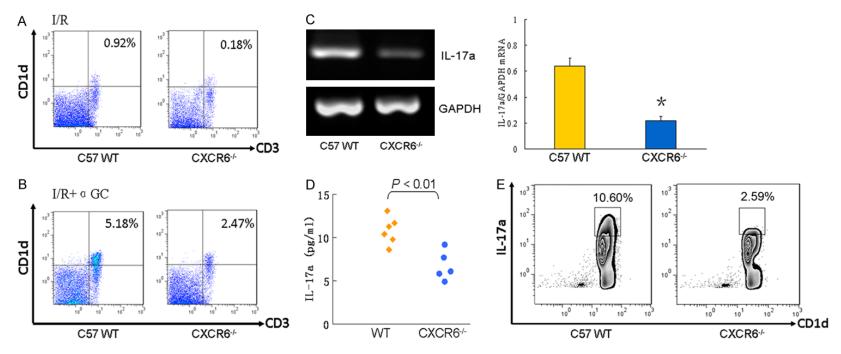
Figure 2. The production of IL-17a by iNKT cells was reduced in CXCR6$^{-/-}$ mice following I/R injury. A. Representative flow cytometric analysis of cardiac mononuclear cell (MNC) suspensions from C57 WT or CXCR6$^{-/-}$ mice. B. With α-GalCer induction for 24 h before I/R injury. C. RT-PCR and Quantitative assessment of IL-17a mRNA in LV tissue from WT and CXCR6$^{-/-}$ mice after I/R injury. D. Detection of serum IL-17a levels in WT and CXCR6$^{-/-}$ mice after I/R injury using ELISA kit. E. Spleen monocytes from WT or CXCR6$^{-/-}$ mice were pooled isolated by anti-CD11b antibody, then cells were cultured and stimulated by α-GC for 48 h. CD1d$^+$IL-17a$^+$ cells were measured by FACS. The numbers shown upside of the blocks represented the percentage of IL-17a producing iNKT cells which indicated by the contour diagrams.

stained and subjected to flow cytometric analysis as previously described [18].

Serum IL-17a was determined by ELISA

Serum level of IL-17a were determined by ELISA kits (R&D Systems, Inc.), and absorbance was measured at 450 nm using Bio-Tec Reader, the results were expressed as pg/ml for each samples.

RT-PCR detection for IL-17a

Total RNA was extracted from left ventricular tissues by Two-Step PrimeScript® RT-PCR Kit II (TaKaRa, Inc.). Semi-quantitative RT-PCR analysis was performed on PTC200 (BIO-RAD) with the condition at 95°C for 2 min; at 94°C for 30 s, 54°C for 45 s and 72°C for 45 s, 35 cycles; at 72°C for 5 min. The primers used as follows: 5'-GACTACCTCAACCGTTCCA-3'; 3'-TTTTCTTCC-ACATTTCCCCA-5'. All the bands were scanned and analyzed by ChemiDoc™ XRS system (Bio-Rad, Inc.).

Western blot analysis

Total proteins were isolated from left ventricular tissue or cultured cardiomyocytes and lysed in a buffer containing 50 mM Tris (pH7.4), 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. Protein lyses were separated by electrophoresis on the 10% SDS-PAGE gel and transferred to the PVDF membranes (Millipore Corp.). Then the membrane were blocked for 1 h with 5% bovine serum albumin (BSA), followed by probing with anti-Caspase3 (9662, Cell Signaling) or anti-Bcl-2 (2876, Cell Signaling).

Statistical analysis

Data were expressed as mean ± s.e.m. Student t-tests were performed for unpaired values and one-way ANOVA for multi-group differences. All tests were 2 tailed. A value of *P*<0.05 was considered statistically significant.

Results

Cardiac injury was attenuated in CXCR6^-/- mice following I/R injury

Ischemia for 45 min and followed by reperfusion for 48 h, cardiac autophagy and apoptosis

were examined in risky border zone of left ventricular myocardium, and cardiac function was evaluated in CXCR6^-/- mice and C57BL/6 WT littermates. As shown in **Figure 1A**, I/R injury induced a significant increase of Bax but a reduction of Beclin-1 in WT mice, compared with those in CXCR6^-/- mice. The results of TTC staining reflected that the area at risk (AAR) per LV was similar in WT and CXCR6^-/- mice after I/R injury, but the INF relative to the AAR was decreased by 27.3% (48.6±3.2% vs. 35.3±2.6%, P<0.05) and the INF relative to the entire LV by 30% (29.4±3.6% vs. 20.7±2.5%, P<0.05) in CXCR6^-/- mice when compared with WT mice (**Figure 1B**). Meanwhile, M-mode echocardiography analysis suggested that left ventricular ejection fraction (LVEF) was improved by 15% in CXCR6^-/- mice (**Figure 1C**). Together, these data indicated that CXCR6 deficiency limited the extent of cardiac injury following myocardial I/R.

Inactivation of IL-17a producing iNKT cells in CXCR6^-/- mice after I/R injury

iNKT cells played a key role in I/R-induced organ injury, thus we first assessed the proportion of iNKT cells in ischemic hearts after I/R stress. Flow cytometric analysis showed that CD1d positive T cells were reduced in hearts of CXCR6^-/- mice after I/R when compared with those of C57BL/6 WT mice (**Figure 2A**). Next, we stimulated the mice with α-GC (0.1 μg/g body weight), the iNKT cells specific activator, by intraperitoneal injection. However, the number of iNKT cells remained lower in CXCR6^-/- hearts than those in WT hearts (**Figure 2B**). Recent studies revealed that IL-17a contributed to myocardial I/R injury through stimulating cardiomyocyte apoptosis and neutrophil infiltration [19], and iNKT cells was a major source of IL-17a production after lung I/R injury, thus we further investigated the influence of cardiac iNKT cells on IL-17a producing. A significant elevation of serum IL-17a was observed at 48 h after reperfusion in WT mice, but not in CXCR6^-/- mice (**Figure 2D**). RT-PCR analysis showed that the mRNA level of IL-17a in CXCR6^-/- heart was lower than in WT littermates (**Figure 2C**). Because many sources of T lymphocytes, such as Th17 cells, CD8 T cells, γδ T cells and iNKT cells could produce IL-17a [20-22], we then examined the role of CXCR6 in iNKT-dependent IL-17a production. The spleens from CXCR6^-/- mice and WT control were pooled
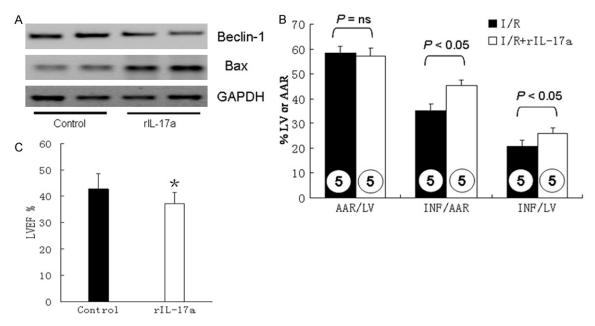
Figure 3. rIL-17a treatment reversed myocardial protection against I/R injury in CXCR6$^{-/-}$ mice. A. Representative Western blots of Beclin-1 and Bax in LV tissue lysates from CXCR6$^{-/-}$ mice with or without treatment of rIL-17a for 2 h before I/R injury. B. Cumulative data of AAR as a percentage of LV and INF as a percentage of AAR or LV for CXCR6$^{-/-}$ mice pretreated with rIL-17a (n=5) or with saline control (n=5). C. Echocardiographic analysis of LVEF from CXCR6$^{-/-}$ mice pretreated with rIL-17a or saline control. *$P < 0.05$, n=5.

and the splenocytes were incubated with α-GC for 48 h. Consistent with the IL-17a mRNA expression patterns in CXCR6$^{-/-}$ and WT hearts, the proportion of IL-17a producing iNKT cells were was only increased in WT splenocytes, but greatly reduced in CXCR6$^{-/-}$ splenocytes (**Figure 2E**). These data indicated that CXCR6 deficiency impaired myocardial infiltration of iNKT cells and IL-17a production in response to I/R stress.

IL-17a induction aggregated myocardial damage in CXCR6$^{-/-}$ mice following I/R

IL-17a is known to cause the pathological progression of ventricular myocytes apoptosis and cardiac remodeling [19, 23]. Therefore, we investigated whether I/R-induced cardiac injury was aggregated cardiac injury and dysfunction in CXCR6$^{-/-}$ mice after treating with recombinant IL-17a (rIL-17a). The cardiac autophagy and apoptosis caused by I/R were determined in LV tissues of CXCR6$^{-/-}$ mice in the presence or absence of rIL-17a induction. As seen in **Figure 3**, rIL-17a significantly suppressed Beclin-1 and enhanced Bax expression in CXCR6$^{-/-}$ LV tissues (**Figure 3A**). Meanwhile, TTC staining quantitative assessment showed that the percentage of INF/LV was enlarged and LVEF was decreased in CXCR6$^{-/-}$ mice after treating with rIL-17a (**Figure 3B**, **3C**). Together, these results sug-

gested that IL-17a-producing iNKT cells played a critical role in cardiac damage and dysfunction in response to I/R stress.

Discussion

In the present study, we demonstrated that IL-17a level was significantly elevated in myocardium post I/R injury, which might be induced by the activation of iNKT cells during reperfusion period. Considering that the activation of CXCR6 was essential for the maturation and recruitment of iNKT cells [10-12], and in the present study, we observed that IL-17a production and myocardial injury were both reduced in CXCR6$^{-/-}$ mice. We thus supported the notion that CXCR6 deficiency alleviated the cardiac infiltration of iNKT cells after I/R stress, and also alleviated IL-17a-dependent myocyte apoptosis and cardiac injury.

Inconsistent with our findings, Tsutsui et al. previously reported that after myocardial infarction, the activation of iNKT cells by α-GC reduced cardiac fibrosis and hypertrophy, and ameliorated ventricular remodeling and heart failure [3]. Another study from the same lab showed that iNKT cells ameliorated myocardial I/R injury by suppressing myocardial apoptosis, infiltration of inflammatory cell and production

of inflammatory cytokines [4]. Both reports indicated that iNKT cells activation prevented myocardial I/R injury and LV remodeling through enhancing the expression of IL-10. However, iNKT cells disrupted Jα18^{-/-} mice also exhibited decreased infarct size to the same extent as C57 WT mice in response to α-GC [24], suggesting that the secretion of IL-10 rather than iNKT cells themselves contributed to the protection against cardiac ischemic injury [25]. The source of IL-10 in response to α-GC treatment remained to be elucidated. Besides iNKT cells, many other immune cells, such as macrophages, Th2 cells and B lymphocytes, were alleged sources of IL-10 [26, 27]. In our previous study, we did not observe a pronounced decrease of IL-10 in CXCR6^{-/-} mice when compared with WT mice during the reperfusion times [18]. Thus, the role of IL-10 might not be critically involved in cardiac I/R injury at least in the model of CXCR6^{-/-} mice.

Instead, iNKT cells stimulation contributed to the secretion of multiple inflammatory cytokines, such as TNF-α, IFN-γ and IL-17a. IL-17a had been linked to the pathogenesis of several inflammatory and cardiovascular diseases [7-9]. Suppression of IL-17a attenuated the development of atherosclerotic lesion in ApoE^{-/-} mice [28]. In contrast, stimulation with IL-17a promoted vascular dysfunction [29]. IL-17a level was enhanced after myocardial I/R injury, which played a fundamental role in post infarct inflammatory and apoptotic responses [30, 31]. In our study, we found that both mRNA transcriptional and serum levels of IL-17a were significantly reduced in CXCR6^{-/-} mice, and the mobilization of IL-17a-producing iNKT cells were impaired in CXCR6 deficient spleens. These data suggested that CXCR6 was essential for iNKT cells activation and IL-17a production. Most importantly, CXCR6 deficiency mediated protection against cardiac I/R injury and LV remodeling was abolished by rIL-17a treatment. Several lines of evidences clearly suggested that iNKT cell influx contributed to organ injury observed in kidney and lung. Li and colleagues reported that renal injury was ameliorated in iNKT cells deficient Jα18^{-/-} mice [17], in which neutrophil infiltration and IFN-γ production was significantly suppressed. In addition, mice lacking IL-17a-producing iNKT cells exhibited lower levels of TNF-α and IL-6 after pulmonary I/R injury, and transfection of WT but not IL-17a^{-/-} iNKT cells abolished the protective effects on pulmonary injury in Jα18^{-/-} mice [8, 9]. Here we further confirmed that the lacking of IL-17a-producing iNKT cells contributed to reduced infarct size and improved cardiac function post I/R injury in CXCR6^{-/-} mice.

Increased cardiomyocyte apoptosis was one of the major reasons for I/R injury-mediated cardiac remodeling and dysfunction [19, 23]. IL-17a enhanced myocyte apoptosis via regulating the ratio of Bax/Bcl-2. I/R-induced Bax was markedly decreased in CXCR6^{-/-} hearts, which balanced cardiomyocyte survival and cardiac function. However, Beclin-1 expression at 24 h after reperfusion was significantly upregulated in CXCR6^{-/-} mice. Accumulation of autophagosomes could be suppressed in Beclin-1^{-/-} mice [32], which led to the detrimental effect on ischemic damage especially during the period of reperfusion. Interaction between Beclin-1 and Bcl-2 family proteins in response to I/R stress accelerated the phosphorylation of mTOR pathway [33], which promoted the recovery from cardiac I/R injury [34]. In this study, we found that disruption of CXCR6 promoted the expression of Beclin-1, which was highly associated with reduced infarct size and improved LV function. Notably, both increased Beclin-1 and decreased Bax could be reversed by rIL-17a pretreatment. These data support the hypothesis that CXCR6 deficiency-mediated cardioprotection after I/R stress was due to attenuated IL-17a production rather than enhanced IL-10 expression, at least partly through inactivation of IL-17a-producing iNKT cells.

In conclusion, CXCR6 is required for iNKT cells activation and myocardial infiltration during cardiac I/R stress, which promotes cardiac dysfunction, LV remodeling and cardiomyocyte apoptosis through enhanced production of IL-17a cytokines. Therefore, CXCR6 may represent a novel therapeutic target for cardiac ischemic diseases.

Acknowledgements

Disclosure of conflict of interest

None.

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