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Long pentraxin PTX3 attenuates ischemia reperfusion injury in a cardiac transplantation model

Hongfei Zhu,[1] Dan Cui,[2] Kebin Liu,[1] Li Wang,[1] Lili Huang[1] and Jinjie Li[1]

1 Department of Anesthesiology and The State Key Laboratory Breeding Base of Basic Science of Stomatology (Hubei-MOST) & Key Laboratory of Oral Biomedicine Ministry of Education, School & Hospital of Stomatology, Wuhan University, Wuhan, China

2 Public Health School, Wuhan University, Wuhan, China

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Correspondence
Hongfei Zhu, Department of Anesthesiology and The State Key Laboratory Breeding Base of Basic Science of Stomatology (Hubei-MOST) & Key Laboratory of Oral Biomedicine Ministry of Education, School & Hospital of Stomatology, Wuhan University, Luoyu Road 237#, Wuhan 430079, China.
Tel.: +0086-18971319201;
fax: +0086-27-87686305;
e-mail: hongfeizhu1973@163.com

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Summary

Ischemia reperfusion (IR) injury is a major issue in cardiac transplantation, and inflammatory processes play a major role in myocardial IR injury. Long pentraxin-3 (PTX3) is a member of a phylogenetically conserved group of acute-phase reactants that are involved in inflammation and innate immunity. In our study, hearts of C57Bl/6 mice were flushed and stored in cold Bretschneider solution for 8 h and then transplanted into syngeneic recipient. We found that both mRNA and protein levels of PTX3 were increased following myocardial IR injury; neutralizing antibody against PTX3 aggravated cardiomyocyte apoptosis and recruitment of neutrophils and macrophages. Troponin T (TnT) production on 24 h after myocardial IR injury was reduced by exogenous PTX3 administration and increased by PTX3 neutralization in comparison with control. Cardiac output at 60 mmHg of afterload pressure was also increased in hearts with exogenous PTX3 administration and decreased with PTX3 neutralization (PTX3: 58.4 ± 7.4 ml/min; Control: 24.5 ± 3.8 ml/min; Anti-PTX3: 11.6 ± 1.7 ml/min; $P < 0.05$). Furthermore, PTX3 restricted expansion of γδ T cell that was the major source of IL-17A and down-regulated expression of IL-23 and IL-17A. In conclusion, PTX3 played a protective role in cardiomyocyte IR injury. PTX3 ameliorated cardiomyocyte apoptosis and infiltration of neutrophil and macrophage and then improved hemodynamic performance. This was associated with restricted γδ T-cell expansion and decreased IL-23/IL-17A expression.

Introduction

Cardiac transplantation is the last resort for patients with end-stage heart failure. Ischemia reperfusion (IR) injury is a major issue in cardiac transplantation. IR injury is associated with increased primary organ dysfunction and subsequent delayed organ function after cardiac transplantation. In the long term, this correlates with increased episodes of acute and chronic rejections [1].

Pentraxin 3 (PTX3) is a member of a superfamily of conserved proteins characterized by a cyclic multimeric structure and a conserved c-terminal domain [2,3]. Levels of PTX3, produced by a range of cell types, including myeloid dendritic cells, endothelial cells, epithelial cells, mononuclear phagocytes, smooth muscle cells, adipocytes, fibroblasts, synovial cells, and chondrocytes, are very low in serum and tissues of healthy subjects but rapidly increased in response to a variety of inflammatory stimuli [4,5]. Previous paper indicated that PTX3 played a cardioprotective role in acute myocardial infarction in mice [6], but the role of PTX3 in cardiac transplantation has not been investigated, and the mechanisms involved are still largely unknown.

Interleukin (IL)-17A is a member of the IL-17 family, which includes six structurally related isoforms: IL-17A, IL-17B, IL-17C, IL-17D, IL-17E, and IL-17F [7]. IL-17A was secreted by different cells, including Th17 cells, γδ T cells, NK cells, NKT cells, and neutrophils [8,9]. IL-17A is a critical mediator of neutrophils recruitment and migration through induction of granulopoiesis and neutrophil

chemokines [10]. Previous studies have indicated that exogenous PTX3 could down-regulate IL-17A production in mice chronic granulomatous disease model [11]. However, the impact of PTX3 on IL-23/IL-17 axis in myocardial IR injury is still unknown.

In this study, we demonstrated that PTX3 played a protective role in cardiomyocyte IR injury. PTX3 ameliorated cardiomyocyte apoptosis and infiltration of neutrophil and macrophage and then improved hemodynamic performance. This was associated with restricted expansion of γδ T cell and decreased expression of IL-23/IL-17A.

Materials and methods

Animals

Inbred male C57Bl/6 mice, used as donors and recipients, were from the Center of Experimental Animals, Wuhan University, China. All the mice were male at 15–20 g in weight, which were housed in specific pathogen-free facility with regular food and water adlibitum. Experiments were approved by the Institutional Animal Care and Use Committee at Wuhan University (Wuhan, China).

Heterotopic cardiac transplant and post-transplant therapies

Syngeneic heart transplantation was performed by a modified nonsuture cuff technique previously described by Heron *et al.* [12]. Hearts were stored in cold Bretschneider solution for 8 h before transplantation with consecutive *in vivo* reperfusion for 8, 24, or 48 h [1]. For neutralization of endogenous PTX3, 0.2 mg of anti-mouse PTX3 mAb (Sigma-Aldrich, St. Louis, MO, USA) was administered i.v. 5 min prior to reperfusion. Recombined PTX3 was injected with 5 mg/kg at 0, 6, and 12 h after reperfusion [13]. For deletion of γδ T cells, the mice were injected i.v. with 0.5 mg of an anti-γδTCR mAb (ATCC, Manassas, VA, USA) 5 min prior to reperfusion. For neutralization of endogenous IL-17A or IL-23, 0.2 mg of neutralizing rabbit anti-mouse IL-17A (Biolegend, San Diego, CA, USA) or neutralizing rabbit anti-mouse IL-23p19 (eBioscience, San Diego, CA, USA) was administered i.v. 5 min prior to reperfusion. Control rabbit IgG was used as isotype control [14].

Function assessment

To assess graft function, transplanted hearts were evaluated using an isolated working heart apparatus, as previously described [1]. After 8 h of ischemia and 24 h of *in vivo* reperfusion, hearts were excised and mounted on the isolated working heart apparatus. Hearts were perfused with a preload pressure of 8 mmHg and exposed to an afterload pressure of 60 mmHg while being paced at 250 beats per min. Pressure–volume ratio was performed as follows: afterload pressure was increased stepwise by 10 mmHg from 10 mmHg up to 180 mmHg of afterload pressure every 10 s.

Serum analysis of cardiac troponin T

Analysis of cardiac serum TnT levels was performed as follows: 1 ml of heparinized blood was centrifuged to obtain plasma and stored at −30 °C until assayed. TnT was measured using the cardiac reader system according to the manufactures' instruction.

Myocardial apoptosis

Terminal deoxynucleotidyl-transferase-mediated dUTP nick-end labeling (TUNEL) staining was performed as previously described [14]. Hearts were fixed in 4% paraformaldehyde, embedded in paraffin, cut into 5-μm thickness sections and treated as instructed in the In Situ Cell Death Detection kit (Roche Diagnostics GmbH, Mannheim, Germany). Total nuclei were stained with DAPI. Cardiac caspase-3 activity was measured as previously described [6] using a caspase colorimetric assay kit following the manufacturer's instructions (Chemicon, Temecula, CA, USA). The absorbance of the p-nitroaniline cleaved by caspase was measured at 405 nm using a microplate reader (ELx800; Bio-Tek Instruments, Highland Park, Winooski, VT, USA). Results were standardized to the sham group for comparison of the fold change in caspase-3 activity.

Myeloperoxidase assay

On 24-h post-transplantation, tissue samples from cardiac isografts were assessed for MPO activity [14]. Samples were homogenized in hexadecyltrimethyl ammonium bromide (Sigma-Aldrich) and dissolved in potassium phosphate. After centrifugation, supernatants were collected and mixed with o-dianisidine dihydrochloride (Sigma-Aldrich) and H_2O_2 in phosphate buffer. The activity of MPO was measured spectrophotometrically at 470 nm using microplate reader (ELx800; Bio-Tek Instruments) and expressed as units per 100 mg tissue. Myeloperoxidase standards (Sigma-Aldrich) were measured concurrently with the tissue samples.

FACS analysis

Accordingly, previously study reported by Victoria Gorbacheva *et al.*, infiltrated cells in the isograft were isolated [15]. For measurement of cardiac IL-17A-producing leukocytes, $CD45^+$ cells were isolated using anti-CD45 microbeads (Miltenyi Biotec, Bergisch-Gladbach, Germany) and then stained with intracellular cytokine combined with various surface markers as previously described [16]. For

detection, the number of cardiac infiltrating neutrophils, cells were stained with PerCP-cy5.5 anti-mouse CD45, PE-cy7 anti-mouse CD11b, PE anti-mouse Ly-6G/Gr-1, and measured by FACScalibur flow cytometry.

Western blotting

The protein level of PTX3 was determined by Western blotting. Protein extracted from cells or tissue was separated on 10% SDS–polyacrylamide electrophoresis gels and transferred to nitrocellulose membranes (Pierce, Rockford, IL, USA). After being blocked with 5% nonfat milk in TBS for 3 h, the membranes were incubated with indicated primary antibodies (0.2 μg/ml) at 4 °C overnight, followed by incubation with HRP-conjugated secondary antibody (1:5000) for 3 h. β-actin was used as a loading control for comparison between samples.

Real-time PCR

Total RNA was extracted from cultured cells or tissues using Trizol (Invitrogen, Carlsbad, CA, USA) and reverse transcribed into cDNA using the PrimeScript RT reagent kit (Takara Biotechnology, Dalian, China) according to the manufacturer's instructions. mRNA levels of target genes were quantified using SYBR Green Master Mix (Takara Biotechnology) with ABI PRISM 7900 Sequence Detector system (Applied Biosystems, Foster City, CA, USA). Each reaction was performed in duplicate, and changes in relative gene expression normalized to 18sRNA levels were determined using the relative threshold cycle method. Primer sequences were shown in Table 1.

Protein detection

Serum PTX3 and IL-6 levels were determined by ELISA by following the manufacture's protocols (RD Systems, Abingdon, UK).

Statistics

Data are presented as means ± SEM. Differences were evaluated using unpaired Student's t test between two groups and one-way ANOVA for multiple comparisons, followed by a *post hoc* Student–Newmann–Keuls test when necessary. All analyses were carried out using SPSS 13.0 (SPSS, Chicago, IL, USA), and statistical significance was set at $P < 0.05$.

Results

PTX3 increased after myocardial IR injury

We investigated PTX3 levels in serum and myocardium at different reperfusion time points after IR injury. Both PTX3 mRNA and protein levels were significantly increased on 24 h after reperfusion, and then began to decrease, although it remain at a high level compared with the sham group until 48 h after IR injury (Fig. 1).

PTX3 ameliorated myocardial IR injury

We first detected cardiac TnT production and found that on 24 h post-transplantation, the production of TnT was reduced by exogenous PTX3 administration and increased by PTX3 neutralization in comparison with control (PTX3*:* 4.63 ± 0.45 ng/ml; Control: 7.16 ± 0.77; Anti-PTX3: 10.66 ± 0.96 ng/ml; $P < 0.05$; Fig. 2a). To further evaluating the functional status of hearts exposed to ischemia, hearts were excised after 24 h of reperfusion and mounted on a Langendorff apparatus to assess the pressure–volume ratio. For comparison, pressure–volume ratio was also performed in healthy hearts. The results showed that ischemic hearts were functionally impaired compared with healthy hearts, PTX3 neutralization resulted in a markedly decreased hemodynamic performance, and exogenous PTX3 administration induced significant improvement of hemodynamic performance compared with control hearts.

Table 1. Primers used for real-time PCR.

Gene	Forward (5′-3′)	Reverse (5′-3′)
IL-17A	TGTGAAGGTCAACCTCAAAGTCT	GAGGGATATCTATCAGGGTCTTCAT
KC	GCTGGGATTCACCTCAAGAA	CTTGGGGACACCTTTTAGCA
MIP-2	CGCCCAGACAGAAGTCATAG	TCCTCCTTTCCAGGTCAGTTA
LIX	GGTCCACAGTGCCCTACG	GCGAGTGCATTCCGCTTA
IL-23p19	AGCGGGACATATGAATCTACTAAGAGA	TCCTAGTAGGGAGGTGTGAAGTTG
IL-6	TGATGCACTTGCAGAAAACA	ACCAGAGGAAATTTTCAATAGGC
IL-10	ATCGATTTCTCCCCTGTGAA	TGTCAAATTCATTCATGGCCT
IL-12	AGCAGTAGCAGTTCCCCTGA	AGTCCCTTTGGTCCAGTGTG
MR	ATATATAAACAAGAATGGTGGGCAGT	TCCATCCAAATGAATTTCTTATCC
Arg1	GGACTGGACCCATCTTTCA	ATTACCCTCCCGAGCAACT
PTX3	CCTGCTTTGTGCTCTCTGGT	TCTCCAGCATGATGAACAGC
18S RNA	GCAATTATTCCCCATGAACG	AGGGCCTCACTAAACCATCC

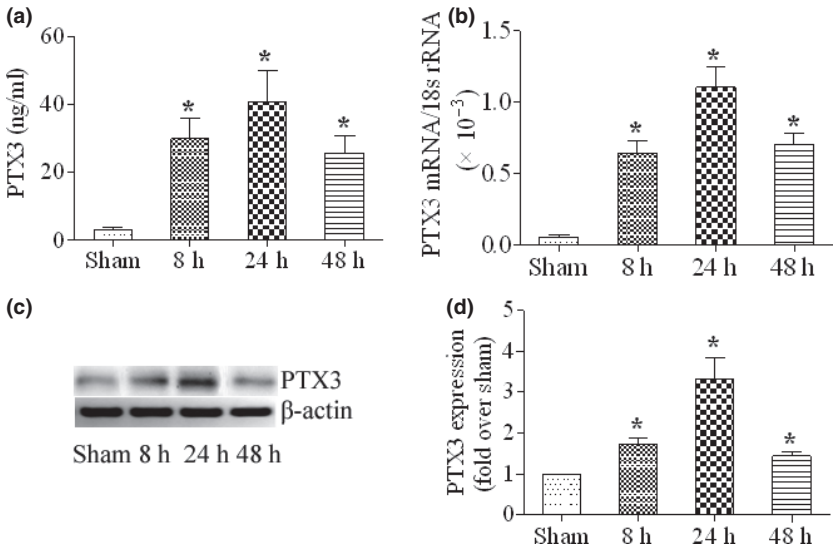

Figure 1 Levels of PTX3 in serum and cardiac isografts increased following myocardial IR injury. Levels of PTX3 were measured by ELISA in serum (a) and by real-time PCR (b) and Western blotting (c, d) in cardiac isografts from sham and cardium IR injury group for different times (n = 6). Asterisks on top of a line section indicate statistically significant difference between this two groups as * for $P < 0.05$.

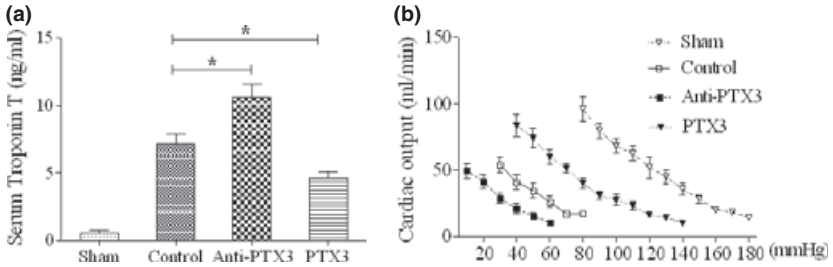

Figure 2 PTX3 increased hemodynamic performance of cardiac isografts and decreased serum TnT post-transplantation. (a) Serum TnT was measured in sham, Control, Anti-PTX3 mAb, PTX3 treated recipients 24 h post-transplantation (n = 6). (b) Hearts were evaluated for hemodynamic function in a pressure–volume relationship 24 h post-transplantation. Asterisks on top of a line section indicate statistically significant difference between this two groups as * for $P < 0.05$.

Cardiac output at 60 mmHg of afterload pressure was significantly increased in hearts with exogenous PTX3 administration and decreased in hearts with PTX3 neutralization (PTX3: 57.8 ± 7.1 ml/min; Control: 24.2 ± 3.6 ml/min; Anti-PTX3: 11.8 ± 1.8 ml/min; $P < 0.05$; Fig. 2b).

PTX3 reduced cardiomyocyte apoptosis and recruitment of neutrophil and macrophage

Apoptosis contributes significantly to myocardial IR injury. We carried out terminal deoxynucleotidyl-transferase-mediated dUTP nick-end labeling (TUNEL) of cardiac isografts from different experiment groups at 24 h post-transplantation. And we found that anti-PTX3 mAb treatment remarkably increased the number of TUNEL-positive cardiomyocytes compared with control group, while exogenous PTX3 administration significantly decreased the number of TUNEL-positive cardiomyocytes in comparison with control group (Fig. 3a and b). Caspase 3 activity determined by a caspase colorimetric assay from cardiac isografts was concomitantly up-regulated by anti-PTX3 mAb and down-regulated by exogenous PTX3 treatment (Fig. 3c).

Neutrophil infiltration is a hallmark of inflammatory injury after myocardial IR injury [17]. Therefore, we investigated the function of PTX3 on neutrophil recruitment. As determined by myeloperoxidase activity and fluorescence-activated cell sorting analysis of $CD11b^{+}Gr\text{-}1^{+}$ neutrophils, myocardial IR injury induced a surge in neutrophil recruitment to myocardium and anti-PTX3 mAb treatment increased neutrophil recruitment, while exogenous PTX3 treatment decreased neutrophil recruitment (Fig. 3d and e). The CXC glutamic acid-leucine-arginine chemokines KC, MIP-2, and LIX are potent neutrophil chemoattractants.

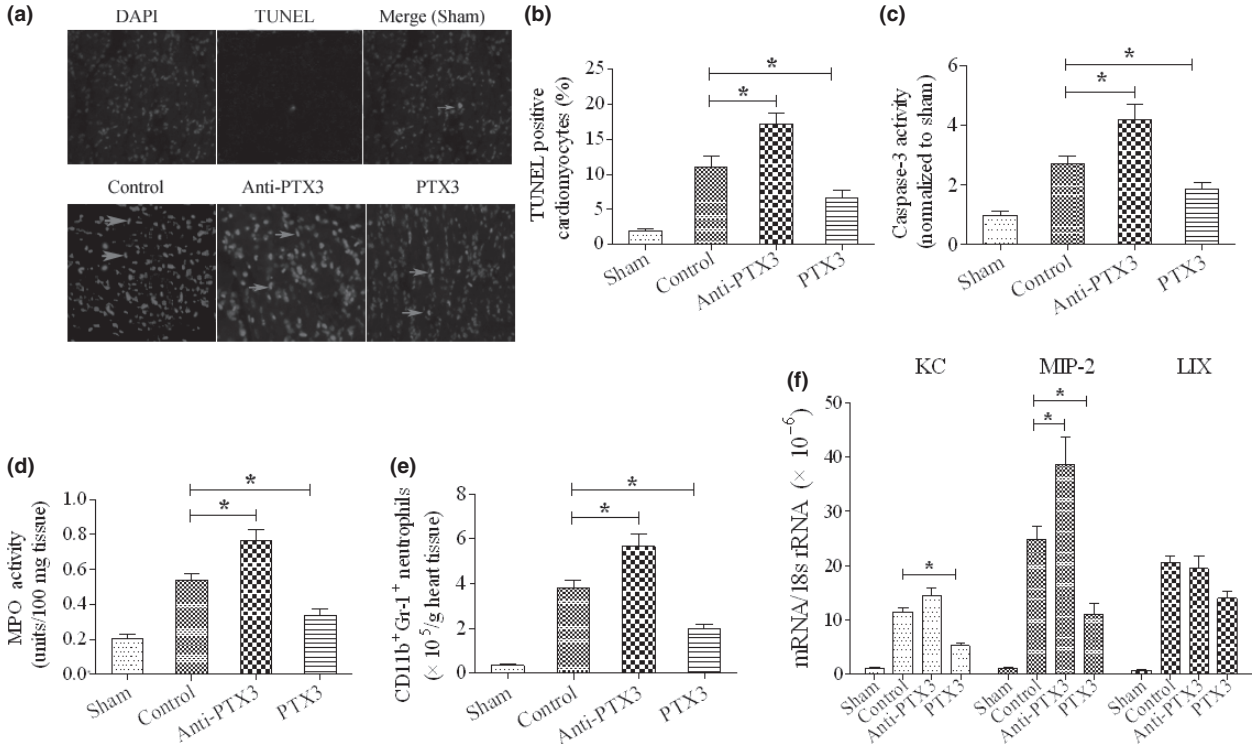

Figure 3 PTX3 attenuated cardiomyocyte apoptosis and cardiac neutrophil recruitment. (a) Representative photographs of TUNEL stained cardiac isograft sections 24 h post-transplantation. Total nuclei by 4′,6-diamidino-2-phenylindole (DAPI) staining (blue), apoptotic nuclei were identified by TUNEL staining (red). Arrows indicate apoptotic cardiomyocytes. (b) Percentages of TUNEL-positive nuclei over total number of nuclei. (c) Caspase 3 activity in cardiac isograft was assessed 24 h post-transplantation, and the values were normalized to sham. (d) Cardiac myeloperoxidase activity in tissue samples. (e) The number of $CD11b^+Gr\text{-}1^+$ neutrophils infiltrated in myocardium was analyzed by flow cytometry. (f) Lipopolysaccharide-induced CXC chemokine (LIX), cytokine-induced neutrophil chemoattractant (KC), and macrophage inflammatory protein-2 (MIP-2) mRNA levels were analyzed by real-time PCR. Asterisks on top of a line section indicate statistically significant difference between these two groups as * for $P < 0.05$; $n = 6$.

Myocardial IR injury caused a significant induction of mRNA level of all the three chemokines. Moreover, after myocardial IR injury, exogenous PTX3 treatment reduced expression of chemokines KC and MIP-2 (Fig. 3f).

Macrophage migration is an important factor in IR injury in heart [18]. And macrophages display two different phenotypes, namely classically (M1) and alternatively (M2) activated macrophages [19]. So we studied the expression of macrophage after IR injury. We found that the mRNA expression of some M1 and M2 mononuclear phagocyte markers displayed increased expression of the M1 marker IL-12 and the M2 markers mannose receptor (MR) and arginase-1 (Arg1) with PTX3 neutralization, and exogenous PTX3 treatment has opposite effect (Fig. 4).

PTX3 down-regulate IL-23/IL-17A expression in myocardial IR injury

PTX3 shares similarities with the classic short pentraxin that is a prototypic acute-phase protein produced in the liver in response to inflammatory signals, most prominently IL-6, which serves as a marker of inflammation and infection [20]. Therefore, we investigated the expression of IL-6 in myocardium and serum. The result showed that anti-PTX3 mAb treatment enhanced IL-6 expression in myocardium and serum, and exogenous PTX3 decreased IL-6 expression (Fig. 5a and b). Next, we checked the expansion of γδ T cell and expression of IL-23/IL-17A and found that expansion of γδ T cell, which was the major source of IL-17A, was restricted by PTX3 (Fig. 5c). Both IL-23 and IL-17A expression were also decreased with PTX3 administration and increased with PTX3 neutralization (Fig. 5d). Furthermore, inhibition of IL-23 or IL-17A or γδ TCR significantly reduced TnT, MPO expression, and cardiomyocyte apoptosis after myocardial IR injury, respectively (Fig. 5e–g).

Discussion

This study revealed a crucial role for PTX3 in cardiomyocyte IR injury. PTX3 ameliorated cardiomyocyte apoptosis and infiltration of neutrophil and macrophage and then

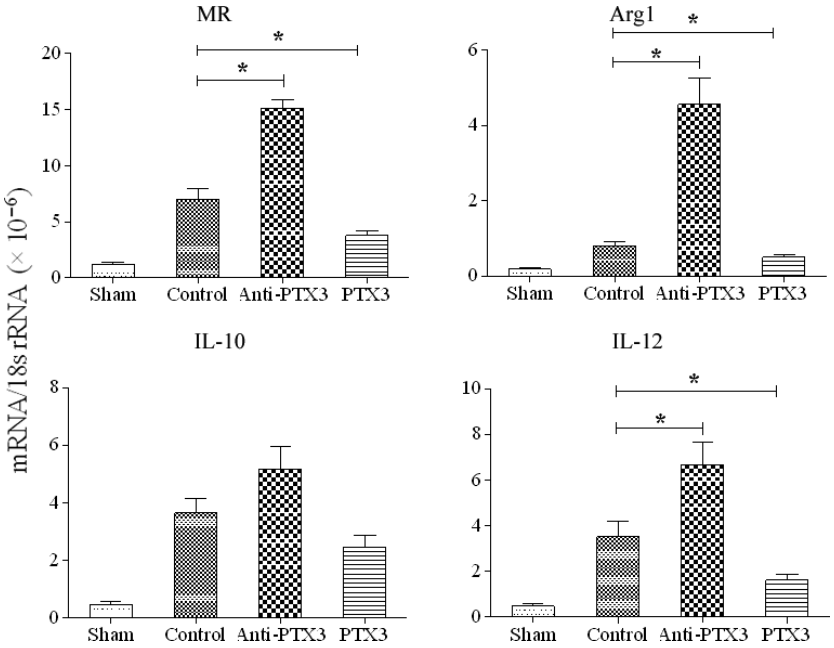

Figure 4 PTX3 decreased macrophage marker mRNA expression after myocardium IR injury. Macrophage markers mannose receptor (MR), Arginase-1 (Arg1), IL-10, and IL-12 were analyzed by real-time PCR ($n = 6$). Asterisks on top of a line section indicate statistically significant difference between this two groups as * for $P < 0.05$.

improved hemodynamic performance. This was associated with restricted expansion of γδ T cell and decreased expression of IL-6 and IL-23/IL-17A.

Ischemia reperfusion triggers a vigorous inflammatory response, augmented by the generation and release of various cytokines that ultimately exacerbates tissue injury, although the precise mechanism of the ischemia reperfusion injury has not been fully revealed [21]. Innate immune activation plays a role in the propagation of IR injury in transplanted solid organs. IR injury involves toll-like receptor (TLR) signaling pathways, complements activation, and natural killer cell migration in cardiac transplantation models and may lead to decreased allograft tolerance and the later development of accelerated cardiac allograft vasculopathy [22,23]. Long pentraxin-3 (PTX3) is a member of a phylogenetically conserved group of acute-phase reactants that are involved in inflammation and innate immunity. In our experiment, we found that increased PTX3 expression was associated with cardiac IR injury in a mouse cardiac transplantation model. And our further studies with PTX3 blockade and recombinant PTX3 were consistent in terms of an anti-inflammatory role of PTX3 in IR injury. Neutralization of PTX3 aggravated all aspects of IR injury and exogenous PTX3 ameliorated IR injury.

Apoptosis has been proposed to be an important mechanism for a significant amount of cell death in reperfused ischemic myocardium [24]. And it could be regulated by oxygen free radicals, cytokines, and neutrophil accumulation [25]. Our experiment showed that PTX3 could regulate cardiomyocyte apoptosis, as confirmed by the change of TUNEL-positive cardiomyocytes and caspase-3 activity. Neutrophil recruitment and macrophage migration play a major role in myocardial damage after IR injury [18,26]. Neutrophil chemotaxis and activation might be strongly regulated by CXC chemokines. And KC, MIP-2, LIX are rodent homologs of CXCL1, CXCL2, and CXCL5 [27]. Our result further proved that decreased infiltration of neutrophil and macrophage contribute to attenuated IR injury by PTX3 treatment.

IL-6 is a proinflammatory cytokine that plays a key role in both innate and adaptive immune response [28], and it is essential in the induction of Th17 cells in both human and mice [29]. γδ T cell comprises about 5% of the overall T-cell population, and they differ from conventional αβ T cells in that they express invariant γ and δ chains as part of their T-cell receptor [9]. γδ T cells are a component of the innate immune cell population and play important roles during physiological processes, such as defense against pathogens, tumor surveillance, ischemia reperfusion injury, and regulation of immune response through cytokine production [30]. Recent research indicated that γδ T cells were recognized as the main source of IL-17A [31,32], and additional immune cell populations also shown to secrete IL-17A, including $CD4^+$ T cells, $CD8^+$ T cells, NK cells,

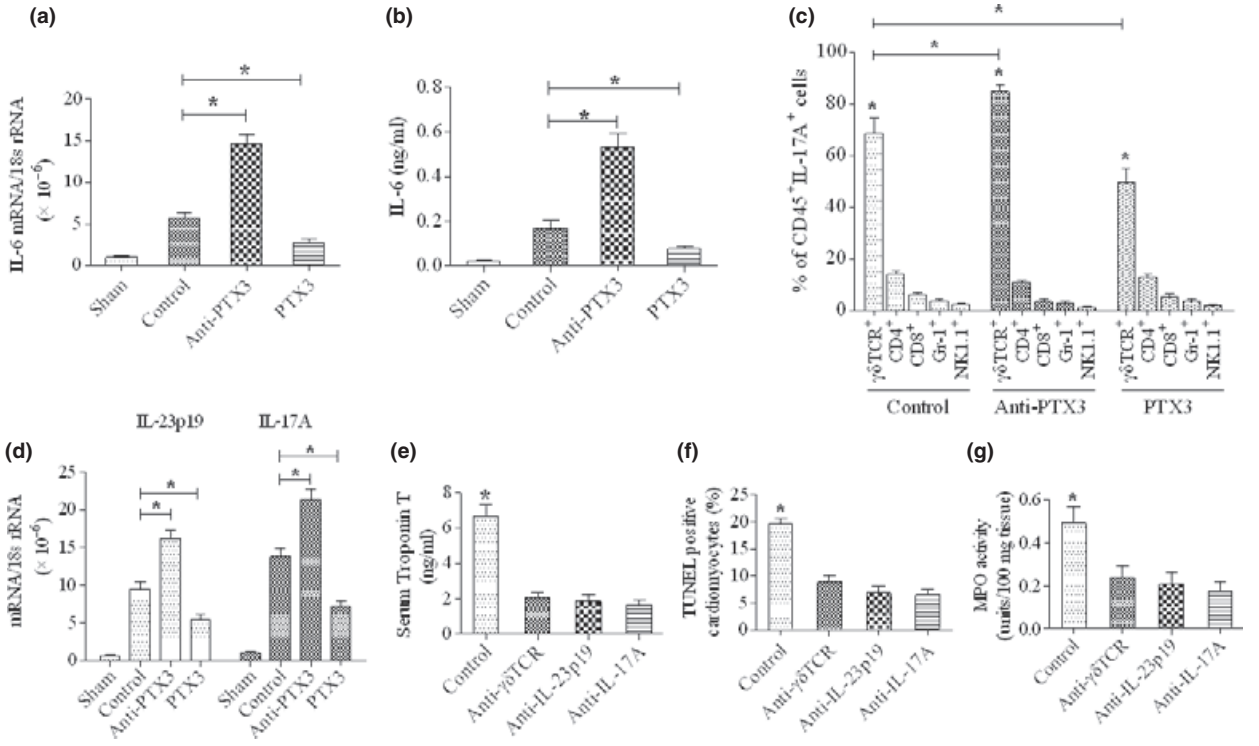

Figure 5 PTX3 restricted γδ T-cell expansion and inhibited IL-23/IL-17A expression. (a) IL-6 mRNA level was analyzed by real-time PCR in cardiac isografts 24 h post-transplantation. (b) IL-6 level in serum was analyzed by ELISA 24 h post-transplantation. (c) Infiltrated IL-17A$^+$ leukocytes in cardiac isografts 24 h post-transplantation from Control, Anti-PTX3, and PTX3 groups were analyzed by flow cytometry. CD45$^+$ cells were isolated and restimulated. The IL-17A$^+$CD45$^+$ cells were further analyzed for γδTCR, CD4, CD8, NK1.1, and Gr-1 expression to detect the cellular source of IL-17A. (d) IL-23p19 and IL-17A mRNA levels in cardiac isografts 24 h post-transplantation from Control, Anti-PTX3, and PTX3 groups were analyzed by real-time PCR. (e) Serum cTnT was measured in Control, Anti-γδTCR, Anti-IL-23p19, and Anti-IL-17A group 24 h post-transplantation. (f) Percentages of TUNEL-positive nuclei over total number of nuclei in cardiac isograft from Control, Anti-γδTCR, Anti-IL-23p19, and Anti-IL-17A group 24 h post-transplantation. (g) Cardiac myeloperoxidase activity in cardiac isograft from Control, Anti-γδTCR, Anti-IL-23p19, and Anti-IL-17A group 24 h post-transplantation. Asterisks on the top of an error bar indicate statistically significant differences this group and other groups, whereas asterisks on top of a line section indicate statistically significant difference between these two groups as * for $P < 0.05$; $n = 6$.

NKT cells, and neutrophils [9]. The heterodimeric cytokine IL-23, which was secreted mainly by activated DCs and macrophages in response to TLR activation, stimulate T-cell differentiation and function in linking innate and adaptive immunity [33]. IL-23 contributes to autoimmunity and host defense through IL-23/IL-17-dependent pathways [34]. Recent study showed that an IL-23/IL-17 pathway was activated in kidney ischemia–reperfusion injury [35]. We first detected IL-6 expression and found that expression of IL-6 in both myocardium and serum was increased with anti-PTX3 mAb treatment and decreased with exogenous PTX3 administration. This was consistent with a recent study reporting that PTX3-KO mice undergoing coronary artery ligation and reperfusion showed greater no-reflow areas, increased neutrophil infiltration, and IL-6 expression [6]. However, another study showed that IL-6 circulation and production in the heart were increased by PTX3 in a pressure overload-induced left ventricular dysfunction model [36]. These different results indicated that the molecule may have different functions in different settings. In our experiment, the result indicated that γδ T cell was the major source of IL-17A, and expression of IL-23 and IL-17A was restricted by PTX3 treatment. Furthermore, neutralizing antibody to γδ TCR, IL-23, or IL-17A ameliorated IR injury after cardiac transplantation. These suggested that restricted γδ T-cell expansion and inhibited IL-23/IL-17A expression were contributable to the alleviated myocardium IR injury induced by PTX3.

In summary, our study provides evidence that PTX3 played a protective role in cardiomyocyte IR injury, and this was associated with restricted expansion of γδ T cell and decreased expression of IL-23/IL-17. Although further investigations are needed to fully clarify the precise molecular and cellular mechanism involved in the immunoregulation, PTX3 may be exploited as a novel therapeutic agent to attenuate cardiomyocyte apoptosis, neutrophils, and macrophage accumulation and IR injury in cardiac transplantation.

Authorship

HZ: designed research. KL, LW: performed study. KL, LW, LH: collected data, analyzed data and wrote the paper.

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