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ORIGINAL ARTICLE

The protective role of interleukin-18 binding protein in a murine model of cardiac ischemia/reperfusion injury

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Conflicts of interest
The authors declare no conflict of interests.

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Summary

IL-18, a proinflammatory cytokine, is produced by macrophages, epithelial cells, T cells, neutrophils, NK-T cells, and B cells, and has been implicated in the pathophysiology of a variety of inflammatory diseases including ischemia/reperfusion (IR) injury, transplant rejection, and autoimmune disease. Recent study indicated that neutralization of IL-18 with anti-IL-18 antibody or IL-18-binding protein (IL-18BP) ameliorates IR-induced myocardial injury. However, the mechanism needs to be further investigated. In our current study, syngeneic heterotopic heart transplantation was performed by a modified non-suture cuff technique. We found that IL-18BP treatment ameliorated cardiomyocyte necrosis and infiltration of $CD4^+$ T cells, neutrophils, and macrophages. IL-18BP-treated mice exhibited decreased expression of inflammatory cytokines including IL-1β, IL-23, IL-18, and IL-17. IL-18BP treatment suppressed Th17 differentiation *in vivo* and *in vitro*. Adoptive transfer of T cells from IL-18BP-treated mice showed alleviated cardiac IR injury when compared with that transferred from control mice. Furthermore, the decreased infiltration of mononuclear cells and production of troponin T (TnT) induced by IL-18BP treatment were both abrogated by additional administration of recombinant mouse IL-17 (rmIL-17). These data revealed a protective role of IL-18BP in cardiac IR injury. Above all, IL-18BP ameliorates cardiac IR injury in part through suppression of Th17 differentiation.

Introduction

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 [1]. In the long term, this correlates with increased episodes of acute and chronic rejections. Thus, the development of more effective drugs or interventions to protect the myocardium from reperfusion injury is required to provide greater clinical benefits for patients with ischemic heart disease.

IL-18, a proinflammatory cytokine, is produced by macrophages, epithelial cells, T cells, neutrophils, NK-T cells, and B cells, and has an active role in inflammatory incidents and the immune system [2–4]. IL-18 has been implicated in the pathophysiology of a variety of inflammatory diseases including ischemia/reperfusion injury, transplant rejection, and autoimmune disease [5–7]. IL-18 binds to the IL-18 receptor (IL-18R), a heterodimer consisting of a ligand-binding IL-18Rα subunit and a signaling IL-18Rβ subunit. IL-18R is expressed on Th1 lymphocytes as well as on accessory cells [8,9]. Recent studies showed that neutralization of IL-18 reduced inflammation [10,11]. Moreover, results from Millward *et al.* [12] indicated that IL-18-binding protein (IL-18BP), the endogenous inhibitor of the Th1-promoting cytokine IL-18, regulates Th17 responses in the CNS. Inhibition of IL-18 has been demonstrated to ameliorate IR-induced myocardial injury [5]. However, the mechanism needs to be further investigated. Therefore, in our current study, we neutralize the effect of IL-18 with IL-18BP in a syngeneic cardiac transplantation model. Then, we further

investigated the role of Th17 cells in the attenuated cardiac IR injury induced by IL-8BP.

Materials and methods

Mouse cardiac IRI model

Animal facilities and protocols were approved by the Laboratory Animal Care and Use Committee of Zhejiang University. T-cell-deficient athymic male mice (B6.Cg-*Foxn1*^{nu}, *nu/nu*) and their C57Bl/6 WT male littermates (6–8 weeks of age, weighing 20 to 25 g) were purchased from The Jackson Laboratory (Bar Harbor, ME, USA). Syngeneic heterotopic heart transplantation was performed by a modified non-suture cuff technique previously described by Heron *et al.* [13]. Briefly, the donor heart was heterotopically transplanted to the neck vessels of the recipient: The aortic root was anastomosed to the right common carotid artery and the pulmonary artery to the right jugular vein of the recipient. Hearts were stored in cold Bretschneider solution for 8 h before transplantation with consecutive *in vivo* reperfusion for 6 or 24 h [14]. In some recipients, 75 μg/kg of IL-18BP (R&D Systems, Minneapolis, MN, USA) in 0.9% saline (1 ml) was administrated intraperitoneally 30 min before transplantation [15]. In some mice, 5 μg of recombinant mouse IL-17 [rmIL-17 (R&D Systems) was intraperitoneally injected 5 min prior to reperfusion. There are six recipients in each group.

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 (Roche Diagnostics, New York, USA) according to the manufactures' instruction.

Myocardial apoptosis

Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) staining was performed as previously described [16]. Cardiac caspase-3 activity was measured as previously described using a caspase colorimetric assay kit following the manufacturer's instructions (Chemicon, Temecula, CA, USA) [17,18].

Histopathology and immunohistochemistry

Cardiac graft tissues were stained with hematoxylin–eosin (HE). For immunohistochemical staining, 5-μm sections of cryostat frozen tissues were applied to poly-L-lysine microscope slides (Sigma-Aldrich, Saint Louis, MO, USA) and fixed with cold acetone. To quantify leukocyte infiltration, sections were stained with rat anti-mouse CD4, CD8, Ly6G, F4/80, and RORγt antibody (Abcam, Cambridge, MA, USA) followed by goat anti-rat biotin conjugate. The number of $CD4^+$ and $CD8^+$ T cells, macrophages, neutrophils, and $ROR\gamma t^+$ cells was assessed in 10 fields per slide at a magnification of ×400, and the results were expressed as cells per high-power field.

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 18s RNA levels were determined using the relative threshold cycle method. The expression levels in the sham animals were not different between genotypes.

FACS analysis

For detection of the ratio of Th17 cells, isolated cells were stained with anti-CD4 and IL-17. The entire Abs used for flow cytometry was obtained from eBioscience (San Diego, CA, USA) or BD Biosciences (San Jose, CA, USA). Flow cytometry was performed on a FACSCalibur and analyzed using CellQuest (BD Pharmingen, Houston, USA) or WINMDI 2.8 software.

Adoptive transfer studies

Twenty-four hours after C57Bl/6 WT mice underwent transplantation of a C57Bl/6 WT heart with administration of IL-18BP, spleens that were collected from C57Bl/6 recipients were minced on a nylon mesh and filtered through a cell strainer (70 μm). The obtained cell suspension underwent red blood cell lysis using a red blood cell lysis buffer (eBioscience). T cells were enriched by mouse T-cell enrichment columns (R&D Systems) according the manufacturer's instructions. After enrichment treatment, the purity of the T-cell suspension was >90%. Approximately 3×10^6 enriched T cells were injected intraperitoneally into *nu/nu* mouse. Then, the *nu/nu* recipients underwent transplantation of a *nu/nu* heart [19].

$CD4^+$ T-cell activation and polarization

Purified $CD4^+$ T cells from C57Bl/6 WT mice were activated by 5 μg/ml plate-bound anti-CD3 and 2 μg/ml sol-

uble anti-CD28. For propagation under Th17 condition, 2.5 ng/ml rTGF-β1, 30 mg/ml rIL-6, 10 μg/ml anti-IFN-γ, and 10 μg/ml anti-IL-4 ± 20 ng/ml IL-18BP were provided. All antibodies used were purchased from eBioscience. All cytokines used were purchased from Peprotech (Rocky Hill, NJ, USA).

Statistical analysis

Data are expressed as means ± SEM.; the *t*-test and one-way analysis of variance were used for comparisons of means between experimental groups. Kaplan–Meier curve was used to estimate graft survival time. Differences were considered to be significant at $P < 0.05$. We analyzed the data using GraphPad Prism software (GraphPad Software, La Jolla, CA, USA).

Results

IL-18BP ameliorates myocardial IR injury

We first examined the myocardial IR injury by histological examination (Fig. 1a). Either on 6 or 24 h after reperfusion, biopsies of grafts in IL-18BP-treated group revealed significantly fewer infiltrations of mononuclear cells in comparison with biopsies from control group. We next detected cardiac TnT production and found that on 6 h postreperfusion, the production of TnT was decreased in IL-18BP-treated mice in comparison with control mice (IL-18BP group: 2.00 ± 0.29 ng/ml; Control group: 3.50 ± 0.46; $P < 0.05$); on 24 h after reperfusion, the production of TnT in control mice was further increased and significantly higher than that in IL-18BP-treated mice (IL-18BP group: 2.92 ± 0.36 ng/ml; Control group: 5.22 ± 0.55; $P < 0.05$) (Fig. 1b). The cardiac graft survival in IL-18BP-treated animals was significantly prolonged (MST = 90 days in IR + IL-18BP group, $P < 0.05$ as compared with IR group, MST = 72 days) (Fig. 1c).

IL-18BP reduces leukocyte infiltration and alters cytokine expression

We investigated the infiltration of inflammatory cells, that is, $CD4^+$ and $CD8^+$ T cells, neutrophils, and macrophages, in the cardiac grafts on 6 and 24 h postreperfusion. The number of myocardial $CD4^+$ T cells, neutrophils, and macrophages in the IL-18BP-treated mice on 24 h postreperfusion was significantly decreased compared to those in the control group. Although a significant differ-

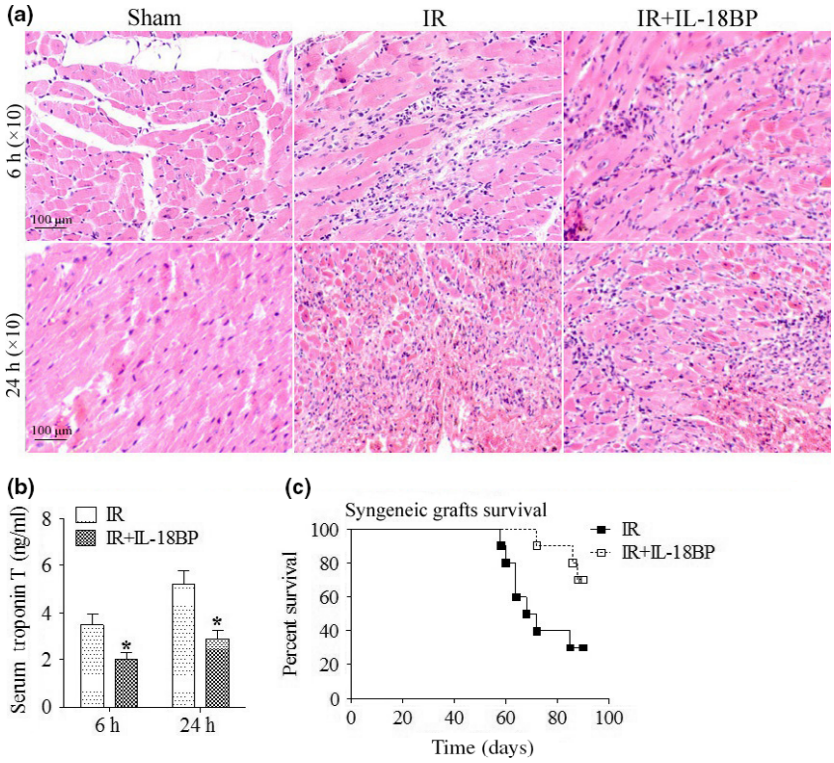

Figure 1 IL-18BP ameliorates myocardial IR injury. (a) HE staining of cardiac grafts from Sham, IR, and IR+IL-18BP groups on 6 and 24 h postcardiac IR injury; (b) serum TnT was measured in Sham, IR, and IR+IL-18BP groups on 6 and 24 h postcardiac IR injury; (c) indicating the survival of heart grafts ($n = 10$). *$P < 0.05$. $N = 6$.

ence was not observed, the number of $CD8^+$ T cells was decreased in the IL-18BP-treated mice on 24 h when compared to the control group. On 6 h postcardiac IR injury, only the number of neutrophils in the IL-18BP-treated mice was markedly decreased compared to those in the control group (Fig. 2a). Apoptosis contributes significantly to myocardial IR injury. We carried out terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) of cardiac grafts from different experiment groups at 6 or 24 h postreperfusion. We found that IL-18BP-treated mice remarkably decreased the number of TUNEL-positive cardiomyocytes compared with control mice (Fig. 2b). Caspase 3 activity determined by a caspase colorimetric assay from cardiac grafts was concomitantly downregulated by IL-18BP treatment (Fig. 2c).

We next measured cardiac mRNA with real-time quantitative PCR. Results indicated that the IL-18BP treatment significantly downregulated the expressions of proinflammatory cytokines (IL-1β, IL-23, IL-18 and IL-17) on 6 and 24 h when compared to that in control group. Either on 6 or 24 h postreperfusion, the cytokine mRNA expressions of TNF-α and IFN-γ were not significantly different between the two groups (Fig. 3).

IL-18BP regulates Th17 cell differentiation *in vivo* and *in vitro*

IL-1 family cytokines are key co-regulators of $CD4^+$ T-cell fate, and the role of IL-1β in Th17 cell differentiation is mirrored by the contribution of IL-33 and IL-18 to Th2

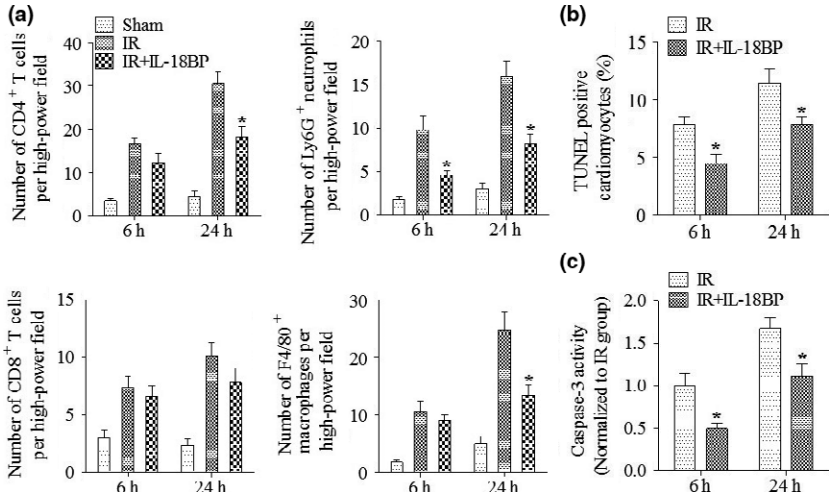

Figure 2 IL-18BP reduces leukocyte infiltration. (a) Indicating the numbers of $CD4^+$ and $CD8^+$ T cells, neutrophils, and macrophages in IR and IR+IL-18BP groups on 6 and 24 h postcardiac IR injury; (b) percentages of TUNEL-positive nuclei over total number of nuclei; (c) caspase 3 activity in cardiac graft was assessed on 6 and 24 h post-transplantation, and the values were normalized to IR group. $*P < 0.05$. $N = 6$.

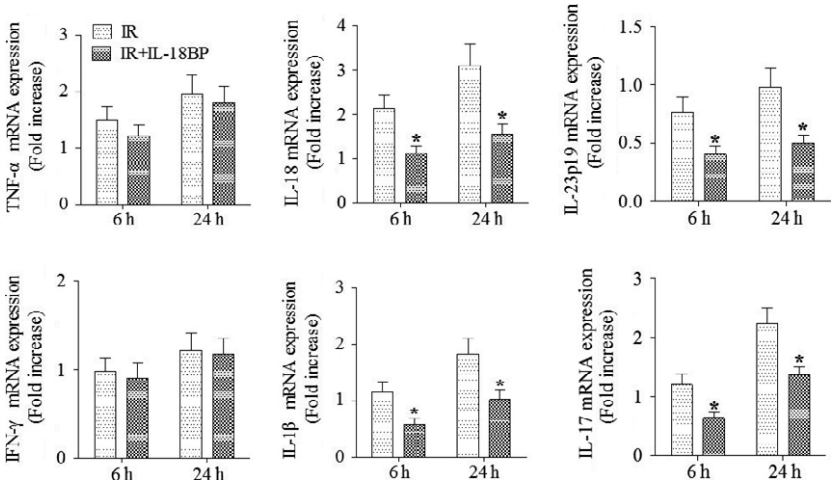

Figure 3 IL-18BP alters cytokine expression. The mRNA expression of TNF-α, IFN-γ, IL-1β, IL-23p19, IL-18, and IL-17 was measured by RT-PCR. $*P < 0.05$. $N = 6$.

and Th1 subsets, respectively [20]. Previous papers have indicated the important role of IL-17 in cardiac IR injury [21,22]. In our current study, we found that the expression of RORγt in cardiac graft on 24 h post-transplantation was significantly decreased in IL-18BP-treated mice when compared with that in control mice (Fig. 4a and b). The frequency of $CD4^+IL\text{-}17^+$ Th17 cells in spleen on 24 h post-transplantation was also markedly decreased in IL-18BP-treated mice in comparison with that in control mice (Fig. 4c and d). Next, we performed parallel *in vitro* studies. We utilized a Th17 cell differentiation system whereby culture of naive $CD4^+$ T cells in the presence of anti-CD3, anti-CD28, rTGF-β1, rIL-6, anti-IFN-γ, and anti-IL-4 results in efficient Th17 cell polarization. Under this polarizing condition, addition of IL-18BP significantly suppressed Th17 cell differentiation from naive $CD4^+$ T cells (Fig. 5a and b).

IL-18BP treatment attenuates cardiac IR injury in part dependent on suppressed Th17 differentiation

To determine whether IL-18BP treatment was indeed an attenuating factor in cardiac IR injury, we transferred 3×10^6 T cells from IL-18BP-treated mice with cardiac IR injury into *nu/nu* mice. Then, the nu/nu recipients were underwent transplantation of a *nu/nu* heart. Results showed that the adoptive transfer of T cells from IL-18BP-treated mice led to a significant decrease in mononuclear cell infiltration, RORγt expression, and serum TnT production at 24 h post-transplantation when compared to that from control mice (Fig. 6a and b). Furthermore, the cardiac graft survival in mice transferred with T cells from IL-18BP-treated mice was significantly prolonged when compared to that from control mice (MST = 84 days in Group

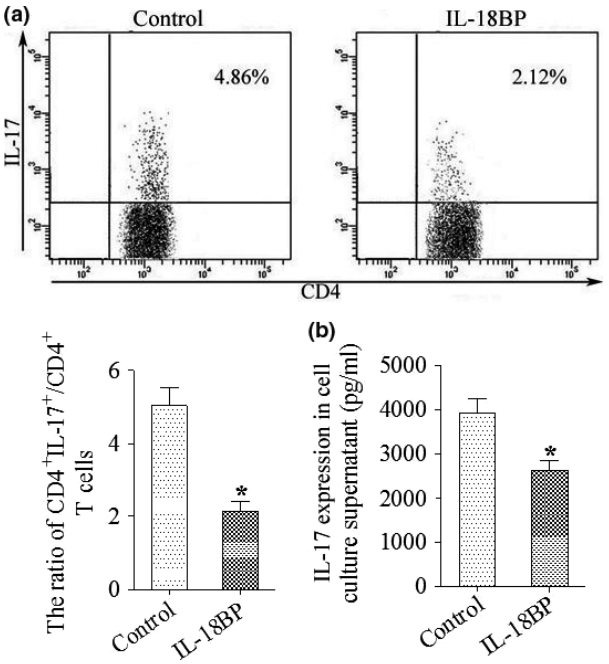

Figure 5 IL-18BP inhibits Th17 cell differentiation *in vitro*. (a) The frequencies of Th17 cells in $CD4^+$ T cells were determined by flow cytometry. Th17 cells were gated with $CD4^+IL\text{-}17^+$. Representative FACS pictures from a single case are shown; (b) indicating the IL-17 level in cell culture supernatant which was detected by ELISA. $^*P < 0.05$. $N = 6$.

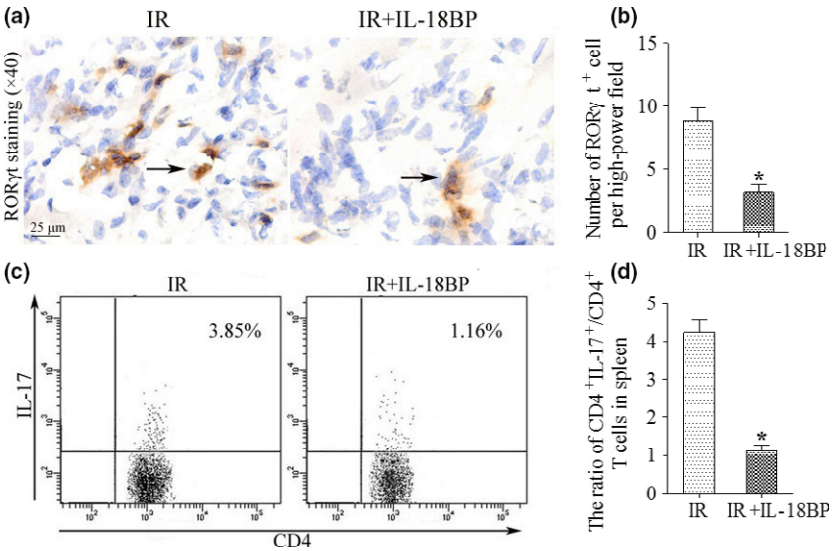

Figure 4 IL-18BP inhibits Th17 cell differentiation *in vivo*. (a) Representative image of RORγt staining in cardiac graft on 24 h after transplantation; (b) indicating the number of RORγt-positive cells in cardiac graft on 24 h after transplantation; (c) representative frequencies of $CD4^+IL\text{-}17^+$ cells in spleen on 24 h after transplantation; (d) indicating the frequencies of Th17 cells in spleen on 24 h after transplantation. $^*P < 0.05$. $N = 6$.

A, $P < 0.05$ as compared with Group B, MST = 90 days) (Fig. 6c).

Next, we further investigated the role of IL-17 in cardiac IR injury. Results indicated that the decreased infiltration of mononuclear cells in cardiac graft induced by IL-18BP treatment was abrogated by additional administration of rmIL-17 (Fig. 7a). The decreased production of TnT induced by IL-18BP treatment was also reversed by additional rmIL-17 treatment (Fig. 7b). Furthermore, the prolonged cardiac graft survival in IL-18BP-treated animals was abolished by rmIL-17 (MST = 90 days in IR + IL-18BP group, $P < 0.05$ as compared with IR + IL-18BP + rmIL-17 group, MST = 80 days) (Fig. 7c).

Discussion

This study revealed a crucial role for IL-1BP in cardiomyocyte IR injury. IL-18BP ameliorated cardiomyocyte necrosis and infiltration of $CD4^+$ T cells, neutrophils, and macrophages. IL-18BP-treated mice exhibited decreased expression of inflammatory cytokines including IL-1β, IL-23, IL-18, and IL-17. IL-18BP treatment suppressed Th17 differentiation *in vivo* and *in vitro*. Adoptive transfer of T cells from IL-18BP-treated mice showed alleviated cardiac IR injury when compared with that transferred from control mice. Furthermore, the decreased infiltration of mononuclear cells and production of TnT induced by IL-18BP treatment was both abrogated by additional administration of rmIL-17.

Invading inflammatory cells play an important role in cardiac IR injury, capable of releasing degrading enzymes and producing large amount of ROS [23], and the inflammatory response is further enhanced by the myocardium through the release of chemoattractant factors and many other mediators in which necrotic cell death is known to play an important role [24,25]. In our current study, the number of $CD4^+$ T cells, neutrophils, and macrophages in IL-18BP-treated mice was decreased significantly compared to those in control group on 24 h after reperfusion. The mechanism by which IL-18 promotes cardiac damage in this setting seems to involve the generation of inflammatory cytokines in the promotion of $CD4^+$ T cells, neutrophils, and macrophages.

Apoptosis has been proposed to be an important mechanism for a significant amount of cell death in reperfused ischemic myocardium [26]. And it could be regulated by oxygen free radicals, cytokines, and neutrophil accumulation [27]. Our experiment showed that IL-18BP could regulate cardiomyocyte apoptosis, as confirmed by the change of TUNEL-positive cardiomyocytes and caspase-3 activity.

Previous papers have reported that IL-17 is linked to the pathogenesis of several cardiovascular diseases, including atherosclerosis [28–30], hypertension, viral myocarditis, and dilated cardiomyopathy [31–33]. Recently, IL-17 has also been found to contribute to brain, kidney, and intestine I/R injury [34–36] . IL-18, a member of the IL-1 cytokine superfamily, has an impressive ability to induce Th1-Tc1 differentiation and immune responses. IL-18 was initially discovered as an IFN-γ-inducing factor, which, together with IL-12, promoted the development of Th1 cells [37]. However, IL-18 has pleiotropic effects and can also induce Th2 responses in the absence of IL-12 [38]. IL-

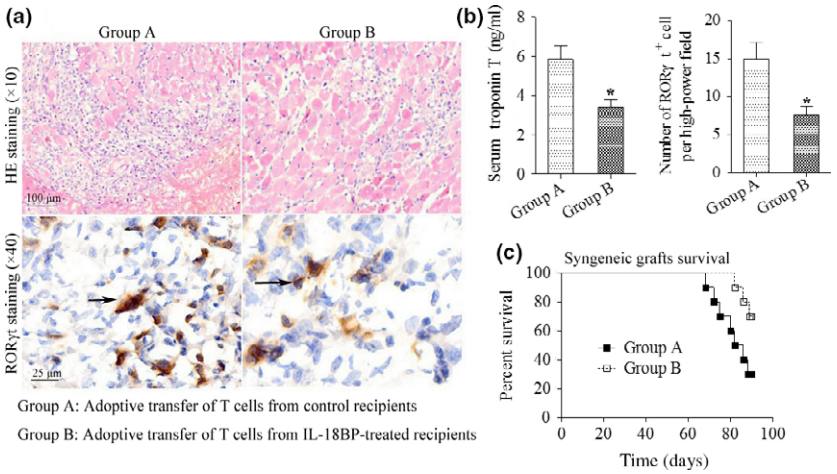

Figure 6 Effect of T cells transfer on cardiac IR injury on 24 h post-transplantation. 3×10^6 T cells from IL-18BP-treated mice with cardiac IR injury were transferred into *nu/nu* mice. Then, the nu/nu recipients were underwent transplantation of a *nu/nu* heart. (a) HE and RORγt staining of cardiac grafts from Group A and Group B on 24 h postcardiac IR injury; (b) serum TnT and the positive RORγt staining were measured in Group A and Group B on 24 h postcardiac IR injury; (c) indicating the survival of heart grafts ($n = 10$). Group A indicates the mice transferred with T cells from control recipients, and Group B indicates the mice transferred with T cells from IL-18BP-treated recipients. *$P < 0.05$. $N = 6$.

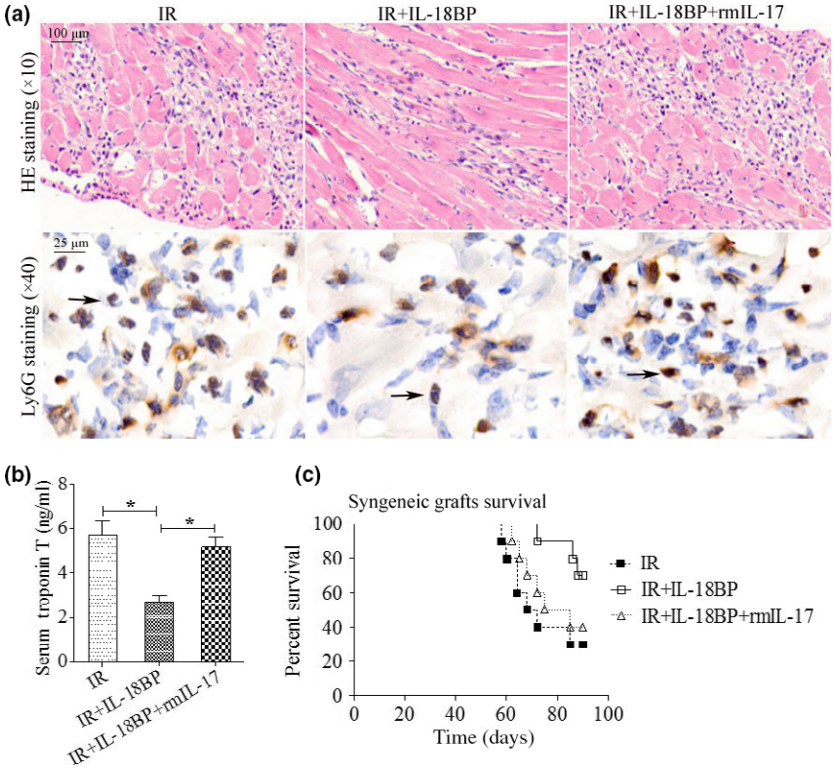

Figure 7 IL-17 contributes to cardiac IR injury. (a) HE and Ly6G staining of cardiac grafts from IR, IR+IL-18BP, and IR+IL-18BP+rmIL-17 groups on 24 h postcardiac IR injury; (b) serum TnT was measured in IR, IR+IL-18BP, and IR+IL-18BP+rmIL-17 groups on 24 h postcardiac IR injury; (c) indicating the survival of heart grafts (n = 10). *P < 0.05. N = 6.

18 also plays a key role in the generation of Th17 responses in diseases characterized by autoimmunity [39]. Blockade of IL-18 signaling with a soluble form of the IL-18R accessory protein suppressed IFN-γ and IL-4 production by CD3 T cells and decreased circulating $CD4^+CD25^+Foxp3^+$ regulatory T cells but enhanced Th17 differentiation and increased IL-17 production [40]. Moreover, IL-$18^{-/-}$ mice were found to be susceptible to IL-17-mediated experimental autoimmune uveitis, and IL-18 was found to be redundant in an Ag-induced arthritis model [41,42]. In our current study, IL-18BP treatment exhibited lower production of IL-17 *in vivo* and *in vitro*. Adoptive transfer of T cells from IL-18BP-treated mice showed alleviated cardiac IR injury when compared with that transferred from control mice. Furthermore, the decreased infiltration of mononuclear cells and production of TnT induced by IL-18BP treatment were both abrogated by additional administration of rmIL-17. All of these indicated that IL-18BP ameliorates cardiac IR injury in part through suppression of Th17 differentiation.

In summary, our data document a protective role of IL-18BP in the cardiac inflammatory response against IR injury. IL-18BP ameliorates cardiac IR injury in part through suppression of Th17 differentiation. Although further investigations are needed to fully clarify the precise molecular and cellular mechanism involved in the immunoregulation, IL-18BP may be a novel therapeutic strategy to protect cardiac from IR injury.

Authorship

HG and XL: designed the study. HG, MX, LX, XZ and YY: performed the study. XL: wrote the study.

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