TRANSPLANT INTERNATIONAL

Transplant International ISSN 0934-0874

ORIGINAL ARTICLE

Necrostatin-1 inhibits Hmgb1-IL-23/IL-17 pathway and attenuates cardiac ischemia reperfusion injury

Anbin Zhang,[1] Xiaogang Mao,[2] Lin Li,[2] Yunjie Tong,[1] Yanli Huang,[2] Yanli Lan[2] and Hong Jiang[2]

1 Department of Rheumatology and Immunology, Xiangyang Central Hospital, Hubei University of Arts and Science, Hubei, China
2 Department of Gynaecology and Obstetrics, Xiangyang Central Hospital, Hubei University of Arts and Science, Hubei, China

Keywords
Hmgb1, interleukin-23/interleukin-17, ischemia reperfusion injury, necrostatin-1.

Correspondence
Hong Jiang, Jinzhou Road 136#, Xiangyang 441021, Hubei, China.
Tel.: 0086-13971961433;
fax: 0086-27-87686305;
e-mail: hongjiangxyhb@163.com

Conflicts of interest
The authors have no conflict of interest.

Anbin Zhang and Xiaogang Mao contributed equally to this paper.

Received: 25 November 2013
Revision requested: 13 January 2014
Accepted: 5 May 2014
Published online: 29 August 2014

doi:10.1111/tri.12349

Summary

Ischemia reperfusion (IR) injury is a major issue in cardiac transplantation and inflammatory processes play a major role in myocardial IR injury. Necrostatin-1 (Nec-1) is a small molecule capable of inhibiting RIP1 kinase activity and attenuates inflammation-mediated tissue injury. In our study, hearts of C57Bl/6 mice were flushed and stored in cold Bretschneider solution for 8 h and then transplanted into syngeneic recipients. We found that Nec-1 decreased cardiomyocyte necrosis and recruitment of neutrophils and macrophages. Troponin T (TnT) production on 24 h after myocardial IR injury was reduced by Nec-1 administration. Cardiac output at 60 mmHg of afterload pressure was significantly increased in hearts with Nec-1 administration and the cardiac allograft survival in Nec-1-treated animals was significantly prolonged (MST = 90 days in IR + Nec-1 group, $P < 0.05$ as compared with IR group, MST = 83.5 days). Nec-1 treatment attenuated ROS generation and increased expression of NOS2 and COX-2. The expression of Hmgb1, IL-23, and IL-17A were also decreased with Nec-1 administration. Furthermore, the decreased TnT expression induced by Nec-1 was abrogated with exogenous Hmgb1 administration. In conclusion, Nec-1 played a protective role in cardiomyocyte IR injury, and this was associated with inhibited Hmgb1-IL-23/IL-17 pathway.

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. 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 [1].

Cell death by necrosis is often associated with inflammation. Although necrosis was once thought to be an unregulated form of cell injury because of trauma, recent evidence indicates that necrosis is a highly regulated process that involves a dedicated molecular circuitry [2,3]. In necrosis, the loss of plasma membrane integrity often coincides or precedes the exposure of phosphatidyl serine [4]. This morphological signature of necrosis distinguishes it from apoptosis, in which the early exposure of phosphatidyl serine and other “eat-me” signals prompt their clearance by professional phagocytes before membrane leakage and release of inflammatory cellular adjuvants [5]. Thus, necrosis is a more inflammatory form of cell death than apoptosis. Recent work shows that the receptor interacting protein (RIP) kinases RIP1 and RIP3 play essential roles in TNF-induced necrosis [6,7]. Necrostatin-1 (Nec-1), a small molecule capable of inhibiting RIP1 kinase activity, was known to inhibit necroptosis and efficiently prevented necrotic cell death after ischemic brain injury [8]. Even more, recent paper indicating that administration of Nec-1 at the onset of

reperfusion inhibits RIP1-dependent necrosis *in vivo*, leading to infarct size reduction and preservation of cardiac function [9]. However, the role of Nec-1 in cardiac transplantation model is still not known, and the mechanism needs to be further investigated.

In this study, we demonstrated a crucial role for Nec-1 in cardiomyocyte IR injury. Nec-1-ameliorated cardiomyocyte necrosis and infiltration of neutrophil and macrophage and then improved hemodynamic performance. Nec-1 treatment attenuated ROS generation and increased NOS2 and COX-2 expression. The expression of Hmgb1, IL-23, and IL-17A were also decreased with Nec-1 administration. Furthermore, the decreased TnT expression induced by Nec-1 was abrogated with exogenous Hmgb1 administration, and the reduced production of IL-17A and IL-23 was also abolished by rHmgb1.

Materials and methods

Animals

Inbred male C57Bl/6 mice, used as donors and recipients, were from the Center of Experimental Animals, HUST, and China. All the mice were male at 15 to 20 g in weight, which were housed in specific pathogen-free facility with regular food and water *ad libitum.*

Heterotopic cardiac transplant and post-transplant therapies

Syngeneic heterotopic heart transplantation was performed by a modified nonsuture cuff technique previously described by Heron [10]. 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 24 h [11]. Mice were received either phosphate-buffered saline (PBS) or 3.5 mg/kg necrostatinn-1 dissolved in PBS via a tail vein injection 5 min prior to reperfusion [9]. 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 and For inhibition of Hmgb1, mice were treated with glycyrrhizin (TCI, Shanghai, China) at 5 mg/mouse 5 min prior to reperfusion [12]. For animal experiments using recombinant Hmgb1 (rHmgb1), animals received an intravenous bolus (tail vein) injection of 100 ng/ml of rHmgb1 (R&D Systems, Minneapolis, MN, USA). Animals received rHmgb1 injection 1 h after IR [13]. There are six animals in each group.

Function assessment

To assess graft function, transplanted hearts were evaluated using an isolated working heart apparatus, as previously described [10]. 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 minute. 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 were 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 manufacturers' instruction.

Propidium iodide and TUNEL staining

Animals received ip injections of 10 mg/kg propidium iodide (PI) to label necrotic cells 1 h before termination. After termination, hearts were explanted and snap-frozen in liquid nitrogen. Frozen sections of 7 μm were cut and counterstained with Hoechst after which quantification of necrotic cells was performed. Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) staining was performed as previously described [14].

Histopathology and immunohistochemistry

Cardiac graft tissues were stained with hematoxylin–eosin (HE). For immunohistochemical staining, 5 μm sections of cryostat-frozen tissue 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 neutrophil antibody or anti-mouse F4/80 antibody (Abcam, Cambridge, MA, USA) followed by goat anti-rat biotin conjugate.

Myeloperoxidase assay

On 24 h post-transplantation, tissue samples from cardiac isografts were assessed for MPO activity. 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, New York, NY, USA) and expressed as units per 100 mg tissue. Myeloperoxidase standards (Sigma-Aldrich) were measured concurrently with the tissue samples.

FACS analysis

According to previous study reported by Victoria Gorbacheva *et al.*, infiltrated cells in the isograft were isolated [15]. For detection of the number of cardiac infiltrating cells, cells were stained using the following fluorochrome-labeled Abs: anti-Ly6G and F4/80. The entire Abs used for flow cytometry was obtained from eBioscience or BD Biosciences (San Jose, CA, USA). Flow cytometry was performed on a FACSCalibur and analyzed using CellQuest (BD Pharmingen, San Diego, CA, USA) or WINMDI 2.8 software (Beijing, China).

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. The 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.

Detection of reactive oxygen species

In situ ROS levels were assessed using dihydroethidium (DHE) as described previously in sections after 24 h of reperfusion. The fluorescence reaction was carried out by incubating cardiac sections with DHE (10 μM) for 30 min at 37 °C [16].

Western blotting

The protein levels of RIP1/3 and p-RIP1/3 were 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. All lanes were probed for β-actin as loading controls and protein was detected using ECL (enhanced chemiluminescence) detection kit (GE Healthcare, Hong Kong, China).

Statistics

Data are presented as means ± SEM. Kaplan–Meier curve was used to estimate graft survival time. 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 performed using SPSS 13.0 (SPSS, Chicago, IL, USA), and statistical significance was set at $P < 0.05$.

Results

Nec-1 inhibited RIP1/3 and p-RIP1/3 expression after myocardial IR injury

RIP1 kinase activity and subsequent recruitment of RIP3 to RIP1 are necessary for the induction of programmed necrosis [17]. Therefore, we first investigated RIP1/3 levels in myocardium at 24 h after IR injury. Results showed that RIP1 and RIP3 protein levels in myocardium from control group (PBS-treated) were significantly increased on 24 h after reperfusion. RIP1, but not RIP3 expression was markedly increased in Nec-1-treated animals compared with Sham group and both RIP1 and RIP3 expression were significantly decreased in Nec-1-treated animals compared with control group (Fig. 1a). Next, we investigated the expression of RIP1 and RIP3 phosphorylation. In the control group, the expression of p-RIP1 and p-RIP3 were clearly increased after cardial IR injury, compared with Nec-1 treatment (Fig. 1b).

Nec-1-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 Nec-1 administration in comparison with IR group (IR + Nec-1*:* 1.72 ± 0.23 ng/ml; IR: 3.78 ± 0.50; $P < 0.05$) (Fig. 1c). For 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, Nec-1 administration induced significant improvement of hemodynamic performance compared with IR injury hearts. Cardiac output at 60 mmHg of afterload pressure was significantly increased in hearts with Nec-1 administration (IR+Nec-1: 66.9 ± 7.25 ml/min; IR: 39.0 ± 3.1 ml/min;

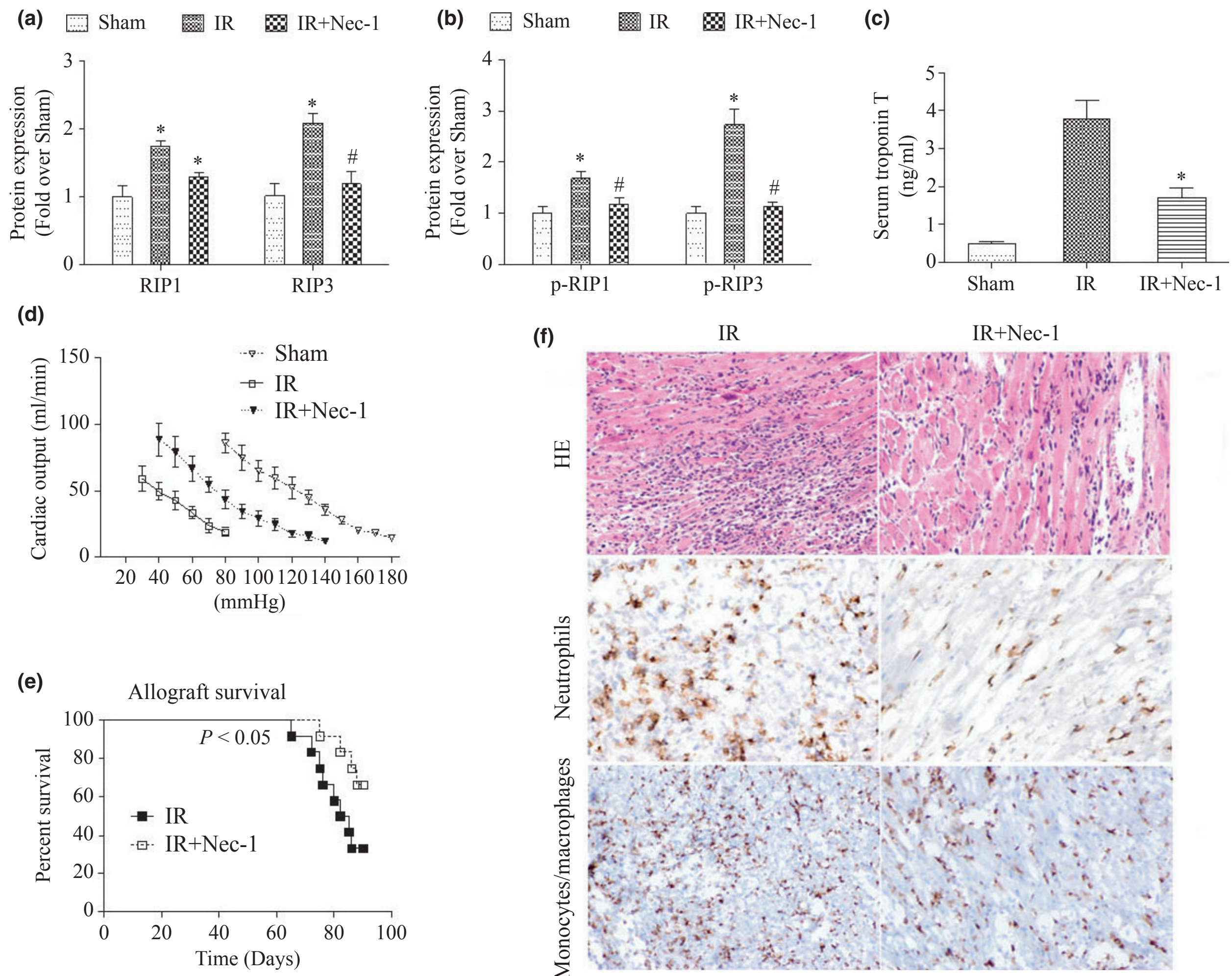

Figure 1 Nec-1 inhibited p-RIP1/3 expression and increased hemodynamic performance of cardiac isografts post-transplantation. (a) RIP1 and RIP3 protein expression in myocardium was measured by Western blotting at 24 h post-transplantation ($n = 6$). (b) p-RIP1 and p-RIP3 protein expression in myocardium was measured by Western blotting at 24 h post-transplantation ($n = 6$). (c) Serum TnT was measured in sham, IR, IR+Nec-1 treated recipients 24 h post-transplantation ($n = 6$). (d) Hearts were evaluated for hemodynamic function in pressure–volume relationship 24 h post-transplantation ($n = 6$). (e) Indicating the survival of heart allografts ($n = 12$). (f) Inflammatory cells infiltration were detected by immunologic staining. $^{*}P < 0.05$ vs. all other groups. $^{\#}P < 0.05$ vs. IR group.

$P < 0.05$) (Fig. 1d). The cardiac allograft survival in Nec-1-treated animals was significantly prolonged (MST = 90 days in IR + Nec-1 group, $P < 0.05$ as compared with IR group, MST = 83.5 days) (Fig. 1e).

Nec-1-prevented necrotic cell death and reduced leukocytes infiltration

We examined whether decreased RIP1/3-phosphorylation by Nec-1 affected the amount of necrosis after myocardial IR injury. Nec-1 administration resulted in less PI-positive cells in cardiac at 24 h post-transplantation when compared with control (Fig. 2a and b). We also carried out terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) of cardiac isografts from different experiment groups at 24 h post-transplantation. We found that Nec-1 administration did not alter the number of TUNEL-positive cardiomyocytes in comparison with control group (Fig. 2c).

Neutrophil infiltration is a hallmark of inflammatory injury after myocardial IR injury [18]. Therefore, we investigated the function of Nec-1 on neutrophil recruitment. As determined by myeloperoxidase activity and fluorescence activated cell sorting analysis of $Ly6G^{+}$ neutrophils, myocardial IR injury induced a surge in neutrophil recruitment to myocardium and netrin-1 treatment decreased neutrophil recruitment (Figs 1f and 2d–e). Macrophage migration is an important factor in

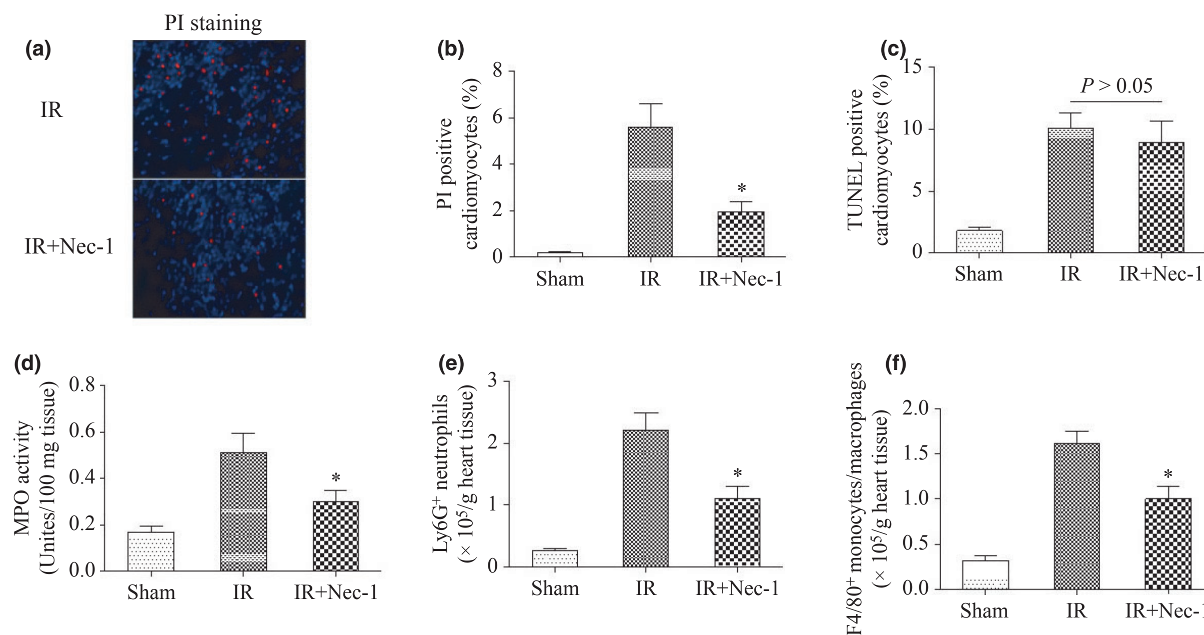

Figure 2 Nec-1-prevented necrotic cell death and attenuated cardiac neutrophil recruitment. (a) Representative photographs of propidium iodide (PI, red) and nuclear (Hoechst, blue) staining 24 h post-transplantation. (b) Percentages of PI-positive cells over total number of nuclei. (c) Percentages of TUNEL-positive nuclei over total number of nuclei. (d) Cardiac myeloperoxidase activity in tissue samples. (e) The number of Ly6G$^+$ neutrophils infiltrated in myocardium was analyzed by flow cytometry. (f) The number of F4/80$^+$ monocytes/macrophages infiltrated in myocardium was analyzed by flow cytometry. *$P < 0.05$ vs. all other groups. $n = 6$.

IR injury in heart [19]. We found that myocardial IR injury was characterized by a drastic increase in the infiltration of F4/80$^+$ monocytes/macrophages compared with sham-operated mice. This increase in cardiac monocytes/macrophages was significantly reduced in Nec-1-treated mice (Figs 1f and 2f).

Nec-1 attenuates ROS generation and changes the expression of oxidative stress genes

Previous paper demonstrated that RIP1/3 kinases regulate ROS production [20]. Therefore, we assessed ROS production at 24 h post-transplantation using DHE fluorescence intensity. Nec-1 treatment significantly reduced fluorescent intensity (22.96 ± 1.94 vs. 13.80 ± 1.53, $P < 0.05$), suggesting lower levels of ROS after cardiac IR injury when necroptosis is inhibited (Fig. 3a). We performed a PCR array on genes both involved in oxidative stress and cardiac disease [9]. The result showed that Nec-1 treatment significantly decreased both CYBA (p22phox subunit of NADPH oxidase) and TXNIP (thioredoxin interacting protein) mRNA levels after 24 h of cardiac IR injury. In contrast, nitric oxidase synthase 2 (NOS2), cyclo-oxygenase 2 (COX-2), glutathione peroxidase 1 (GPX1) and GRB-associated binding protein 1 (GAB1) mRNA levels were significantly increased (Fig. 3b).

Nec-1 downregulate IL-23/IL-17A dependent on inhabiting Hmgb1 expression in myocardial IR injury

Interleukin-17A is a critical mediator of neutrophils recruitment and migration through induction of granulopoiesis and the production of neutrophil chemokines [21]. Previous papers have indicated that IL-17A contributes to cardiac IR injury, and IL-23 was required for IL-17A production [12]. Therefore, we investigated the expression of IL-23 and IL-17A. Result showed that both IL-23 and IL-17A expression were decreased with Nec-1 administration. And inhibition of IL-23 or IL-17A significantly reduced expression of TnT and MPO after myocardial IR injury (Fig. 4).

Hmgb1, a highly conserved nuclear protein, served as an early mediator of inflammation and cell injury and plays a key role in many pathogenic states including myocardial IR [22]. In our experiment, Hmgb1 expression was markedly decreased with Nec-1 administration. Hmgb1 blockade significantly decreased expression of TnT and MPO and reduced production of IL-23/IL-17A (Fig. 5). To further investigate the role of Hmgb1 in attenuated IR injury induced by Nec-1, we administrated animal with rHmgb1 in addition of Nec-1. The result showed that the decreased TnT expression induced by Nec-1 was abrogated with exogenous Hmgb1 administration. And the reduced

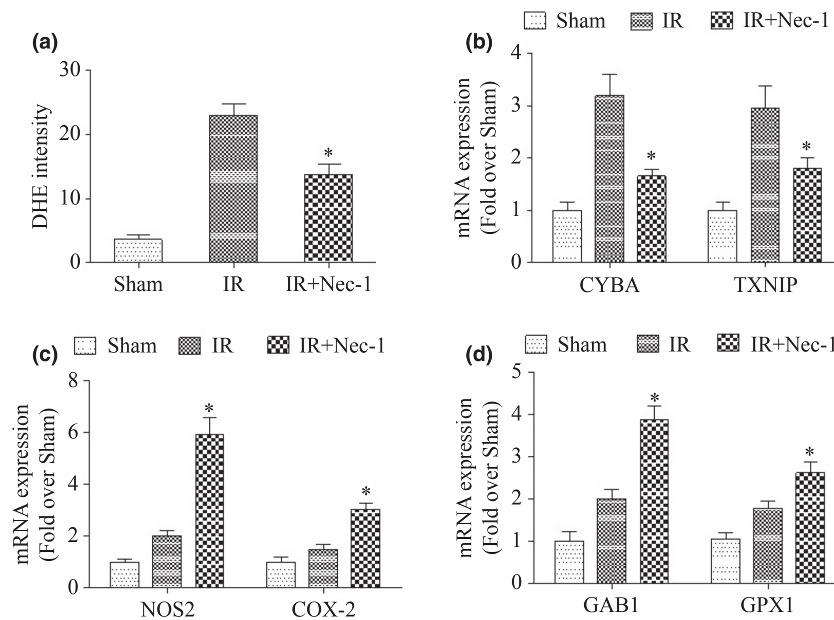

Figure 3 Nec-1 reduced ROS generation and changed the expression of oxidative stress genes after IR injury. (a) DHE fluorescence intensity in myocardial from sham, IR, IR + Nec-1 groups was measured. (b) Cardiac mRNA levels of genes both involved in oxidative stress and cardiac disease were measured by RT-PCR. *$P < 0.05$ vs. all other groups. $n = 6$.

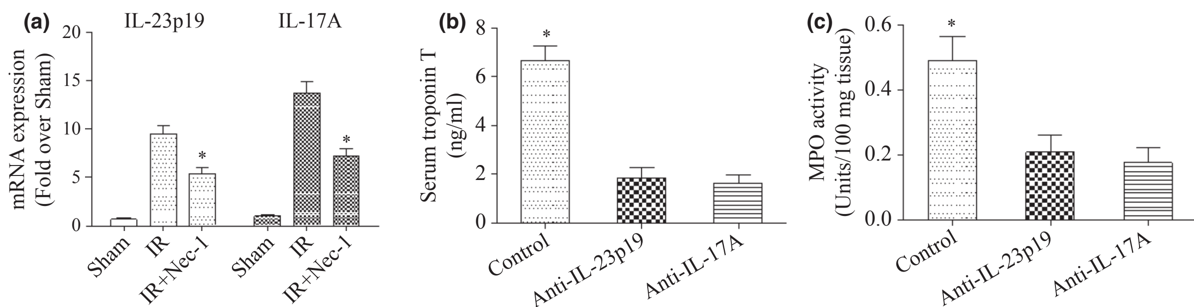

Figure 4 Nec-1 reduced IL-23 and IL-17A expression. (a) IL-23p19 and IL-17A mRNA levels in cardiac isografts 24 h post-transplantation from Sham, control, Nec-1-treated groups were analyzed by real-time PCR. (b) Serum cTnT was measured in control, Anti-IL-23p19 and Anti-IL-17A group 24 h post-transplantation. (c) Cardiac myeloperoxidase activity in cardiac isograft from control, Anti-IL-23p19, and Anti-IL-17A group 24 h post-transplantation. *$P < 0.05$ vs. all other groups. $n = 6$.

production of IL-23/IL-17A was also abolished by additional rHmgb1 treatment (Fig. 6).

Discussion

This study revealed a crucial role for Nec-1 in cardiomyocyte IR injury. Nec-1-ameliorated cardiomyocyte necrosis and infiltration of neutrophil and macrophage and then improved hemodynamic performance. Nec-1 treatment attenuated ROS generation and increased expression of NOS2 and COX-2. The expression of Hmgb1, IL-23 and IL-17A were also decreased with Nec-1 administration. Furthermore, the decreased TnT expression induced by Nec-1 was abrogated with exogenous Hmgb1 administration, and the reduced production of IL-23/IL-17A was also abolished by additional rHmgb1 treatment.

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 [23]. The emerging role of RIP1 and RIP3-dependent necrosis in many disease pathologies have led to the wide use of Nec-1 and its derivatives by many investigators. Nec-1 was thought to specifi-

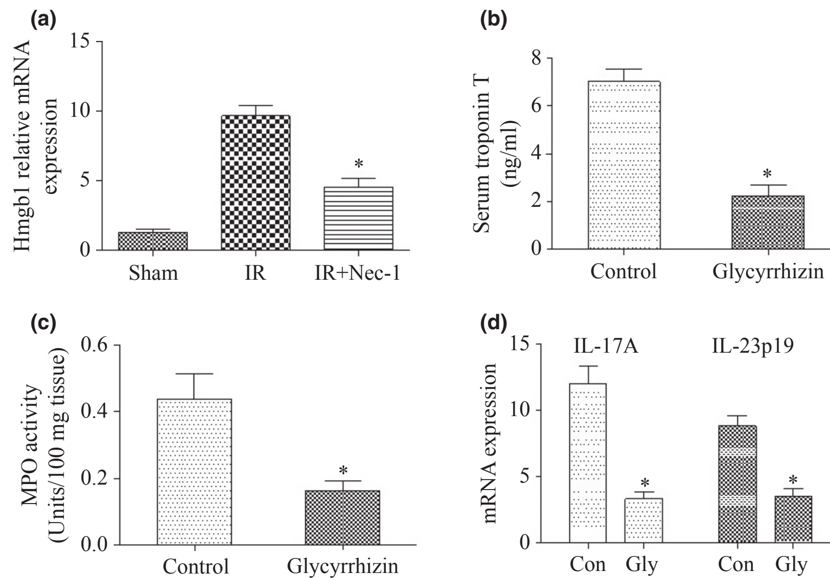

Figure 5 Nec-1 reduced IL-23 and IL-17A expression. (a) Hmgb1 mRNA level in cardiac isografts 24 h post-transplantation from Sham, control, Nec-1-treated groups were analyzed by real-time PCR. (b) Serum cTnT was measured in control, Glycyrrhizin-treated group 24 h post-transplantation. (c) Cardiac myeloperoxidase activity in cardiac isograft from control, Glycyrrhizin-treated group 24 h post-transplantation. (d) IL-23p19 and IL-17A mRNA levels in cardiac isografts 24 h post-transplantation from control, Glycyrrhizin-treated group groups were analyzed by real-time PCR. *$P < 0.05$ vs. all other groups. $n = 6$.

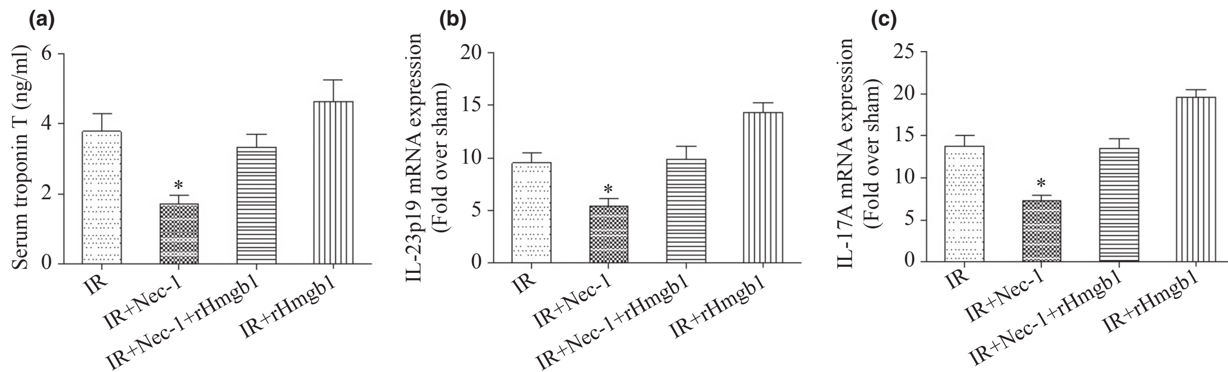

Figure 6 Hmgb1-IL-23/IL-17 pathway contributes to attenuated IR injury induced by Nec-1. (a) Serum cTnT was measured in IR, IR + Nec-1, IR + Nec-1 + rHmgb1, and IR + rHmgb1 groups 24 h post-transplantation. (b) IL-23p19 mRNA levels in cardiac isografts 24 h post-transplantation from IR, IR + Nec-1, IR + Nec-1 + rHmgb1, and IR + rHmgb1 groups were analyzed by real-time PCR. (c) IL-17A mRNA levels in cardiac isografts 24 h post-transplantation from IR, IR + Nec-1, IR + Nec-1 + rHmgb1, and IR + rHmgb1 groups were analyzed by real-time PCR. *$P < 0.05$ vs. all other groups. $n = 6$.

cally inhibit the pro-necrotic kinase function of RIP1 [8]. In this report, we demonstrated that RIP1 and RIP3 and their phosphorylation are increased in the cardiac response to IR injury. Inhibition of RIP1 with Nec-1 significantly inhibited the expression of RIP1 and RIP3 and their phosphorylation. Neutrophil recruitment and macrophage migration play a major role in myocardial damage after IR injury [24]. Decreased inflammatory cells infiltration and prolonged allograft survival in our results further proved attenuated IR injury induced by Nec-1 treatment.

Invading inflammatory cells play an important role in cardiac IR injury, capable of releasing degrading enzymes, and producing large amount of ROS [25]; 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 [26,27]. We observed that inhibition of RIP1/3 phosphorylation upon Nec-1 treatment was associated with decreased myocardial ROS levels after myocardial IR injury. Recent reports showed that increased

NADPH oxidase activity was contributable to IR-induced ROS formation and cell death [28,29]. Furthermore, Inhibition of TXNIP, which was encoded by a mechanically-regulated gene that control cell growth and apoptosis, resulted in enhanced cardiomyocytes survival and attenuated cardiac hypertrophy [30]. GPX1 and GAB1 were also reported to be involved in cell survival and exert a protective role during cardiovascular disease [31,32]. In our experiment, CYBA and TXNIP were both downregulated, while GPX1 and GAB1 increased significantly. All of these were consistent with previous research indicating that Nec-1 treatment altered the expression of genes that are critically involved in oxidative stress regulation and this alteration may be a potential mechanism of attenuated IR injury induced by Nec-1 administration.

Interleukin (IL)-17A, a member of the IL-17 family, was secreted by different cells including Th17 cells, γδ T cells, NK cells, NKT cells, and neutrophils. The role of IL-17 in innate and adaptive auto- and allo-immune responses has been investigated by several groups, but is still not fully understood. Clearly, IL-17 was increased in human lung, liver and kidney rejection and promoted graft inflammation [15,33]. 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 [34]. Previous research from Langrish *et al.* [35] has demonstrated that IL-23 drives a pathogenic T cell population which induces autoimmune inflammation. Recent paper showed that an IL-23/IL-17 pathway was activated in cardiac ischemia reperfusion injury [36]. Hmgb1, a highly conserved nuclear protein, served as an early mediator of inflammation and cell injury and plays a key role in many pathogenic states including myocardial IR. We found that the expression of IL-23/IL-17 and Hmgb1 was markedly decreased with Nec-1 treatment and blockade of IL-23/IL-17 or Hmgb1 showed cardioprotective effect. Furthermore, the cardioprotective effect induced by Nec-1 was abrogated with exogenous Hmgb1 administration and the reduced production of IL-23/IL-17A was also abolished by additional treatment of rHmgb1. All of these showed that the attenuated IR injury by Nec-1 administration was at least partially dependent on reduced expression of IL-23/IL-17 or Hmgb1 and we are the first to demonstrate that the Hmgb1-IL-23/IL-17 pathway played an important role in the cardioprotective effect induced by Nec-1.

In summary, our study provides evidence that Nec-1 played a protective role in cardiomyocyte IR injury and this was associated with inhibited of Hmgb1-IL-23/IL-17 pathway. Although further investigations are needed to fully clarify the precise molecular and cellular mechanism involved in the immunoregulation, Nec-1 may be exploited as a novel therapeutic agent to attenuate IR injury in cardiac transplantation.

Authorship

AZ, XM and HJ: designed research. AZ, XM, LL, YT, LL, YH and YL: performed study. AZ, XM and LL: collected data, analyzed data and wrote the paper.

Funding

This work has no funding.

References

1. Wang J, Takahashi K, Piao H, Qu P, Naruse K. 9-Phenanthrol, a TRPM4 inhibitor, protects isolated rat hearts from ischemia-reperfusion injury. *PLoS ONE* 2013; **8**: e70587.
2. Moquin D, Chan FK. The molecular regulation of programmed necrotic cell injury. *Trends Biochem Sci* 2010; **35**: 434.
3. Vandenabeele P, Galluzzi L, Vanden Berghe T, Kroemer G. Molecular mechanisms of necroptosis: an ordered cellular explosion. *Nat Rev Mol Cell Biol* 2010; **11**: 700.
4. Challa S, Chan FK. Going up in flames: necrotic cell injury and inflammatory diseases. *CMLS* 2010; **67**: 3241.
5. Kono H, Rock KL. How dying cells alert the immune system to danger. *Nat Rev Immunol* 2008; **8**: 279.
6. Cho YS, Challa S, Moquin D, *et al.* Phosphorylation-driven assembly of the RIP1-RIP3 complex regulates programmed necrosis and virus-induced inflammation. *Cell* 2009; **137**: 1112.
7. He S, Wang L, Miao L, *et al.* Receptor interacting protein kinase-3 determines cellular necrotic response to TNF-alpha. *Cell* 2009; **137**: 1100.
8. Degterev A, Huang Z, Boyce M, *et al.* Chemical inhibitor of nonapoptotic cell death with therapeutic potential for ischemic brain injury. *Nat Chem Biol* 2005; **1**: 112.
9. Oerlemans MI, Liu J, Arslan F, *et al.* Inhibition of RIP1-dependent necrosis prevents adverse cardiac remodeling after myocardial ischemia-reperfusion in vivo. *Basic Res Cardiol* 2012; **107**: 270.
10. Heron I. A technique for accessory cervical heart transplantation in rabbits and rats. *Acta Pathol et Microbiol Scand Section A, Pathol* 1971; **79**: 366.
11. Wiedemann D, Schneeberger S, Friedl P, *et al.* The fibrin-derived peptide Bbeta(15-42) significantly attenuates ischemia-reperfusion injury in a cardiac transplant model. *Transplantation* 2010; **89**: 824.
12. Zhu H, Li J, Wang S, Liu K, Wang L, Huang L. Hmgb1-TLR4-IL-23-IL-17A axis promote ischemia-reperfusion injury in a cardiac transplantation model. *Transplantation* 2013; **95**: 1448.

 © 2014 Steunstichting ESOT

13. Rabadi MM, Ghaly T, Goligorksy MS, Ratliff BB. HMGB1 in renal ischemic injury. *Am J Physiol Renal Physiol* 2012; **303**: F873.
14. Liao YH, Xia N, Zhou SF, *et al.* Interleukin-17A contributes to myocardial ischemia/reperfusion injury by regulating cardiomyocyte apoptosis and neutrophil infiltration. *J Am Coll Cardiol* 2012; **59**: 420.
15. Gorbacheva V, Fan R, Li X, Valujskikh A. Interleukin-17 promotes early allograft inflammation. *Am J Pathol* 2010; **177**: 1265.
16. Liu X, Gai Y, Liu F, *et al.* Trimetazidine inhibits pressure overload-induced cardiac fibrosis through NADPH oxidase-ROS-CTGF pathway. *Cardiovasc Res* 2010; **88**: 150.
17. Christofferson DE, Li Y, Hitomi J, *et al.* A novel role for RIP1 kinase in mediating TNFalpha production. *Cell Death Dis* 2012; **3**: e320.
18. Chandrasekar B, Colston JT, de la Rosa SD, Rao PP, Freeman GL. TNF-alpha and H_2O_2 induce IL-18 and IL-18R beta expression in cardiomyocytes via NF-kappa B activation. *Biochem Biophys Res Commun* 2003; **303**: 1152.
19. Liu A, Fang H, Dirsch O, Jin H, Dahmen U. Early release of macrophage migration inhibitory factor after liver ischemia and reperfusion injury in rats. *Cytokine* 2012; **57**: 150.
20. Zhang DW, Shao J, Lin J, *et al.* RIP3, an energy metabolism regulator that switches TNF-induced cell death from apoptosis to necrosis. *Science* 2009; **325**: 332.
21. Ley K, Smith E, Stark MA. IL-17A-producing neutrophil-regulatory Tn lymphocytes. *Immunol Res* 2006; **34**: 229.
22. Scaffidi P, Misteli T, Bianchi ME. Release of chromatin protein HMGB1 by necrotic cells triggers inflammation. *Nature* 2002; **418**: 191.
23. Steffens S, Montecucco F, Mach F. The inflammatory response as a target to reduce myocardial ischaemia and reperfusion injury. *Thromb Haemost* 2009; **102**: 240.
24. Bettelli E, Korn T, Oukka M, Kuchroo VK. Induction and effector functions of T(H)17 cells. *Nature* 2008; **453**: 1051.
25. Vinten-Johansen J. Involvement of neutrophils in the pathogenesis of lethal myocardial reperfusion injury. *Cardiovasc Res* 2004; **61**: 481.
26. Prasad A, Stone GW, Holmes DR, Gersh B. Reperfusion injury, microvascular dysfunction, and cardioprotection: the "dark side" of reperfusion. *Circulation* 2009; **120**: 2105.
27. Morgan MJ, Kim YS, Liu ZG. TNFalpha and reactive oxygen species in necrotic cell death. *Cell Res* 2008; **18**: 343.
28. Borchi E, Parri M, Papucci L, *et al.* Role of NADPH oxidase in H9c2 cardiac muscle cells exposed to simulated ischaemia-reperfusion. *J Cell Mol Med* 2009; **13**: 2724.
29. Heymes C, Bendall JK, Ratajczak P, *et al.* Increased myocardial NADPH oxidase activity in human heart failure. *J Am Coll Cardiol* 2003; **41**: 2164.
30. Yoshioka J, Imahashi K, Gabel SA, *et al.* Targeted deletion of thioredoxin-interacting protein regulates cardiac dysfunction in response to pressure overload. *Circ Res* 2007; **101**: 1328.
31. Nakaoka Y, Nishida K, Narimatsu M, *et al.* Gab family proteins are essential for postnatal maintenance of cardiac function via neuregulin-1/ErbB signaling. *J Clin Investig* 2007; **117**: 1771.
32. Cao C, Huang X, Han Y, *et al.* Galpha(i1) and Galpha(i3) are required for epidermal growth factor-mediated activation of the Akt-mTORC1 pathway. *Science Signal* 2009; **2**: ra17.
33. Vanaudenaerde BM, De Vleeschauwer SI, Vos R, *et al.* The role of the IL23/IL17 axis in bronchiolitis obliterans syndrome after lung transplantation. *Am J Transplant* 2008; **8**: 1911.
34. Sun J, Walsh M, Villarino AV, *et al.* TLR ligands can activate dendritic cells to provide a MyD88-dependent negative signal for Th2 cell development. *J Immunol* 2005; **174**: 742.
35. Langrish CL, Chen Y, Blumenschein WM, *et al.* IL-23 drives a pathogenic T cell population that induces autoimmune inflammation. *J Exp Med* 2005; **201**: 233.
36. Zhu H, Liu K, Wang L, Huang L. Long pentraxin PTX3 attenuates ischemia reperfusion injury in a cardiac transplantation model. *Transplant Int* 2014; **27**: 87.