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The spleen contributes importantly to myocardial infarct exacerbation during post-ischemic reperfusion in mice via signaling between cardiac HMGB1 and splenic RAGE

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Abstract

The spleen plays a critical role in post-infarct myocardial remodeling. However, the role of the spleen in exacerbating myocardial infarction (MI) during acute ischemia/reperfusion (I/R) injury is unknown. The present study tests the hypothesis that splenic leukocytes are activated by substances released from ischemic myocardium to subsequently exacerbate myocardial injury during reperfusion. The left coronary artery in C57BL/6 mice underwent various durations of occlusion followed by 60 minutes of reperfusion (denoted as min/min of I/R) with or without splenectomy prior to I/R injury. Splenectomy significantly decreased myocardial infarct size (IS) in 40'/60' and 50'/60' groups ($p < 0.05$); however, it had no effect on IS in 10'/60', 20'/60' and 30'/60' groups ($p = \text{NS}$). In the 20'/60' group, infusion of 40-minute ischemic heart homogenate (40-IHH) upon reperfusion increased IS by >3-fold versus infusion of 10-IHH ($p < 0.05$).

Splenectomy abolished the infarct exacerbating effect of 40-IHH, which was restored by splenic leukocyte adoptive transfer (SPAT). Furthermore, depletion of HMGB1 in the 40-IHH group abolished its infarct exacerbating effect ($p < 0.05$), and 40-IHH failed to increase IS in both RAGE^{-/-} mice and splenectomized wild-type mice with SPAT from RAGE^{-/-} mice. The injection of 40-IHH significantly increased formyl peptide receptor 1 (FPR1) expression in sham spleens when compared to 10-IHH-treated sham & control mice. cFLFLF, a specific FPR1 antagonist, reduced myocardial neutrophil infiltration and abrogated the infarct exacerbating effect of 40-IHH during reperfusion.

Conclusions—A cardio (HMGB1) – splenic (RAGE receptor) signaling axis exists and contributes to myocardial infarct exacerbation during reperfusion after prolonged ischemic insults

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DISCLOSURES

None.

by activating splenic leukocytes. The FPR1 is a potential therapeutic target for inhibiting the cardio-splenic axis that augments infarct size during post-ischemic reperfusion.

Keywords

myocardial ischemia; reperfusion injury; spleen; HMGB1/RAGE pathway; neutrophils

INTRODUCTION

Ischemic heart disease remains the single leading cause of death in the United States, accounting for fully one out of every four deaths. Myocardial infarction (MI) and heart failure account for the vast majority of the morbidity and mortality associated with ischemic heart disease[31]. Current therapy for acute myocardial infarction is directed toward rapid restoration of perfusion to salvage as much of the jeopardized myocardium as possible[4, 5, 8]. This may be accomplished through interventional or surgical means, such as by PCI or CABG. Although reperfusion is a primary therapeutic goal, the reversal of ischemia entails additional damage, which is defined as reperfusion injury, and can paradoxically reduce the beneficial effects of reperfusion therapy[5, 20]. Numerous cardioprotective strategies, such as ischemic preconditioning, post-conditioning and remote ischemic preconditioning, have been developed to address myocardial reperfusion injury by acting on several molecular signal transduction pathways[14, 15]. However, clinical trials based on these translational strategies have largely yielded disappointing outcomes, perhaps due to our incomplete understanding of the pathogenesis of ischemia/reperfusion (I/R) injury.

Considerable evidence implicates the acute inflammatory response as a contributor to reperfusion injury[3, 5, 52, 53]. Studies from our group and others have demonstrated that inflammatory responses play a pivotal role in mediating acute I/R injury[49, 52, 53]. However, exactly how these inflammatory responses are triggered and mediated remains unclear. The spleen has critical roles in regulating inflammatory responses under several disease conditions including post-MI left ventricular remodeling. Swirski and colleagues first reported that the spleen serves as a reservoir to release monocytes after MI in animal models[39]. They found that splenectomy performed either prior to coronary ligation or 3 days after could significantly preserve cardiac function and limit left ventricular remodeling 4 weeks after MI[39]. Recently, Ismahil and colleagues reported a cardio-splenic axis in a murine heart failure model during long term follow up (8 weeks) after MI. Their results demonstrated that splenic mononuclear cells mediate myocardial inflammation in failing hearts and promote ventricular remodeling. Splenectomy can thus reduce the inflammatory response and improve heart function[21]. Moreover, the spleen is also been implicated in human studies that suggest the spleen plays an important role in mediating inflammatory responses during post-MI cardiac remodeling[10, 43]. However, whether the spleen contributes importantly to acute myocardial necrosis remains to be established.

In the current study, we hypothesized that prolonged ischemic insults could produce more pro-inflammatory substances, such as damage associated molecular patterns (DAMPs), like high-mobility group box-1 protein (HMGB1), that activate splenic leukocytes (i.e., a cardio-splenic axis), which subsequently exacerbate myocardial IS during reperfusion. Upon

addressing this hypothesis in an *in vivo* mouse model, we found that prolonged ischemia exacerbates myocardial necrosis and increases levels of circulating HMGB1 upon reperfusion. This HMGB1 then activates splenic leukocytes by binding to the receptor for advanced glycation end products (RAGE), which promotes inflammatory responses during reperfusion and exacerbates myocardial IS. Antagonism of formyl peptide receptor 1 (FPR1) proved sufficient to inhibit this reperfusion-induced and spleen-enabled inflammatory response, thus attenuating myocardial IS.

MATERIALS and METHODS

This study conformed to the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health (Eighth Edition, revised 2011) and was conducted under protocols approved by the University of Virginia's Institutional Animal Care and Use Committee.

Animals and experimental protocols

C57BL/6 wild type (WT) mice (9–13 weeks of age, purchased from The Jackson Laboratory) and RAGE knockout (RAGE^{-/-}) mice[38] were randomly assigned to either I/R injury groups or sham surgery groups. RAGE^{-/-} mice were backcrossed onto C57BL/6 for 10 generations and thus were congenic with C57BL/6. Acute splenectomy (SPLX) was performed 10 minutes before myocardial ischemia with or without adoptive transfer of splenic leukocytes after splenectomy. Isolation of splenic leukocytes was performed using the gentleMACS single splenocyte isolation system according to the manufacturer's instructions (Miltenyi Biotec, San Diego, CA). Viability (>95%) of the splenocytes was confirmed with Trypan Blue staining. As described previously[40], splenic leukocyte adoptive transfer (SPAT) was performed by injecting 5×10^6 isolated splenocytes (in ~100 μ l PBS) into splenectomized mice via left external jugular vein 5 minutes after removing the spleen. Supernatant from ischemic heart tissue homogenate (IHH) was acquired from ischemic heart tissue at the end of 10 minutes (10-IHH) or 40 minutes (40-IHH) of ischemia. IHH was injected 5 minutes before reperfusion via left external jugular vein, and the dose of 10 μ g of total IHH protein per gram mouse body weight was selected based on pilot studies that examined the effect of 3 different doses on infarct size exacerbation (Online Resource Figure I). In cFLFLF-treated mice, cFLFLF was administered (0.5mg/kg, i.v.) 5 minutes before reperfusion.

Splenectomy

As described previously[40], mice were anesthetized with sodium pentobarbital (80 mg/kg i.p.) and orally intubated. Artificial respiration was maintained with a FiO₂ of 0.80, 120 strokes per minute, and a 0.2 to 0.5mL stroke volume. A small midline abdominal incision was made and the peritoneal cavity entered. The spleen was located and brought to the incision. The hilum was clamped and ligated with 3-0 silk and the spleen excised. The incision was closed in two layers using 5-0 Prolene. The mice then underwent further procedures as described. The donor mice for splenic leukocytes were euthanized by cervical dislocation under deep anesthesia with death confirmed by absence of electric activity in ECG.

Myocardial I/R injury and measurement of infarct size

In previous work, we established that myocardial infarct size (IS) as measured by late-gadolinium enhanced (LGE) MRI at 60 minutes of reperfusion attains 95% of the size measured by the same method at 24 hours post-reperfusion in mice[40, 54]. We therefore used 60 minutes of reperfusion in the present study. The left coronary artery (LCA) of WT or RAGE^{-/-} mice was ligated for durations of 10 to 50 minutes to impose graded levels of ischemia (I) followed by 60 minutes of reperfusion (R) as detailed previously[52–55]. The duration of I/R is denoted as minutes/minutes. Briefly, mice were anesthetized with sodium pentobarbital (80mg/kg, i.p.) and orally intubated. An additional dose of pentobarbital (40mg/kg, i.p.) was applied shortly after reperfusion. The adequacy of the anesthesia was confirmed by hind limb pinch reflex every 15 minutes. The heart was exposed through a left thoracotomy. The LCA was identified under a dissecting microscope. A 7-0 silk suture was placed around the LCA at a level 1 mm inferior to the left auricle. Ischemia was induced by securing a suture over a piece of PE-60 tubing placed parallel to the LCA, and reperfusion was achieved by removing the tube. Successful ligation of the LCA was confirmed by blanching in the ischemic zone and by ST segment elevation in the ECG. ECG was monitored perioperatively using PowerLab instrumentation (ADInstruments, Colorado Springs, CO). The mice were euthanized 60 minutes after reperfusion, and explanted hearts were cannulated through the ascending aorta for perfusion with 3ml of 1.0% TTC (Sigma-Aldrich, St. Louis, MO). The LCA was then re-occluded with the same suture used for coronary occlusion prior to 10% Phthalo blue perfusion to determine risk region (RR), which was statistically similar among all groups (Online Resource Figure II). The left ventricle was then cut into 5 to 7 transverse slices that were weighed and digitally photographed to determine IS as a percent of RR[52, 53].

Peripheral white blood cell counting

Blood samples (40µl per mouse) were obtained by puncturing the left external jugular vein at 60 minutes after reperfusion. Cell counts were performed using a HemaVet Hematology System (CDC Technologies, Oxford, CT).

Preparation of ischemic heart homogenate supernatant (IHH) and immunoprecipitation

At the end of 10 or 40 minutes of LCA occlusion or 10 min after thoracotomy without LCA occlusion (sham), hearts were harvested and placed on ice. The ischemic area was located on the anterolateral aspect of the left ventricle based on its pale color with clear demarcation. The full thickness of the ischemic zone was then cut, using a No. 11 scalpel blade, 0.5 mm inside of the demarcation line. The ischemic myocardium was then suspended in PBS (10µl/mg tissue) and homogenized with an electric homogenizer. The supernatant was acquired after centrifuging at 14,000rpm for 10 minutes at 4°C. Protein concentration of the IHH supernatant was determined by BCA protein assay kit for dilution to 5 mg/ml in PBS prior to storage at 4°C.

Western analysis

HMGB1 levels in heart homogenates and plasma were assessed by Western blot analysis as previously described[55]. Briefly, heart tissue was homogenized in PBS. Plasma samples were

obtained by centrifuging blood samples at 1600g for 20 minutes. The total protein concentrations were determined by BCA protein assay (Thermo Scientific, Rockford, IL). Twenty micrograms of protein were separated by 10% SDS-PAGE. After transfer, nitrocellulose membranes (Bio-Rad, Hercules, CA) were probed with primary antibodies against HMGB1 (Abcam, Cambridge, MA) at a 1:2,000 dilution and secondary antibodies (Promega, Madison, WI) at a 1:5,000 dilution in blocking solution (0.5% BSA in TBS-T). Proteins were visualized with enhanced chemiluminescent substrate (Thermo Scientific, Rockford, IL) followed by densitometry using a Fluorchem 8900 imaging system (Alpha Innotech, Santa Clara, CA). β -actin was used as a loading control for HMGB1 measurements in IHH samples.

Immunofluorescence staining

Spleens and hearts were fixed in 4% paraformaldehyde in PBS (pH 7.4) for 1 hour at room temperature and then incubated in 30% sucrose overnight at 4°C before freezing in OCT. Frozen sections were cut and blocked with CD16/32 (eBiosciences, San Diego, CA) in 10% normal serum for 1 hour at room temperature. Spleen sections were co-incubated with goat anti-FPR (Santa Cruz, Dallas, Texas), rat Alexa Fluor-594 labeled anti-CD11b (BioLegend San Diego, CA) and rat FITC-labeled anti-Ly6G antibodies (Cat. 11-9668-80, eBiosciences, San Diego, CA) overnight at 4°C. The anti-FPR sections were then incubated with donkey anti-goat Alexa Fluor 660-conjugated secondary antibodies for 1 hour at room temperature. Alexa Fluor 594- and FITC-labeled rat IgG (Serotec, Raleigh, NC) were used as negative controls for CD11b and Ly6G staining, respectively. Goat IgG (Santa Cruz, Dallas, Texas) was used as a negative control for FPR staining. Heart sections were incubated with anti-Ly6G for neutrophil staining. ProLong Gold anti-fade reagent with DAPI (Life Technologies, Grand Island, NY) was used to mount the specimens. All images were acquired under the same parameters for each fluorochrome using an Olympus BX-41 Microscope (Olympus, America, Inc., Center Valley, PA) with a Retiga-2000R camera (QImaging, Surrey, BC). Neutrophils were counted on 10 high-power fields per section. Fluorescence intensity of FPR expression in at least 100 nuclei per sample was determined with ImageJ software (NIH).

Quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR) analysis

Splenic leukocytes and bone marrow cells were harvested from mice with 40-IHH or vehicle pre-treatment. mRNA levels of IL-1 β , INF- γ , TNF- α and MCP-1 were assessed by qRT-PCR. In brief, total RNA was isolated from cells using the RNeasy Mini Kit (QIAGEN China Co., Ltd, Shanghai) according to the manufacturer's instructions. cDNAs were synthesized using RevertAidTM First Strand cDNA Synthesis Kit (Fermentas China Co., Ltd, Shenzhen). qPCR was performed with Maxima[®] SYBR Green qPCR Master Mix (Fermentas China Co., Ltd, Shenzhen). Actin was used as a housekeeping gene. mRNA levels were quantified using the 2^(-Ct) relative quantification method as previously described[42]. The primer sequences are provided in Online Resource Table I.

Immunoprecipitation

Depletion of HMGB1 protein in IHH was achieved by immunoprecipitation with a Dynabeads[®] Protein A Immunoprecipitation Kit according to the manufacturer's

instructions (Life Technologies, Grand Island, NY). Briefly, the HMGB1 antibody (10 μ g in 200 μ l binding buffer) was pre-incubated with Dynabeads® Protein A for 15 minutes at room temperature. Then, 100 μ l of 40-IHH was mixed with the Dynabeads for 1 hour at room temperature, and the supernatant was collected for use. Western blot analysis was used to confirm efficient HMGB1 depletion. Rabbit IgG was used as a negative control for this analysis.

Statistical analysis

All data are presented as mean \pm SEM (standard error of the mean). Changes in peri-operative white blood cell counts and heart rates were analyzed using repeated measures ANOVA followed by Bonferroni pairwise comparisons. All other data were compared using one-way ANOVA followed by t-test for unpaired data with Bonferroni correction.

RESULTS

Splenectomy attenuates myocardial I/R injury resulting from prolonged ischemic insult

C57BL/6 mice underwent various durations of LAD occlusion followed by 60 min of reperfusion with or without acute splenectomy (SPLX) prior to myocardial I/R. There were no differences in risk region (RR) size among these groups (Online resources Figure IIA). As shown in the Figure 1A, SPLX significantly decreased myocardial infarct size (IS) in the 40'/60' group (I/R, min/min; 49.6 \pm 1.4% vs. 35.7 \pm 1.9%, p<0.05) and the 50'/60' group (56.4 \pm 1.3% vs. 44 \pm 2.0%, p<0.05). However, SPLX had no effect on IS in the 10'/60', 20'/60' and 30'/60' groups (p=NS). The sham procedure of laparotomy without splenectomy had no effect on IS in any of the ischemic groups (data not shown). Acute splenic leukocyte adoptive transfer (SPAT) completely restored IS in splenectomized mice after 40'/60' IR injury (50.9 \pm 1.2% vs. 35.7 \pm 1.9%, p<0.05) to the level of control mice (50.9 \pm 1.2% vs. 49.6 \pm 1.4%, p=NS) (Figure 1B and 1C). However, SPAT did not increase IS in SPLX+20'/60' mice (7.0 \pm 1.6% vs. 7.3 \pm 1.4, p=NS, Figure 1C).

Homogenates from hearts subject to prolonged ischemia exacerbate IS in mice subjected to brief ischemia by acting on splenic leukocytes

Based on the above results, we hypothesized that substance(s) derived from hearts subjected to prolonged ischemia might be released to serve as messengers between the heart and spleen during reperfusion by activating splenocytes. To test this hypothesis, supernatants from ischemic heart homogenates (IHH) were acquired from mouse hearts undergoing either 10 (10-IHH) or 40 (40-IHH) minutes of ischemia without reperfusion. Recipient WT mice underwent 20'/60' I/R, and IHH at a dose of 10 μ g protein per g mouse weight was injected IV into the recipient mice immediately before reperfusion. In the recipient mice, IHH had no effect on heart rates after reperfusion (Table 1). The risk regions (RRs) were similar among all groups. Treatment with 40-IHH significantly exacerbated myocardial IS in the 20'/60' group (7.3 \pm 1.3% versus 33.3 \pm 2.5, p<0.05) (Figure 2). However, treatment with 10-IHH had no effect on IS (7.3 \pm 1.3% versus 9.6 \pm 2.3%, p=NS) (Figure 2). The exacerbating effect of 40-IHH on IS disappeared in splenectomized versus normal 20'/60' mice (9.9 \pm 2.6% versus 33.3 \pm 2.5%, p<0.05) but was restored by SPAT (Figure 2).

HMGB1 from 40-IHH activates splenocytes during reperfusion

Consistent with previous reports[2], we found that HMGB1 levels in mice treated with 40-IHH were significantly increased compared with 10-IHH or sham IHH; whereas there was no difference between 10-IHH and sham IHH (Figure 3A). Depletion of HMGB1 in 40-IHH by immunoprecipitation, as confirmed by Western blot analysis (Figure 3B), completely abrogated the exacerbating effect of 40-IHH on IS (Figure 3C). The immunoprecipitation negative control using rabbit IgG did not negate the IS exacerbating effect of 40-IHH.

The infarct exacerbating effect of 40-IHH is lost in RAGE^{-/-} mice

It has been reported that the HMGB1-RAGE pathway plays important roles in myocardial reperfusion injury[2]. Hence, we next investigated whether the presence of RAGE on splenocytes was required for the HMGB1-induced exacerbation of I/R injury. As shown in Figure 3D, 40-IHH failed to increase IS in RAGE^{-/-} mice. Moreover, 40-IHH failed to exacerbate IS in splenectomized WT mice reconstituted with RAGE^{-/-} splenocytes.

40-IHH activates splenic leukocytes and induces neutrophilia during reperfusion

40'/60' I/R injury caused significant neutrophilia and induced more myocardial neutrophil infiltration than 20'/60' I/R or sham surgery (Figure 4). Neutrophilia in the 40'/60' group was significantly attenuated by splenectomy (Figure 4A). 40-IHH significantly increased IL-1 β , INF- γ , TNF- α and MCP-1 gene expression in splenic leukocytes but not in bone marrow cells (Online Resource Figure III). The injection of 40-IHH significantly augmented neutrophilia and myocardial neutrophil infiltration in the 20'/60' I/R group, which was attenuated by splenectomy. Conversely, neutrophil accumulation in the 40'/60' I/R group was significantly attenuated by splenectomy (Figure 4B). Immunofluorescence staining revealed that splenic leukocytes in the marginal zone of spleens from 40-IHH treated mice displayed elevated levels of FPR1 expression, which is a key receptor that regulates neutrophil chemoattraction (Figure 5). Confocal imaging further showed that the elevated FPR1 expression was primarily localized to Ly6G⁺ cells (Figure 5). These experiments were conducted with the Ly6G monoclonal antibody produced by clone 1A8, which has been reported to be specific for neutrophils since it does not cross-react with Ly6C[9].

The formyl peptide receptor (FPR) antagonist, cFLFLF, abrogates the infarct exacerbating effect of 40-IHH

We next examined whether cFLFLF, a selective FPR1 antagonist[12], could reduce myocardial I/R injury. cFLFLF was administered at a dose of 0.5mg/kg as an i.v. bolus 5 minutes before reperfusion. This dose was selected because it had been used in a previous study for *in vivo* tracking of activated neutrophils[47]. cFLFLF significantly reduced circulating neutrophils and myocardial neutrophil infiltration in 40-IHH treated 20'/60' I/R mice (Figures 6A and 6B). Myocardial infarct sizes in 40-IHH treated 20'/60' I/R mice and 40'/60' I/R mice were significantly reduced by pretreatment with cFLFLF (Figure 6C and Online Resource Figure IV).

DISCUSSION

The present study represents the first demonstration, to the best of our knowledge, that the cardio-splenic axis plays a pivotal role in exacerbating myocardial infarct size during post-ischemic reperfusion. Our results show that prolonged ischemia elevates myocardial HMGB1 levels, and that HMGB1 is released into the bloodstream during reperfusion to activate splenocytes via RAGE stimulation. The activation of splenic leukocytes subsequently activates neutrophils, which transmigrate to the myocardium during reperfusion to exacerbate myocardial infarct size. Blocking FPR1, a key receptor regulating neutrophil chemoattraction, upon reperfusion blocks this cardio-splenic signaling axis and abrogates the reperfusion-induced exacerbation of infarct size.

The spleen exacerbates myocardial infarct size during reperfusion after prolonged ischemic insult

A role for the spleen in mediating I/R injury has been reported in the kidney[46] and brain[56]. Leuschner and colleagues[26] reported recently that splenectomy limited myocardial inflammatory responses and reduced IS after I/R in ApoE^{-/-} mice, which have significantly larger infarct sizes than wild-type mice due to chronic inflammatory stimulation. Recently, we found that splenectomy had no effect on myocardial infarct size after 30 min of ischemia followed by reperfusion; however, it was sufficient to abolish the hyperglycemia-induced exacerbation of myocardial infarct size [40]. Collectively, these findings indicate that the spleen may not contribute importantly to myocardial reperfusion injury unless a strong pro-inflammatory stimulus is present. In this study, we found that 40-IHH significantly increased IL-1 β , INF- γ , TNF- α and MCP-1 gene expression in splenic leukocytes but not in bone marrow cells in mice without I/R injury (Online Resource Figure III). Our current study clearly demonstrates that the spleen contributes to acute myocardial post-ischemic reperfusion injury; however, its role becomes significant in mice only after relatively long ischemic insults (>30 minutes, Figure 1).

HMGB1 from ischemic myocardium is necessary for spleen-mediated infarct exacerbation during reperfusion

Historically, the immune system is understood to protect the body against foreign insults; however, recent evidence has shown that the innate immune system is alerted following tissue injury with the release of endogenous ligands. An increasing body of evidence supports the contention that innate immune responses contribute importantly to myocardial I/R injury[11, 17, 24, 52, 53]. I/R injury results in the release of endogenous ‘danger’ signals referred to as danger associated molecular patterns (DAMPs) by necrotic or activated cells that trigger innate immune responses[29]. DAMPs include mediators such as heat shock proteins[23], HMGB1[48], hyaluronic acid[22], S100[35], DNA fragments[51] and fibronectin fragments[36]. As an important DAMP, HMGB1 has been shown to play critical roles in sterile tissue injury such as stroke[25] and I/R injury of the liver[30], kidney[45] and heart[2, 18]. HMGB1 can mediate the chemotaxis of leukocytes[19, 50]. Andrassy and colleagues reported that myocardial HMGB1 levels were elevated during ischemia[2]. Consistent with these results, we found that HMGB1 levels were significantly higher in supernatants of 40-IHH versus 10-IHH. The gentle homogenization process employed in our

study was conducted without membrane lysis buffer to avoid destroying the nuclear or mitochondrial membranes of viable cardiomyocytes, and thus the elevated HMGB1 in IHH is primarily derived from necrotic cardiomyocytes (Figure 3). We found that 40-IHH tripled the infarct size in mice as compared to 20'/60' I/R injury. More importantly, depletion of HMGB1 in 40-IHH supernatants completely abolished its infarct exacerbating effect (Figures 2 and 3). Moreover, 40-IHH significantly increased cytokine gene expressions in splenic leukocytes, but not in bone marrow cells (Online Resource Figure III). These results strongly suggest that HMGB1 is released from the myocardium after prolonged ischemia to activate splenocytes (but not bone marrow cells), which in turn exacerbate inflammation and myocardial infarct size during reperfusion. It has been reported that HMGB1 can be actively secreted by inflammatory cells, but that process is usually delayed by 6–8 hours after the initiation of injury[44]. Thus, the HMGB1 released by ischemic myocardium is most likely passively released from necrotic cardiomyocytes.

Myocardial HMGB1 signaling through splenic RAGE creates a cardio-splenic axis that activates splenic leukocytes

RAGE has been reported to play an important role in HMGB1-induced inflammatory responses[19]. An HMGB1-RAGE pathway has previously been implicated in mediating myocardial I/R injury[1, 2]. In the present study, infusion of 40-IHH upon reperfusion failed to exacerbate myocardial infarct size (IS) in RAGE^{-/-} mice or in splenectomized wild-type mice after reconstitution with RAGE^{-/-} splenocytes (Figure 3D). In contrast, 40-IHH effectively increased IS in splenectomized mice after reconstitution of wild-type splenocytes (Figure 1C). However, depletion of HMGB1 from 40-IHH negated its infarct exacerbating effect (Figure 3). These results demonstrate that HMGB1 released from ischemic myocardium activates splenic leukocytes during reperfusion by binding to splenocyte RAGE. Of note, it has been reported that RAGE^{-/-} cardiomyocytes have significantly reduced hypoxia-reoxygenation injury in a cell culture model[37] and in a perfused isolated heart model[7]. However, the current study clearly demonstrates that, in intact mice, it is the RAGE present on splenocytes and the cardio-splenic axis that mediates HMGB1-induced I/R injury in vivo.

Neutrophil infiltration is critical in the acute phase of myocardial infarction[16, 32]. A recent study demonstrated that activation of the HMGB1-RAGE pathway triggers recruitment of neutrophils, but not macrophages, to sites of necrosis, and that the newly recruited neutrophils mediate subsequent amplification of injury[19]. We found that 40-IHH significantly increased FPR1 expression on splenic leukocytes (Figure 5) and facilitated both the exodus of neutrophils into the bloodstream and their infiltration into injured myocardium (Figure 4). Increased concentrations of HMGB1 (and possibly other FPR1 ligands) in the necrotic myocardium secondary to prolonged ischemia may exert strong chemotactic signals to attract circulating neutrophils[6, 19, 33]. These results document considerable cross-talk between the heart and spleen during the acute phase of myocardial I/R injury: 1) cardiomyocytes subjected to prolonged ischemia release HMGB1 into the circulation, 2) HMGB1 activates splenic leukocytes by binding to RAGE, 3) activation of RAGE triggers leukocyte activation in the spleen leading to stimulation of neutrophils and neutrophilia, and

4) circulating neutrophils migrate to the ischemic myocardium during reperfusion, facilitated by elevated myocardial HMGB1 and/or chemokine release (Figure 7).

FPR1 antagonism blocks crosstalk between the heart and spleen

Previous work has demonstrated that neutrophils are the first cell type to accumulate to significant levels in previously ischemic myocardium after I/R, and that these cells impose additional tissue injury via oxidative damage and production of pro-inflammatory cytokines. It has also been reported that the HMGB1-RAGE pathway promotes neutrophil migration[19, 33] in a formyl-methionyl-leucyl-phenyl-alanine (fMLP)-dependent manner[6], and that fMLP is a well-known ligand of FPR. FPRs are members of the seven-transmembrane, G-protein coupled receptor superfamily and are highly expressed on neutrophils and mononuclear phagocytes. FPRs are involved in host defense and in sensing cellular dysfunction[34], and activated monocytes and neutrophils overexpress FPRs[28]. By using a selective FPR1 receptor antagonist, cFLFLF[12, 47], we demonstrated that cFLFLF blocks the downstream arm of the cardio-splenic axis by limiting neutrophil transmigration to the heart and abrogating IHH-induced infarct exacerbation (Figure 6).

Study limitations

The HMGB1-TLR (Toll like receptor) pathway was not investigated in the current study, but it is known to participate in sterile inflammatory responses including myocardial I/R injury. It has been reported that there is crosstalk between RAGE and TLRs in mediating HMGB1-induced inflammatory responses. Thus the relative contributions of the RAGE and TLR signaling pathways to myocardial I/R injury will require further study. Also, our experiments employing the adoptive transfer of splenic leukocytes are not neutrophil-specific and thus other types of splenic immune cells may also participate in the cardio-splenic axis. HMGB1 activates a broad spectrum of splenic leukocytes via RAGE, including splenic dendritic cells. Our previous work showed that myocardial I/R injury is mediated by CD4⁺ T cells[52], and activated CD4⁺ T cells likely enhance splenic neutrophil activation by secreting interferon gamma. The relative contribution of T cell signaling and the roles of splenic monocytes and dendritic cells remain to be defined. Thus, it is entirely possible that other circulating and spleen-resident immune cells may function upstream to enhance the activation of neutrophils. Other DAMPs, such as S100[35], HSP60/70[27] and mtDNA[51] may also contribute importantly to myocardial I/R injury. Their potential roles in activating splenic leukocytes remain to be evaluated. Finally, the FPR gene family is comprised of multiple members; of which FPR1 is the most prominent in activated neutrophils. Our study focused on FPR1 to evaluate splenocyte activation, and cFLFLF has marked selectivity for FPR1, but it can also activate FPR2 at higher concentrations.

In summary, by using a mouse model of acute myocardial I/R injury, we discovered a novel cardio-splenic axis that exacerbates myocardial infarct size during reperfusion injury after prolonged ischemic insult (>30 minutes). Prolonged ischemia is required to achieve the threshold of myocardial necrosis necessary to release high levels of HMGB1, which then enter the bloodstream during reperfusion. The circulating HMGB1 activates splenic leukocytes by engaging RAGE, which ultimately induces the stimulation of neutrophils and neutrophilia. The circulating neutrophils then transmigrate to the previously ischemic

myocardium to exacerbate myocardial infarction. Selective blockade of FPR1 upon reperfusion interrupts this cardio-splenic axis, thus suppressing a major component of reperfusion injury. This study has important clinical implications because it demonstrates that selective FPR1 antagonists have the potential to reduce infarct size in patients with acute myocardial infarction during percutaneous coronary intervention or CAGB. Finally, recent work showing that treatment of the spleen with pulsed ultrasound can reduce renal[13] and myocardial[41] I/R injury opens the possibility that non-invasive imaging could provide a clinically-relevant, drug-free option for reducing the size of myocardial infarction.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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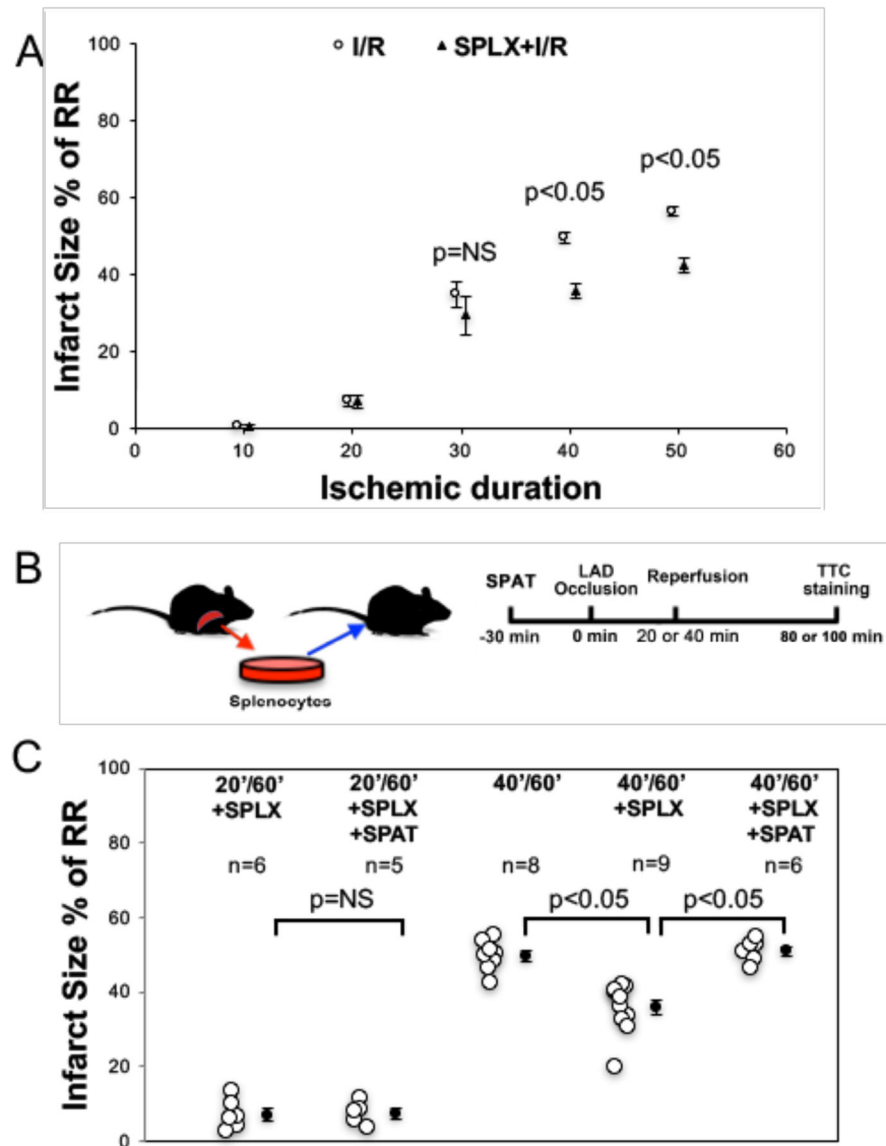


Figure 1. Splenectomy attenuates myocardial ischemia/reperfusion injury after prolonged ischemic insult

A: Splenectomy significantly decreased myocardial infarct size in 40'/60' and 50'/60' groups. It had no effect on 10'/60', 20'/60' and 30'/60' groups. B: Experimental protocol for the adoptive transfer of splenic leukocytes. C: Acute adoptive transfer of splenic leukocytes restored the IS of splenectomized mice in the 40'/60' group to the level of intact control mice. However, it did not increase the IS of splenectomized mice in the 20'/60' group. N = 5 mice/group. I/R: ischemia/reperfusion injury; SPLX: splenectomy; RR: risk region; SPAT: acute splenic leukocyte adoptive transfer.

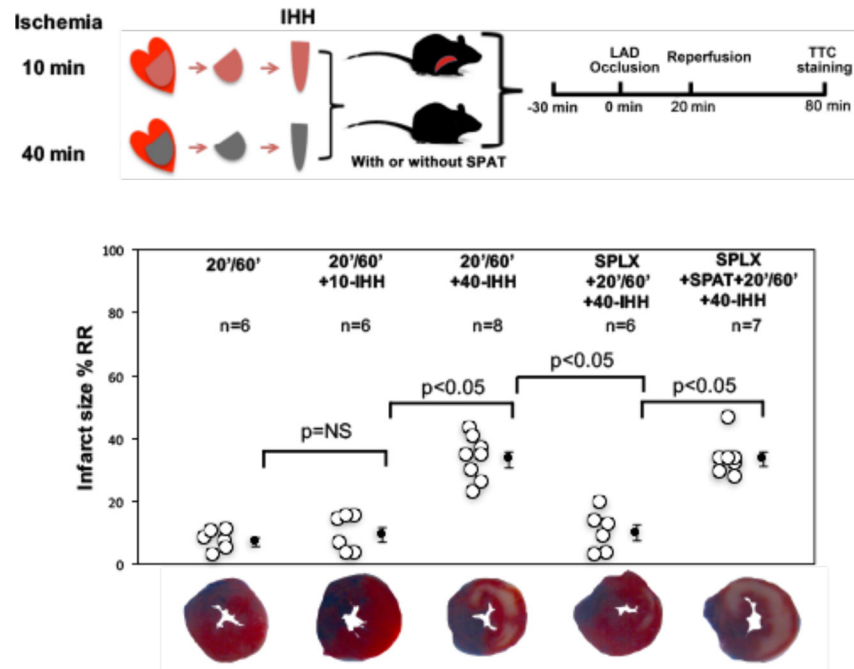


Figure 2. Substances elaborated from the myocardium after prolonged ischemia exacerbate myocardial IS by acting on splenic leukocytes

IHH from 40 minute ischemic myocardium (40-IHH) significantly exacerbated myocardial IS in 20'/60' mice. However, 10-IHH had no effect on IS. The IS exacerbating effect of 40-IHH disappeared in splenectomized mice, but IS could be restored in splenectomized mice by SPAT. N = 5 mice/group. IHH: ischemic heart homogenate.

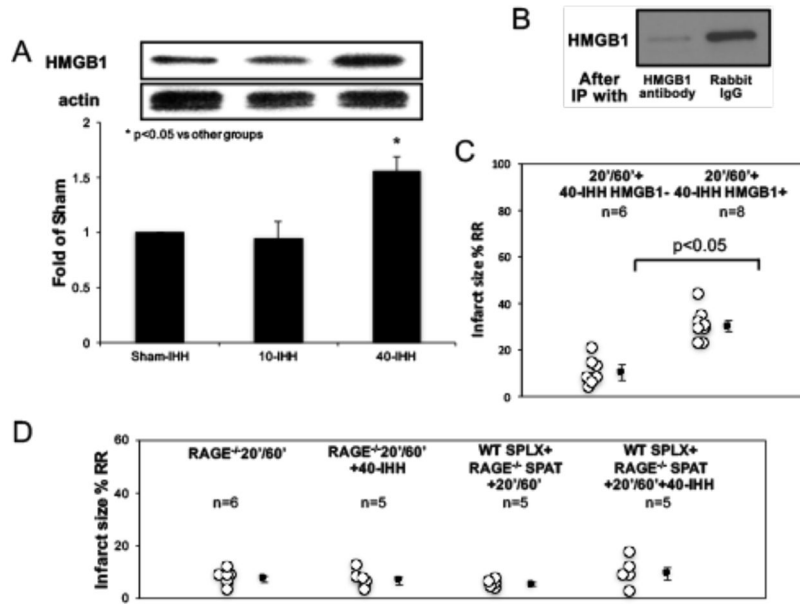


Figure 3. HMGB1 from ischemic myocardium and RAGE receptors on splenocytes mediate the IS exacerbating effects of 40-IHH

A: Western blot analysis showing that 40-IHH had significantly higher HMGB1 levels in heart homogenates than in sham or 10-IHH. B: Immunoprecipitation was used to deplete HMGB1 in heart homogenates, which was confirmed by Western blots. C: Depletion of HMGB1 in 40-IHH completely abrogated its IS exacerbating effect. D: 40-IHH completely failed to increase IS in either RAGE^{-/-} mice or splenectomized WT mice with adoptive transfer of RAGE^{-/-} splenocytes. N = 5 mice/group. IP: immunoprecipitation.

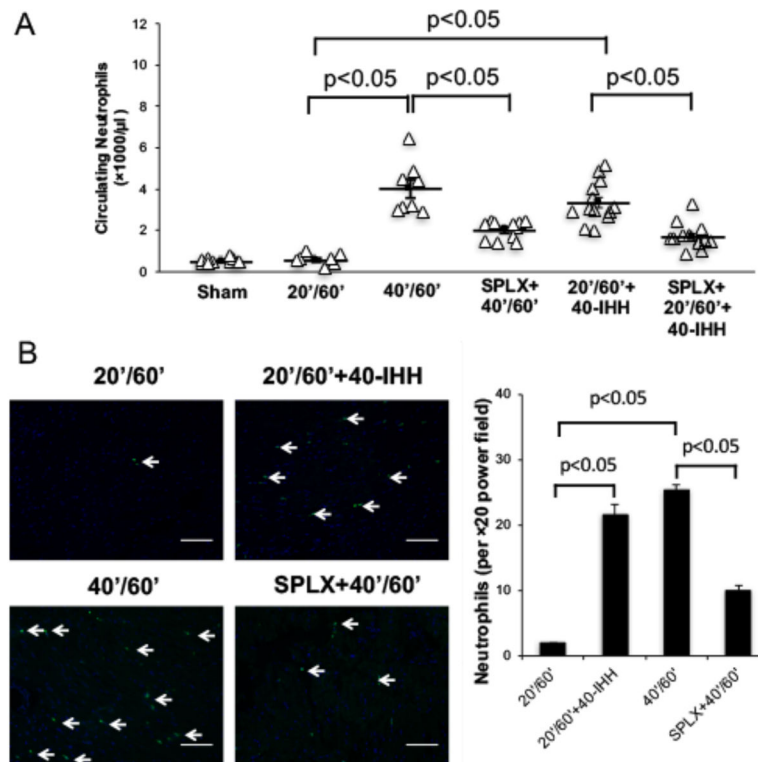


Figure 4. 40-IHH significantly increases both circulating and myocardial tissue neutrophils after IR injury

A: Circulating neutrophils were significantly increased in 40'/60' and 40-IHH treated 20'/60' I/R injury mice as compared to sham or 20'/60' I/R mice. This neutrophilia could be attenuated by splenectomy. B: Myocardial neutrophil infiltration was significantly increased in 40'/60' and 40-IHH treated 20'/60' I/R mice. The increase in neutrophil infiltration could also be attenuated by splenectomy. N = 5 mice/group. Arrows indicate neutrophils. Scale bar: 50μm.

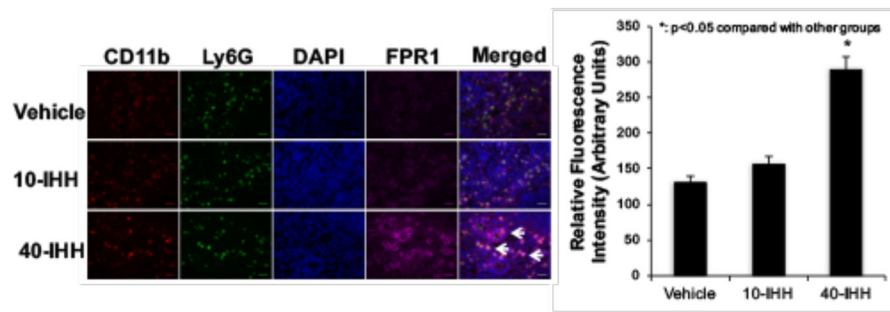


Figure 5. Splenic FPR1 expression levels are significantly increased in 40-IHH treated mice
 Immunofluorescence staining indicated that 40-IHH significantly increases FPR1 expression levels (purple staining) in the splenic marginal zone. Confocal imaging showed that the elevations in FPR1 expression were mainly localized to Ly6G⁺ cells (stained in green). N = 5 mice/group. Arrows indicate Ly6G⁺ cells with elevated FPR1 expression levels. Scale bar: 20 μ m.

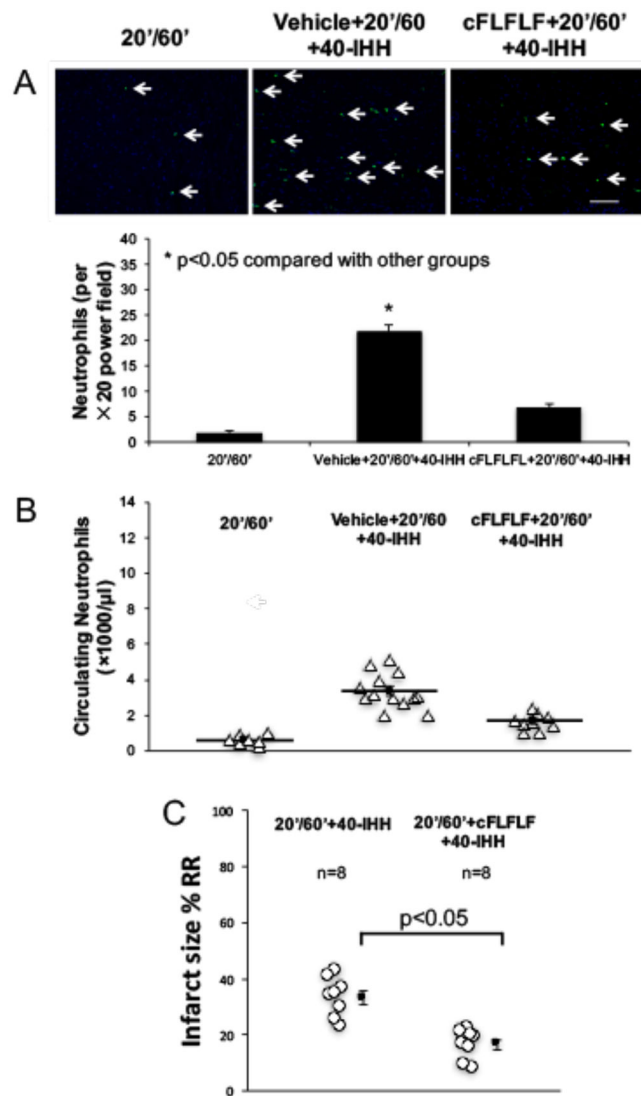


Figure 6. FPR1 receptor blockade attenuates the IS exacerbating effect of 40-IHH

A: cFLFLF significantly decreased myocardial neutrophil infiltration in 40-IHH treated mice (n=3 per group). B: cFLFLF significantly decreased circulating neutrophils in 40-IHH treated mice (N = 5 mice/group). C: cFLFLF significantly attenuated myocardial IS in 40-IHH treated mice (n=8 per group). Arrows indicate neutrophils. Scale bar: 50 μm .

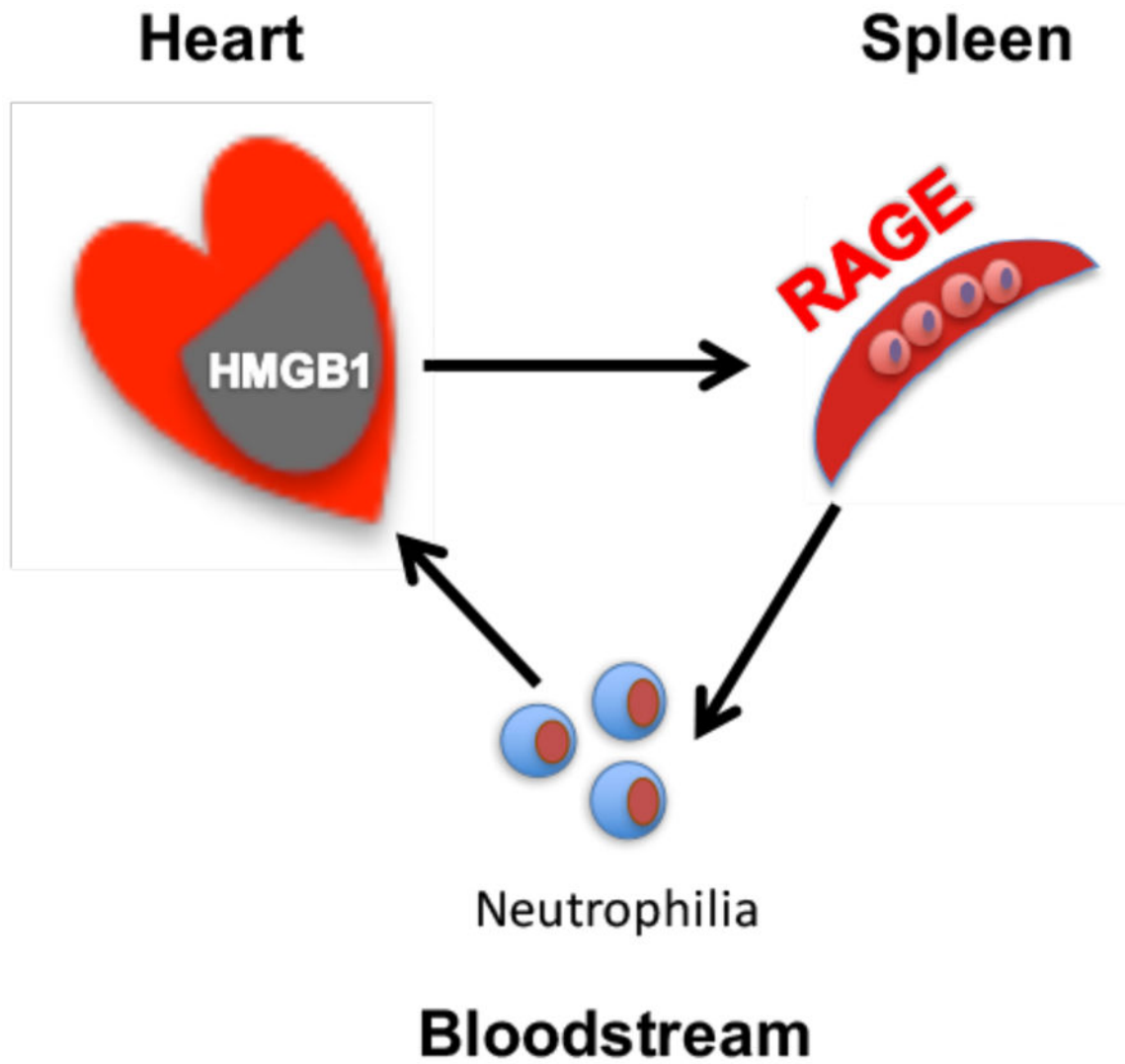


Figure 7. Schematic mechanism of the cardio-splenic signaling axis in acute myocardial I/R injury
 1) Necrotic cardiomyocytes release HMGB1 into the bloodstream upon reperfusion, 2) HMGB1 activates splenic neutrophils by stimulating RAGE, 3) activated splenic neutrophils enter the bloodstream, and 4) circulating splenic neutrophils transmigrate to the ischemic myocardium guided by HMGB1 and/or other chemokine gradients.

Table 1

Heart rates in IHH treated mice (N = 5 mice/group)

Group	Baseline	10 min after ischemia	10 min after reperfusion
20'/60-I/R	424±14	468±12*	484±14*
20'/60'-I/R+10-IHH	423±8	461±6*	496±4*
20'/60'-I/R+40-IHH	429±10	476±4*	496±3*

*p<0.05 compared with baseline.

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