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Original Article

Ischemic preconditioning attenuates acute lung injury after partial liver transplantation

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Abstract: Pulmonary complications frequently occur after liver transplantation and are often life-threatening. Thus, we investigated whether hepatic ischemic preconditioning (IP) attenuates acute lung injury (ALI) after small-for-size liver transplantation. Rat livers were explanted after 9-min ischemia plus 5-min reperfusion, reduced to 50% of original size *ex vivo*, and implanted into recipients with approximately twice the donor body weight, resulting in quarter-size liver grafts (QSG). After QSG transplantation, hepatic Toll-like receptor 4 (TLR4) and tumor necrosis factor-α (TNFα) expression increased markedly and high mobility group box-1 (HMGB1), an endogenous damage-associated molecular pattern molecule (DAMP), was released from QSG into the blood. IP blunted TLR4 and TNFα expression and HMGB1 release from QSG. In the lungs of QSG recipients without IP treatment, nuclear factor-κB (NF-κB) activation and intercellular adhesion molecule (ICAM)-1 expression increased; alveolar septal walls thickened with increased cellularity as neutrophils, monocytes/macrophage and T lymphocytes infiltrated into alveolar septa and alveolar spaces; and pulmonary cleaved caspase-8 and -3 and TUNEL-positive cells increased. In contrast, in the lungs of recipients of ischemic-preconditioned QSG, NF-κB activation and ICAM-1 expression were blunted; leukocyte infiltration was decreased; and alveolar septal wall thickening, caspase activation, and cell apoptosis were attenuated. IP did not increase heat-shock proteins in the lungs of QSG recipients. In conclusion, toxic cytokine and HMGB1 released from failing small-for-size grafts leads to pulmonary adhesion molecule expression, leukocyte infiltration and injury. IP prevents DAMP release and toxic cytokine formation in small-for-size grafts, thereby attenuating ALI.

Keywords: Acute lung injury, damage-associated molecular pattern molecule, heat shock protein, inflammation, ischemic preconditioning, partial liver transplantation

Introduction

Orthotopic liver transplantation remains the only proven therapy for end-stage liver disease [1, 2]. However, pulmonary complications occurring after liver transplantation are often life-threatening [3-7], and some reports indicate that the mortality for acute respiratory distress syndrome exceeds 50% [3, 4, 8]. Extended cold storage, ischemia/reperfusion-induced graft injury, and transfusion of plasma-containing blood products during surgery are documented to increase the occurrence of pulmonary complications [3, 4, 7-11]. Recently, partial liver transplantation has been increasingly utilized to alleviate the severe shortage of donor organs [12-14]. However, small-for-size syndrome occurs after transplantation of partial liver grafts with a relative volume less than 30-40% of the standard recipient liver volume, which leads to slow/no recovery of liver function, more severe post-transplantation complications, and increased mortality [12, 15]. Patients with acute hepatic failure often have pulmonary complications [16]. Moreover, trauma during liver resection/splitting procedures of partial liver transplantation stimulates inflammation responses in the lung [17], and hemodynamic alterations that occur after partial liver transplantation may exacerbate pulmonary complications [18, 19]. Thus, cross-talk between the liver and lung may exist, contributing to increased pulmonary complications after small-for-size liver transplantation. Indeed, our recent work indicates that pulmonary inflammation and injury increased after small-for-size liver transplantation in rats [20]. Because liver

Table 1. Sources of reagents

Reagents	*Sources*
Actin antibody	ICN, Costa Mesa, CA
Cleaved caspase-3 antibody	Abcam, Cambridge, MA
Cleaved caspase-8 antibody	Abcam, Cambridge, MA
Chemiluminescence kit	Pierce Biotec., Rockford, IL
CD4 antibody	Origene Technologies, Rockville, MD
ED1 antibody	Serotek, Raleigh, NC
HMGB1 antibody	Abcam, Cambridge, MA
HMGB1 Elisa kit	My Biosource Inc., San Diego, CA
ICAM-1 antibody	BD Biosciences Pharmingen, San Diego, CA
iScript cDNA synthesis kit	Bio-Rad, Hercules, CA
Lactated Ringer's solution	Abbott Laboratories, North Chicago, IL
MPO polyclonal antibodies	DAKO Corp., Carpinteria, CA
NF-κB p65 antibody	Santa Cruz Biotechnology, Santa Cruz, CA
phosphate buffered saline	Invitrogen Corp. Grand Island, N.Y.
Phospho-NF-κB p65 antibody	Santa Cruz Biotechnology, Santa Cruz, CA
TLR4 antibody	Abcam, Cambridge, MA
TNFα antibody	Abcam, Cambridge, MA
Trizol	Invitrogen, Grand Island, NY
UW cold storage solution	Barr Laboratories Inc. Pomona, NY

HMGB1, high mobility group box-1; ICAM-1, intercellular adhesion molecule-1; MPO, myeloperoxidase; NF-κB, nuclear factor-κB; TLR4, Toll-like receptor-4; TNFα, tumor necrosis factor-α UW, University of Wisconsin.

graft failure is often associated with acute lung injury (ALI), understanding the mechanisms behind this event is essential for preventing or treating pulmonary complications after partial liver transplantation.

Ischemic preconditioning (IP) renders tissues more tolerant to subsequently longer episodes of ischemia and other stresses such as those caused by endotoxin and organ storage for transplantation [21-25]. IP even protects remote organs from injury and inflammation [26-28]. For example, hind limb IP is cardioprotective after ischemia/reperfusion [29, 30] and decreases lipopolysaccharide (LPS)-induced liver injury by inhibiting nuclear factor-κB (NF-κB) activation [31]. Similarly, IP to one-half of the liver protects against reperfusion injury to the other half [25]. Remote IP also increases post-traumatic ganglion cell survival by up-regulating heat-shock protein (HSP)-27 [32] and protects the lung against ischemia/reperfusion- and transfusion-related ALI by modulating endothelial function and neutrophil activation/infiltration. IP also protects against ALI secondary to renal aorta clamping [33, 34]. In previous work, we observed that IP prior to organ retrieval decreases graft injury and prevents graft failure after both full-size and small-for-size liver transplantation in rats [25, 35]. However, uncertainty regarding whether IP of liver graft also decreases ALI after small-for-size liver transplantation prompted us to undertake investigations reported here. Because IP may be beneficial via increasing protective factors and/or decreasing release of detrimental factors, we explored the underlying mechanisms by which IP protects against ALI after small-for-size liver transplantation, including decreasing toxic cytokine formation and hepatic endogenous damage-associated molecular pattern molecule (DAMP) release and increasing protective HSPs in the lung.

Methods

Chemicals

Sources for reagents are listed in **Table 1**.

Ischemic preconditioning and liver transplantation

IP and liver transplantation were performed in male Lewis rats (170-200 g, Charles River, Durham, NC), as described previously [35-37]. Briefly, the donor abdomen was opened under ether anesthesia, and heparin (200 U) was injected into the vena cava. IP was induced 2 min later by clamping the portal vein and hepatic artery for 9 min. Livers were flushed *in situ* with 5 ml UW cold storage solution (0-1°C) via the portal vein 5 min after releasing the vascular clamp, and then livers were explanted. In ice-cold UW solution, liver mass was reduced *ex vivo* to ~50% of original size, as described [35-37], and unreduced and reduced-size livers were stored in UW solution at 0-1°C for 6 h. Reduced-size liver explants were implanted into recipients of greater body weight (350-420 g), which results in a graft weight/standard liver weight of ~25% (quarter-size graft, QSG), as described [35, 36]. Unreduced livers were implanted into recipients of similar body weights as full-size graft controls (FSG). Implantation surgery was performed as described pre-

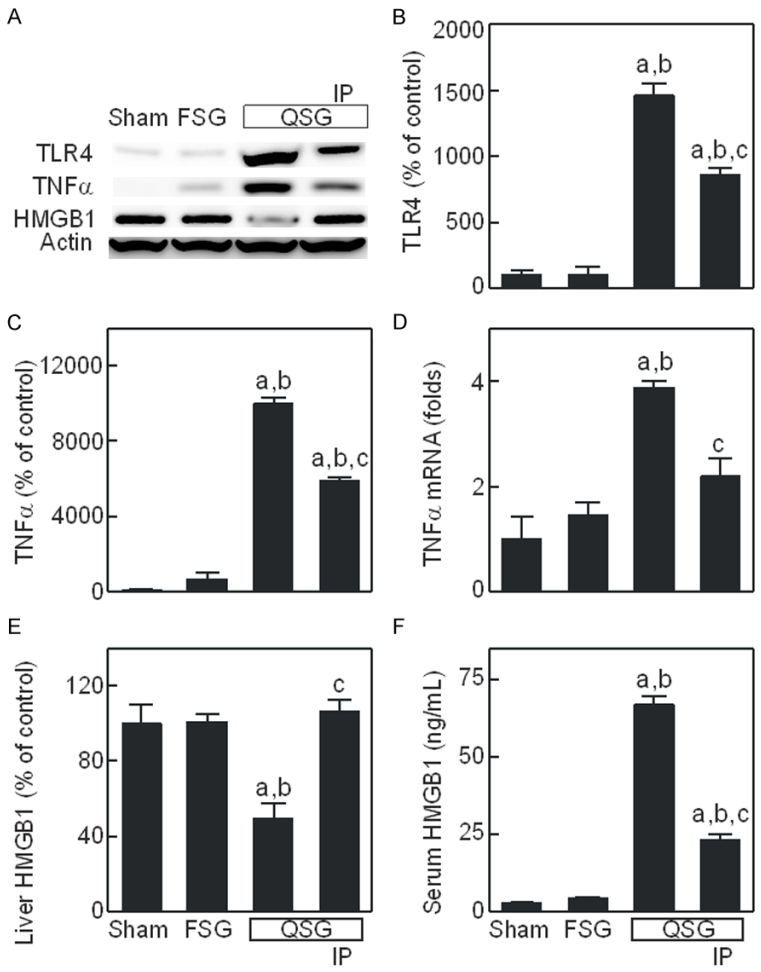

Figure 1. Ischemic preconditioning decreases pro-inflammatory cytokine formation and HMGB1 release from small-for-size liver grafts after transplantation. Liver grafts and sera were collected 38 h after transplantation. Hepatic TLR4, TNFα, HMGB1 and actin were measured by immunoblotting, and representative immunoblots are shown in (A). Densitometric quantification of immunoblots of TLR4, TNFα and HMGB1 is shown in (B, C), and (E), respectively. Hepatic TNFα mRNAs were measured by real-time PCR (D). Serum HMGB1 was measured using ELISA (F). Sham, livers from sham-operated rats; FSG, full-size grafts; QSG, quarter-size grafts; QSG + IP, ischemic-preconditioned QSG. Values are means ± SEM. Group sizes were 3-4 per group. a, P<0.05 vs sham operation; b, P<0.05 vs FSG; c, P<0.05 vs QSG.

viously with the hepatic artery and bile duct reconstructed with intraluminal stents [36]. The ratios of graft weight/standard liver weight were not significantly different between QSG with and without IP (P>0.1 by the Student's t-test). All animals were given humane care in compliance with institutional guidelines using protocols approved by the Institutional Animal Care and Use Committee.

Pulmonary histology

Under pentobarbital (50 mg/kg, *ip*) anesthesia, rat lungs were harvested at 38 h after implantation and processed for histology [38, 39]. With hematoxylin and eosin (H&E)-stained sections, lung microscopic images were acquired [20]. The thicknesses of alveolar septal walls were quantified by computerized image analysis using IPlab 3.7v software (BD Biosciences, Rockville, MD), as described previously [20], and relative alveolar septal thickness was expressed as a ratio of the average thicknesses of different transplantation groups to the sham-operation group.

Immunohistochemical staining

ED1 (CD68), a marker of monocytes and macrophages, myeloperoxidase (MPO), an indicator of neutrophil infiltration, and intercellular adhesion molecule-1 (ICAM-1) were assessed in deparaffinized pulmonary sections to assess inflammation in lung tissues [20]. Apoptosis was measured by terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) using an *in situ* cell death detection kit (**Table 1**). TUNEL-positive cells were quantified as described previously [20].

Immunoblotting

Liver and lung tissues were collected at 38 h after transplantation and processed for immunoblotting [20]. Aliquots of liver and lung supernatants (40 μg of protein) were separated on NuPAGE 4-12% Bis-Tris gels, transferred onto nitrocellulose membranes and immunoblotted with primary antibodies specific for ED-1 (CD68), CD4, HSP27, -32, -60, -72, -90, high mobility group box-1 (HMBG-1), MPO, nuclear factor-κB (NF-κB) p65, phospho-NF-κB p65, tumor necrosis factor-α (TNFα), and toll-like receptor-4 (TLR4) at 1:1,000 and actin at 1:3,000 overnight at 4°C. Horseradish peroxidase-conjugated secondary antibodies were applied, and detection was accomplished with chemiluminescence.

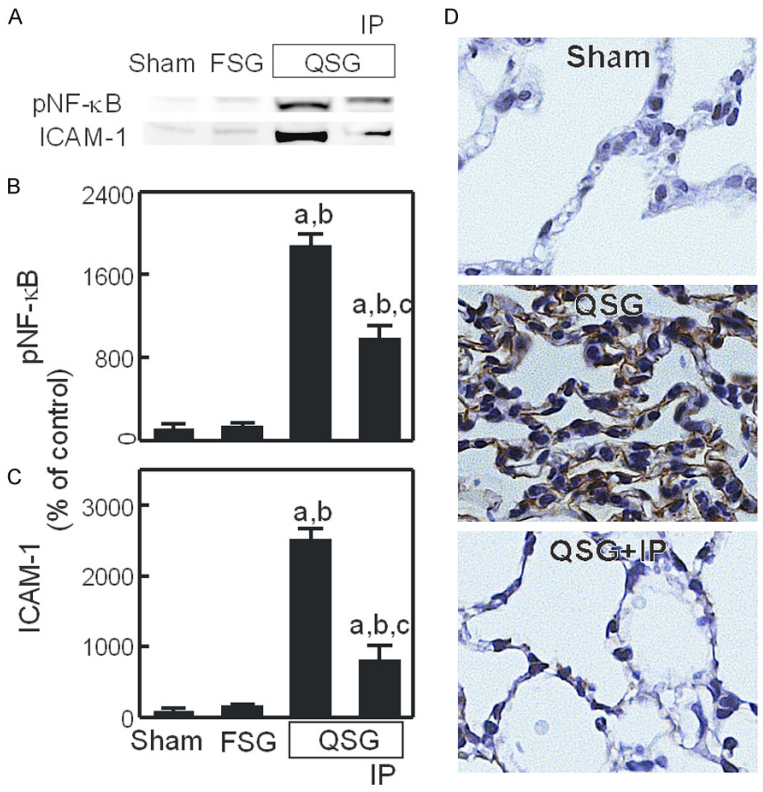

Figure 2. The effects of ischemic preconditioning on pulmonary nuclear factor-κB activation and intercellular adhesion molecule-1 expression after small-for-size liver transplantation. Lungs were harvested 38 h after liver transplantation. Phospho-NF-κB p65 (pNF-κB), ICAM-1, and actin were measured by immunoblotting. Representative immunoblots are shown in (A). Densitometric quantification of pNF-κB and ICAM-1 immunoblots is shown in (B and C), respectively. Representative immunohistochemical staining for ICAM-1 is shown in (D). Sham, lungs from sham-operated rats; FSG, lungs from full-size graft recipients; QSG, lungs from quarter-size graft recipients; QSG + IP, lungs from ischemic-preconditioned quarter-size graft recipients. Values are means ± SEM. Group sizes were 4 per group. a, P<0.05 vs sham operation; b, P<0.05 vs FSG; c, P<0.05 vs QSG.

Detection of tumor necrosis factor-a mRNA by quantitative real-time PCR

Single stranded cDNAs were synthesized from RNA (2 mg) extracted from liver tissue using a Bio-Rad iScript cDNA Synthesis kit, and quantitative real-time PCR (qPCR) was conducted using a CFX96 Real Time-PCR Detection System (Bio-Rad, Hercules, CA) and primer sequences previously described [20].

Serum high mobility group box-1 detection

Under pentobarbital (50 mg/kg, *ip*) anesthesia, blood samples were collected from the vena cava at 38 h after implantation. Serum HMGB1 was measured using an analytical kit (**Table 1**) according to the manufacturer's instructions.

Statistical analysis

Groups were compared using Student's t-test or ANOVA plus a Student-Newman-Keuls *post-hoc* test as appropriate. Data shown are means ± SEM. Group sizes were 3-4 livers in each group for all parameters, as indicated in figure legends. Differences were considered significant at P<0.05.

Results

Ischemic preconditioning blunts pro-inflammatory cytokine formation and HMGB1 release after small-for-size liver transplantation

We previously reported that pro-inflammatory cytokine formation increased after small-for-size liver transplantation [20]. Therefore, we investigated whether IP attenuated pro-inflammatory reactions in small-for-size grafts. Full-size grafts (FSG) and quarter-size grafts (QSG) with and without IP were transplanted. TLR4, which mediates pro-inflammatory endotoxin reactions and leads to toxic cytokine formation, was expressed at low levels in livers of sham-operated rats (**Figure 1A**). Hepatic TLR4 expression did not increase in FSG 38 h after liver transplantation but increased ~15-fold in QSG (**Figure 1A**, **1B**). After IP, TLR4 expression increased only ~9-fold in QSG. Hepatic TNFα protein and mRNA expression also did not increase in FSG but increased markedly in QSG. IP decreased TNFα protein and mRNA expression in QSG by 41% and 43%, respectively (**Figure 1C**, **1D**).

HMGB1 is released during cell stress and injury, acting as a DAMP that promotes inflammatory processes [40, 41]. Previously we reported that QSG transplantation causes mitochondrial dysfunction, increased necrosis and apoptosis,

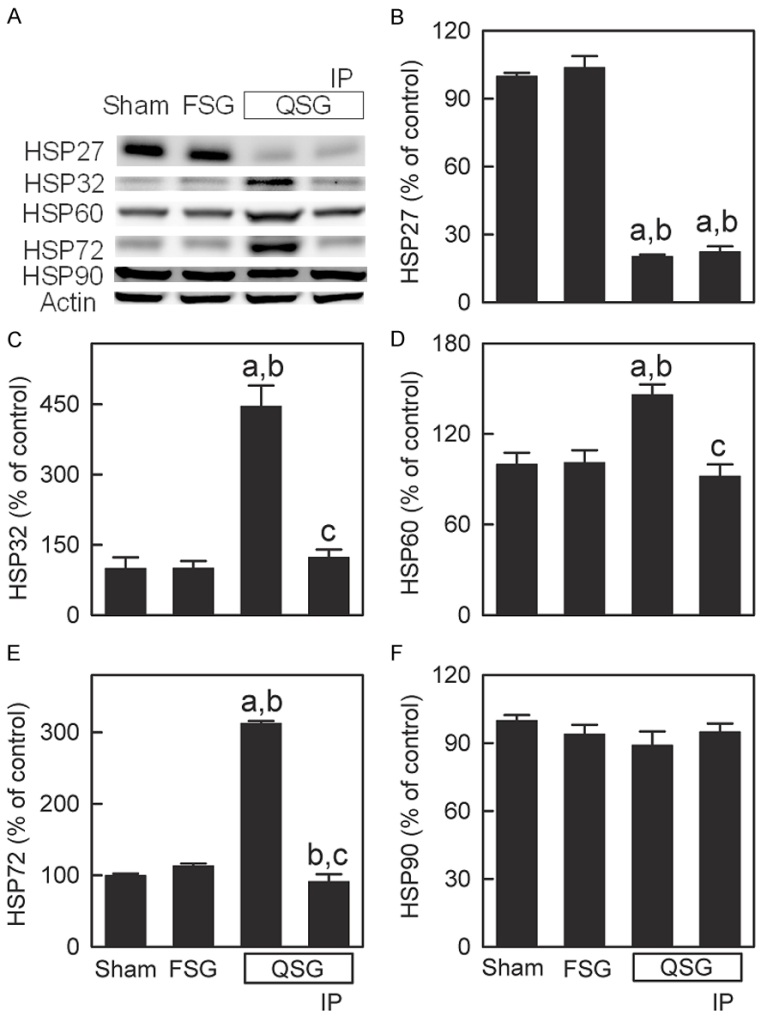

Figure 3. The effects of ischemic preconditioning on pulmonary heat-shock protein expression after small-for-size liver transplantation. Lungs were harvested 38 h after liver transplantation. HSPs and actin were measured by immunoblotting. Representative immunoblots are shown in (A). Densitometric quantification of HSP27, HSP32, HSP60, HSP72 and HSP90 immunoblots is shown in (B-F), respectively. Sham, lungs from sham-operated rats; FSG, lungs from full-size graft recipients; QSG, lungs from quarter-size graft recipients; QSG + IP, lungs from ischemic-preconditioned quarter-size graft recipients. Values are means ± SEM. Group sizes were 4 per group. a, P<0.05 vs sham operation; b, P<0.05 vs FSG; c, P<0.05 vs QSG.

and suppressed liver regeneration [36, 42, 35]. Accordingly, we explored whether DAMP is released from QSG. HMGB1 was detected in sham-operated livers and in FSG but decreased in QSG (**Figure 1A**, **1E**). Serum HMGB1 did not change in FSG but increased ~22-fold in QSG recipients (**Figure 1F**). In contrast, IP blocked the loss of hepatic HMGB1 and decreased serum HMGB1 after QSG transplantation (**Figure 1E**, **1F**).

Ischemic preconditioning decreases NF-κB activation and adhesion molecule expression in recipient lungs after small-for-size liver transplantation

DAMP and pro-inflammatory cytokines released from failing liver grafts has been reported to promote inflammatory reactions in remote organs [43, 44]. Accordingly, we explored activation of NF-κB, which mediates many inflammatory responses of TNFα and HMGB1 [45, 46], in recipient lungs after QSG transplantation. We observed that pulmonary NF-κB p65 expression was similar in all treatment groups (data not shown). However, phospho-NF-κB p65, which was barely detectable in lungs after sham-operation and FSG transplantation, increased ~19-fold after QSG transplantation (**Figure 2A**, **2B**). This effect was blunted by IP (**Figure 2A**, **2B**). Similar changes were observed with ICAM-1 which mediates leukocyte adhesion. ICAM-1 expression in lungs increased ~25-fold after QSG transplantation (**Figure 2A**, **2C**). ICAM-1 expression was primarily in vascular, alveolar and bronchial epithelial cells (**Figure 2D** and not shown). After IP, pulmonary ICAM-1 decreased after QSG transplantation in comparison to QSG without IP (**Figure 2C**, **2D**).

Ischemic preconditioning does not increase heat-shock proteins in recipient lungs after small-for-size liver transplantation

Previous studies show that IP induces HSPs in remote organs and tissues [25, 28, 47, 48]. In rat lungs after QSG transplantation, HSP32, 60 and 72 increased compared to sham operation (**Figure 3A**, **3C-E**). IP before QSG transplantation restored pulmonary HSP 32, 60 and 72

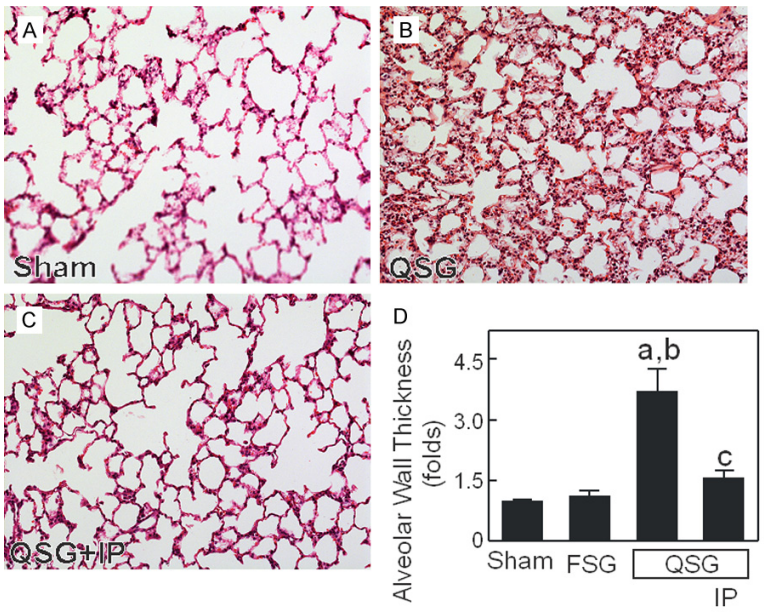

Figure 4. Ischemic preconditioning decreases lung pathological changes after small-for-size liver transplantation. Lungs were harvested 38 h after liver transplantation. Representative images of H&E-stained lung sections are shown in (A-C). In (D), relative alveolar septal thicknesses compared to Sham controls are shown. Sham, lungs from sham-operated rats; FSG, lungs from full-size graft recipients; QSG, lungs from quarter-size graft recipients; QSG + IP, lungs from ischemic-preconditioned quarter-size graft recipients. Values are means ± SEM. Group sizes were 4 per group, a, $P<0.05$ vs sham operation; b, $P<0.05$ vs FSG; c, $P<0.05$ vs QSG.

almost to control levels (**Figure 3**). HSP27 decreased to similar levels in rat lungs after transplantation of QSG with and without IP (**Figure 3A**, **3B**). Pulmonary HSP90 was not altered under any conditions (**Figure 3A**, **3F**).

Ischemic preconditioning decreases alveolar wall thickness and pulmonary inflammation after small-for-size liver transplantation

In FSG recipients, alveolar septa were not thickened compared to sham-operated rats (**Figure 4D**). In lungs of QSG recipients, alveolar septa thickened 3.7-fold and cellularity in alveolar septa increased markedly (**Figure 4B**, **4D**). Leukocytes, including mononuclear and polymorphonuclear cells, increased overtly in the lungs of QSG recipients. Leukocytes were present in blood vessel lumens and infiltrated into the peribronchial, perivascular and intra-alveolar spaces (**Figure 4B** and not shown). With IP, alveolar wall thickness only increased to 1.6-fold after QSG transplantation, and pulmonary leukocyte sequestration was substantially less than after QSG transplantation without IP (**Figure 4C**, **4D**).

Immunohistochemistry revealed 5.6 ED1 (CD68)-positive monocytes and macrophages per high power field (hpf) in the lungs of sham-operated rats (**Figure 5A**, **5B**). ED1-positive cells increased to 18.6/hpf after FSG transplantation and 40.2/hpf after QSG transplantation (**Figure 5A**, **5B**). Most ED1-positive cells in the lungs of QSG recipients were smaller in size compared to those of sham-operated rats and were likely infiltrating monocytes/macrophages. Pulmonary MPO-positive cells (neutrophils) were 3.2/hpf in sham-operated rats, which increased to 7.2/hpf and 138/hpf, respectively, after transplantation of FSG and QSG (**Figure 5C**). After IP, pulmonary ED1-positive cells following QSG transplantation decreased from 40.2/hpf to 20/hpf, and MPO-positive cells decreased from 138/hpf to 23/hpf.

Adaptive immunity has also been reported to affect the progression of ischemia/reperfusion injury [40, 49], and the inflammatory cytokine TNFα promotes migration of CD4+ T lymphocytes after ischemia/reperfusion [50, 51]. Pulmonary CD4 (a T-cell marker) did not increase after FSG transplantation but increased 6.9-fold after QSG transplantation compared to sham operation (**Figure 5D**, **5E**). By comparison after IP, pulmonary CD4 increased only 4.7-fold following QSG transplantation (**Figure 5D**, **5E**).

No histologically overt necrosis was observed in recipient lungs after QSG transplantation (**Figure 4B**), and cleaved caspase-8 and caspase-3 were barely detectable in lung tissues of sham-operated rats and recipients of FSG. However after QSG transplantation, pulmonary cleaved caspase-8 and -3 increased substantially in QSG recipients, which IP blunted (**Figure 6A-C**).

Apoptosis in lung tissue was also detected by TUNEL. TUNEL-positive cells with red nuclear staining were rare (0.3/hpf) in lungs after both

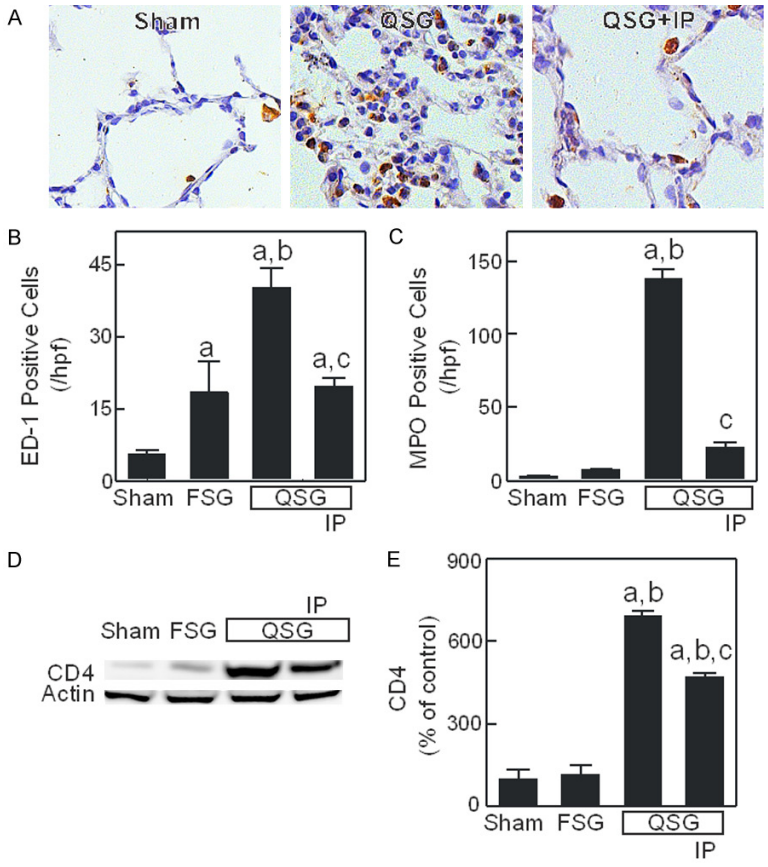

Figure 5. Ischemic preconditioning decreases lung inflammation after small-for-size liver transplantation. Lungs were harvested 38 h after liver transplantation. ED1 and myeloperoxidase (MPO) were assessed by immunohistochemistry. Representative images of ED1 immunohistochemical staining are shown in (A). ED1-positive cells (B) and MPO-positive cells (C) after immunohistochemical staining were counted in 10 random fields per slide using a 40X objective lens. CD4 was detected by immunoblot analysis (D) and quantified by densitometry (E). Sham, lungs from sham-operated rats; FSG, lungs from full-size graft recipients; QSG, lungs from quarter-size graft recipients; QSG + IP, lungs from ischemic-preconditioned quarter-size graft recipients. Values are means ± SEM. Group sizes were 3-4 per group, a, $P<0.05$ vs sham operation; b, $P<0.05$ vs FSG; c, $P<0.05$ vs QSG.

sham operation and FSG transplantation (**Figure 6D**). After QSG transplantation, pulmonary TUNEL increased to 12.2/hpf, which IP decreased to 0.9/hpf (**Figure 6D**). Apoptotic cells were primarily vascular endothelial cells or alveolar epithelial cells.

Discussion

Protection of small-for-size liver grafts by ischemic preconditioning prevents acute pulmonary injury after transplantation

Small-for-size grafts have increased risks of graft failure and postsurgical complications, but exactly how this occurs is uncertain [12, 15]. Pulmonary complications frequently occur after liver transplantation and substantially increase mortality, so prevention/treatment of pulmonary complications is crucial for increasing post-transplantation survival [3, 5, 8]. Our previous data showed that ALI increased markedly in recipients of small-for-size grafts [20]. Moreover, IP of liver grafts prior to harvesting decreased liver injury, improved liver regeneration, and prevented graft dysfunction after small-for-size liver transplantation [35]. Therefore, we explored whether IP also attenuates ALI after small-for-size liver transplantation. In QSG rat recipients, ALI increased markedly (**Figures 4-6**), a finding that was consistent with our previous observations [20]. Interestingly, hepatic IP in rat liver donors significantly decreased lung injury in recipients after QSG transplantation (**Figures 4-6**), suggesting the existence of cross-talk between small-for-size liver grafts and recipient lungs.

Ischemic preconditioning did not increase heat-shock proteins in the lung

Many studies reveal that an increase of HSP expression by IP is associated with protection against injuries from ischemia/reperfusion and other stresses [52-55]. We also observed an increase of HSP90 in QSG after transplantation [35]. Interestingly, remote IP can protect organs from injury and decrease systemic inflammation by increasing HSPs [25-28]. For example, hind limb IP protects the myocardium from infarction after ischemia/reperfusion by increasing HO-1 (HSP32) and HSP27 expression in the heart [28, 47] and protects the spinal cord from ischemia/reperfusion injury by increasing HSP70 [48]. Furthermore, IP of one kidney protects the other kidney from subsequent ischemia/reperfusion injury by increasing HSP25 [55]. Similarly, ischemia to one-half of the liver

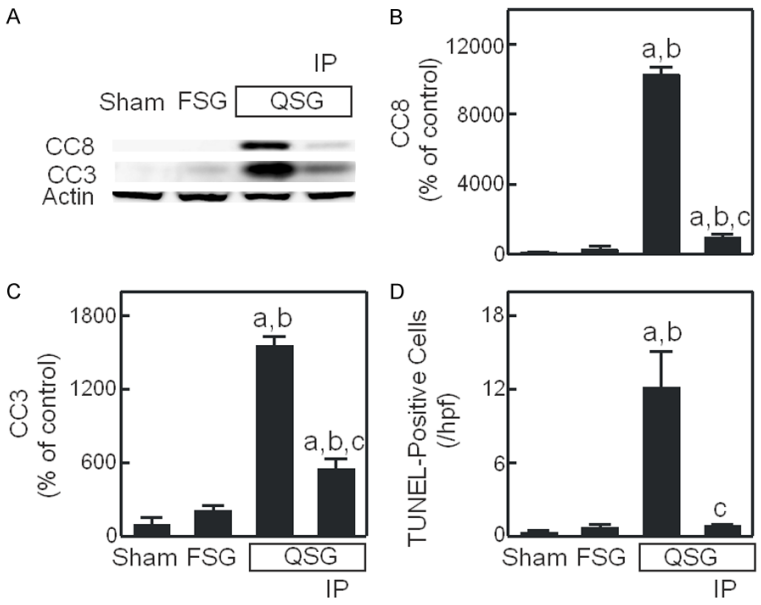

Figure 6. Ischemic preconditioning decreases caspase activation and apoptosis in the lung after small-for-size liver transplantation. Lungs were harvested 38 h after liver transplantation. Cleaved caspase-8 and -3 (CC8 and CC3) and actin were measured by immunoblotting. Representative immunoblots are shown in (A) and quantified by densitometry in (B and C). TUNEL-positive cells were counted in 10 random fields per slide using a 20X objective lens (D). Sham, lungs from sham-operated rats; FSG, lungs from full-size graft recipients; QSG, lungs from quarter-size graft recipients; QSG + IP, lungs from ischemic-preconditioned quarter-size graft recipients. Values are means ± SEM. Group sizes were 4 per group. a, P<0.05 vs sham operation; b, P<0.05 vs FSG; c, P<0.05 vs QSG.

provides protective precondition against ischemia/reperfusion to the other half [25]. Here, we observed protection of QSG recipient lungs by IP of QSG. Possibly, IP grafts release signaling molecules that subsequently increase HSP expression in the lung. Therefore, we explored whether IP of donor livers causes an increase of HSPs in the lungs of recipients. We observed instead that pulmonary HSP27 in recipients of QSG actually decreased to the same extent with and without IP (**Figure 3**). Additionally, HSP32 (HO-1), HSP60 and HSP72 increased more in lungs of recipients of QSG without IP compared to recipients of IP QSG (**Figure 3**). Pulmonary HSP90 was not significantly altered in any groups. Thus, IP to donor liver is unlikely to protect lungs of QSG recipients by increasing pulmonary HSPs.

Ischemic preconditioning decreases toxic cytokine and damage-associated molecular pattern molecule release from small-for-size liver grafts

An alternative possibility is that IP decreases the release of toxic mediators from small-for-size liver grafts. Warm hepatic ischemia/reperfusion causes inflammatory responses in remote organs, including the lung [56], and production of pro-inflammatory cytokines TNFα and IL-1β increases in liver grafts exposed to prolonged cold storage, resulting in ALI after liver transplantation [9]. Due to higher portal blood flow, small-for-size liver grafts are exposed to more endotoxin from the gut, which could stimulate Kupffer cells to produce toxic cytokines. Moreover, free radicals, which also stimulate toxic cytokine production, increase substantially in small-for-size grafts [36]. Indeed, we observed that TNFα expression increased in small-for-size liver grafts (**Figure 1**), consistent with our previous observations [20]. TNFα binds to its receptors to cause pro-inflammatory responses (*e.g.*, NF-κB activation, expression of adhesion molecules, and infiltration of leukocytes) and increase death receptor-mediated caspase activation and apoptosis [46]. IP protects against oxidative stress in QSG [20] which might decrease Kupffer cell activation and toxic cytokine formation. In this study we observed that IP inhibited expression of TLR4, an endotoxin receptor, and decreased TNFα expression in QSG (**Figure 1**).

Moreover, IP decreased HMGB1 release from QSG (**Figure 1**). HMGB1 normally resides in nuclei [57, 58], but with stress, HMGB1 translocates to the cytosol to be released. HMGB1 can be released actively by immune cells (*e.g.*, macrophages) or passively by injured and dying cells. HMGB1 is released under diverse conditions, such as shock, endotoxin-induced lethality, ischemia/reperfusion injury, trauma, sterile inflammation, infection, arthritis, and autoimmune disease [57-60]. HMGB1 is a DAMP molecule that has emerged as one of the most important pro-inflammatory mediators as well as an extracellular signaling factor with roles in pathogenesis to many organs/systems [44, 61-66]. HMGB1 binds to membrane receptors

(TLR4 and advanced glycation end-products receptors), thereby activating NF-κB and promoting inflammatory responses. Administration of recombinant HMGB1 in the mouse trachea causes pulmonary inflammation [67], and serum HMGB1 increases after hepatic ischemia/reperfusion [68, 69], contributing to hepatic warm ischemia/reperfusion-induced acute lung inflammation and injury by binding to pulmonary TLR4 [43]. In patients with acute liver failure, hepatic HMGB1 decreases, whereas plasma HMGB1 increases, indicating HMGB1 release [65]. However, other studies show that although HMGB1 is released from liver during ischemia/reperfusion injury, hepatic HMGB1 increases, possibly due to increased expression [70]. A case report indicates that HMGB1 increases in sera of liver transplantation patients with small-for-size syndrome [71]. In the present work, HMGB1 decreased in QSG but increased in the serum after transplantation, a finding consistent with the conclusion that failing small-for-size grafts were releasing HMGB1. Importantly, IP prevented HMGB1 release from QSG (**Figure 1**).

Conclusions

Collectively, our data indicate that IP of donor livers decreases ALI after small-for-size liver transplantation, possibly by inhibiting pro-inflammatory cytokine formation and release of DAMP from injured and/or failing grafts. Thus, anti-inflammatory treatment and neutralization of DAMP may be effective for prevention of lung injury, thereby improving outcomes of small-for-size liver transplantation.

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Disclosure of conflict of interest

None.

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References

[1] Wertheim JA, Petrowsky H, Saab S, Kupiec-Weglinski JW and Busuttil RW. Major challenges limiting liver transplantation in the United States. Am J Transplant 2011; 11: 1773-1784.

[2] Meirelles Junior RF, Salvalaggio P, Rezende MB, Evangelista AS, Guardia BD, Matielo CE, Neves DB, Pandullo FL, Felga GE, Alves JA, Curvelo LA, Diaz LG, Rusi MB, Viveiros MM, Almeida MD, Pedroso PT, Rocco RA and Meira Filho SP. Liver transplantation: history, outcomes and perspectives. Einstein (Sao Paulo) 2015; 13: 149-152.

[3] Jensen WA, Rose RM, Hammer SM, Jenkins RL, Bothe A Jr, Benotti PN, Dzik WH, Costello P, Khettry U, Trey C. Pulmonary complications of orthotopic liver transplantation. Transplantation 1986; 42: 484-490.

[4] Kotloff RM, Ahya VN and Crawford SW. Pulmonary complications of solid organ and hematopoietic stem cell transplantation. Am J Respir Crit Care Med 2004; 170: 22-48.

[5] Yost CS, Matthay MA and Gropper MA. Etiology of acute pulmonary edema during liver transplantation: a series of cases with analysis of the edema fluid. Chest 2001; 119: 219-223.

[6] Bozbas SS, Eyuboglu FO, Ozturk EF, Gullu AN, Sevmis S, Karakayali H and Haberal M. Pulmonary complications and mortality after liver transplant. Exp Clin Transplant 2008; 6: 264-270.

[7] Gadre S and Kotloff RM. Noninfectious pulmonary complications of liver, heart, and kidney transplantation: an update. Clin Chest Med 2017; 38: 741-749.

[8] O'Brien JD and Ettinger NA. Pulmonary complications of liver transplantation. Clin Chest Med 1996; 17: 99-114.

[9] Jiang A, LIU C, Song Y, Liu F, Li Q, Wu Z, Yu L and Lv Y. NF-kappaB induced the donor liver cold preservation related acute lung injury in rat liver transplantation model. PLoS One 2011; 6: e24960.

[10] Matuschak GM and Martin DJ. Influence of end-stage liver failure on survival during multiple systems organ failure. Transplant Proc 1987; 19 Suppl 3: 40-46.

[11] Colletti LM, Burtch GD, Remick DG, Kunkel SL, Strieter RM, Guice KS, Oldham KT and Campbell DA. The production of tumor necrosis factor alpha and the development of a pulmonary capillary injury following hepatic ischemia/reperfusion. Transplantation 1990; 49: 268-272.

[12] Testa G, Malago M and Broelsch CE. Living-donor liver transplantation in adults. Langenbecks Arch Surg 1999; 384: 536-543.
[13] Gridelli B, Spada M, Petz W, Bertani A, Lucianetti A, Colledan M, Altobelli M, Alberti D, Guizzetti M, Riva S, Melzi ML, Stroppa P and Torre G. Split-liver transplantation eliminates the need for living-donor liver transplantation in children with end-stage cholestatic liver disease. Transplantation 2003; 75: 1197-1203.
[14] Trotter JF, Wachs M, Everson GT and Kam I. Adult-to-adult transplantation of the right hepatic lobe from a living donor. N Engl J Med 2002; 346: 1074-1082.
[15] Sugawara Y, Makuuchi M, Takayama T, Imamura H, Dowaki S, Mizuta K, Kawarasaki H and Hashizume K. Small-for-size grafts in living-related liver transplantation. J Am Coll Surg 2001; 192: 510-513.
[16] Trewby PN, Warren R, Contini S, Crosbie WA, Wilkinson SP, Laws JW and Williams R. Incidence and pathophysiology of pulmonary edema in fulminant hepatic failure. Gastroenterology 1978; 74: 859-865.
[17] von HM, Hulser M, Seibert K, Scheuer C, Dold S, Kollmar O, Wagner M, Menger MD, Schilling MK and Moussavian MR. Split-liver procedure and inflammatory response: improvement by pharmacological preconditioning. J Surg Res 2011; 168: e125-e135.
[18] Hessheimer AJ, Fondevila C, Taura P, Munoz J, Sanchez O, Fuster J, Rimola A and Garcia-Valdecasas JC. Decompression of the portal bed and twice-baseline portal inflow are necessary for the functional recovery of a "small-for-size" graft. Ann Surg 2011; 253: 1201-1210.
[19] Jawan B, Cheung HK, Chen CC, Chen YS, Chiang YC, Wang CC, Cheng YF, Huang TL, Eng HL, Goto S, Pan TL, De Villa V, Liu PP, Wang SH, Lin CL, Lee JH. Repeated hypotensive episodes due to hepatic outflow obstruction during liver transplantation in adult patients. J Clin Anesth 2000; 12: 231-233.
[20] Liu Q, Rehman H, Harley RA, Lemasters JJ and Zhong Z. Small-for-size liver transplantation increases pulmonary injury in rats: prevention by NIM811. HPB Surg 2012; 2012: 270372
[21] Baxter GF and Yellon DM. Delayed myocardial protection following ischaemic preconditioning. Basic Res Cardiol 1996; 91: 53-56.
[22] Peralta C, Closa D, Hotter G, Gelpi E, Prats N and Rosello-Catafau J. Liver ischemic preconditioning is mediated by the inhibitory action of nitric oxide on endothelin. Biochem Biophys Res Commun 1996; 229: 264-270.
[23] Jones NM and Bergeron M. Hypoxic preconditioning induces changes in HIF-1 target genes in neonatal rat brain. J Cereb Blood Flow Metab 2001; 21: 1105-1114.
[24] Nonaka A, Kiryu J, Tsujikawa A, Yamashiro K, Nishijima K, Miyamoto K, Nishiwaki H, Honda Y and Ogura Y. Inhibitory effect of ischemic preconditioning on leukocyte participation in retinal ischemia-reperfusion injury. Invest Ophthalmol Vis Sci 2001; 42: 2380-2385.
[25] Arai M, Thurman RG and Lemasters JJ. Ischemic preconditioning of rat livers against cold storage-reperfusion injury: role of nonparenchymal cells and the phenomenon of heterologous preconditioning. Liver Transpl 2001; 7: 292-299.
[26] Wang Y, Shen J, Xiong X, Xu Y, Zhang H, Huang C, Tian Y, Jiao C, Wang X and Li X. Remote ischemic preconditioning protects against liver ischemia-reperfusion injury via heme oxygenase-1-induced autophagy. PLoS One 2014; 9: e98834
[27] Yang Y, Lang XB, Zhang P, Lv R, Wang YF and Chen JH. Remote ischemic preconditioning for prevention of acute kidney injury: a meta-analysis of randomized controlled trials. Am J Kidney Dis 2014; 64: 574-583.
[28] Zhou C, Li L, Li H, Gong J and Fang N. Delayed remote preconditioning induces cardioprotection: role of heme oxygenase-1. J Surg Res 2014; 191: 51-57.
[29] Li J, Rohailla S, Gelber N, Rutka J, Sabah N, Gladstone RA, Wei C, Hu P, Kharbanda RK and Redington AN. MicroRNA-144 is a circulating effector of remote ischemic preconditioning. Basic Res Cardiol 2014; 109: 423
[30] Brandenburger T, Grievink H, Heinen N, Barthel F, Huhn R, Stachuletz F, Kohns M, Pannen B and Bauer I. Effects of remote ischemic preconditioning and myocardial ischemia on microRNA-1 expression in the rat heart in vivo. Shock 2014; 42: 234-238.
[31] Shin HJ, Won NH and Lee HW. Remote ischemic preconditioning prevents lipopolysaccharide-induced liver injury through inhibition of NF-kappaB activation in mice. J Anesth 2014; 28: 898-905.
[32] Liu X, Sha O and Cho EY. Remote ischemic postconditioning promotes the survival of retinal ganglion cells after optic nerve injury. J Mol Neurosci 2013; 51: 639-646.
[33] Camara-Lemarroy CR. Remote ischemic preconditioning as prevention of transfusion-related acute lung injury. Med Hypotheses 2014; 83: 273-275.
[34] Garbaisz D, Turoczi Z, Fulop A, Rosero O, Aranyi P, Onody P, Lotz G, Rakonczay Z, Balla Z, Harsanyi L and Szijarto A. Therapeutic option for managing lung injury induced by infrarenal aortic cross-clamping. J Surg Res 2013; 185: 469-476.
[35] Rehman H, Connor HD, Ramshesh VK, Theruvath TP, Mason RP, Wright GL, Lemasters JJ and Zhong Z. Ischemic preconditioning pre-

vents free radical production and mitochondrial depolarization in small-for-size rat liver grafts. Transplantation 2008; 85: 1322-1331.
[36] Zhong Z, Connor HD, Froh M, Bunzendahl H, Lind H, Lehnert M, Mason RP, Thurman RG and Lemasters JJ. Free radical-dependent dysfunction of small-for-size rat liver grafts: prevention by plant polyphenols. Gastroenterology 2005; 129: 652-664.
[37] Omura T, Ascher NL and Emond JC. Fifty-percent partial liver transplantation in the rat. Transplantation 1996; 62: 292-293.
[38] Franco-Gou R, Rosello-Catafau J and Peralta C. Protection against lung damage in reduced-size liver transplantation. Crit Care Med 2006; 34: 1506-1513.
[39] Zhong Z, Ramshesh VK, Rehman H, Currin RT, Sridharan V, Theruvath TP, Kim I, Wright GL and Lemasters JJ. Activation of the oxygen-sensing signal cascade prevents mitochondrial injury after mouse liver ischemia-reperfusion. Am J Physiol Gastrointest Liver Physiol 2008; 295: G823-G832.
[40] Zhai Y, Busuttil RW and Kupiec-Weglinski JW. Liver ischemia and reperfusion injury: new insights into mechanisms of innate-adaptive immune-mediated tissue inflammation. Am J Transplant 2011; 11: 1563-1569.
[41] Boros P and Bromberg JS. New cellular and molecular immune pathways in ischemia/reperfusion injury. Am J Transplant 2006; 6: 652-658.
[42] Zhong Z, Theruvath TP, Currin RT, Waldmeier PC and Lemasters JJ. NIM811, a mitochondrial permeability transition inhibitor, prevents mitochondrial depolarization in small-for-size rat liver grafts. Am J Transplant 2007; 7: 1103-1111.
[43] Yang Z, Deng Y, Su D, Tian J, Gao Y, He Z and Wang X. TLR4 as receptor for HMGB1-mediated acute lung injury after liver ischemia/reperfusion injury. Lab Invest 2013; 93: 792-800.
[44] Kang R, Chen R, Zhang Q, Hou W, Wu S, Cao L, Huang J, Yu Y, Fan XG, Yan Z, Sun X, Wang H, Wang Q, Tsung A, Billiar TR, Zeh HJ III, Lotze MT and Tang D. HMGB1 in health and disease. Mol Aspects Med 2014; 9: 2124-2130.
[45] Andersson U and Rauvala H. Introduction: HMGB1 in inflammation and innate immunity. J Intern Med 2011; 270: 296-300.
[46] Luedde T, Kaplowitz N and Schwabe RF. Cell death and cell death responses in liver disease: Mechanisms and clinical relevance. Gastroenterology 2014; 147: 765-783.
[47] Snoeckx LH, Cornelussen RN, Van Nieuwenhoven FA, Reneman RS, Van Der Vusse GJ. Heat shock proteins and cardiovascular pathophysiology. Physiol Rev 2001; 81: 1461-1497.
[48] Selimoglu O, Ugurlucan M, Basaran M, Gungor F, Banach M, Cucu O, Ong LL, Gasparyan AY, Mikhailidis D and Ogus TN. Efficacy of remote ischaemic preconditioning for spinal cord protection against ischaemic injury: association with heat shock protein expression. Folia Neuropathol 2008; 46: 204-212.
[49] Zwacka RM, Zhou W, Zhang Y, Darby CJ, Dudus L, Halldorson J, Oberley L and Engelhardt JF. Redox gene therapy for ischemia/reperfusion injury of the liver reduces AP1 and NF-kappaB activation. Nat Med 1998; 4: 698-704.
[50] Hanschen M, Zahler S, Krombach F and Khandoga A. Reciprocal activation between CD4+ T cells and Kupffer cells during hepatic ischemia-reperfusion. Transplantation 2008; 86: 710-718.
[51] Caldwell CC, Tschoep J and Lentsch AB. Lymphocyte function during hepatic ischemia/reperfusion injury. J Leukoc Biol 2007; 82: 457-464.
[52] Ananthan J, Goldberg AL and Voellmy R. Abnormal proteins serve as eukaryotic stress signals and trigger the activation of heat shock genes. Science 1986; 232: 522-524.
[53] Kozutsumi Y, Segal M, Normington K, Gething MJ and Sambrook J. The presence of malfolded proteins in the endoplasmic reticulum signals the induction of glucose-regulated proteins. Nature 1988; 332: 462-464.
[54] Miller MJ. Preconditioning for cardioprotection against ischemia reperfusion injury: the roles of nitric oxide, reactive oxygen species, heat shock proteins, reactive hyperemia and antioxidants--a mini review. Can J Cardiol 2001; 17: 1075-1082.
[55] Park KM, Chen A and Bonventre JV. Prevention of kidney ischemia/reperfusion-induced functional injury and JNK, p38, and MAPK kinase activation by remote ischemic pretreatment. J Biol Chem 2001; 276: 11870-11876.
[56] Miranda LE, Capellini VK, Reis GS, Celotto AC, Carlotti CG Jr and Evora PR. Effects of partial liver ischemia followed by global liver reperfusion on the remote tissue expression of nitric oxide synthase: lungs and kidneys. Transplant Proc 2010; 42: 1557-1562.
[57] Huang W, Tang Y and Li L. HMGB1, a potent proinflammatory cytokine in sepsis. Cytokine 2010; 51: 119-126.
[58] Andersson U and Tracey KJ. HMGB1 is a therapeutic target for sterile inflammation and infection. Annu Rev Immunol 2011; 29: 139-162.
[59] Schierbeck H, Lundback P, Palmblad K, Klevenvall L, Erlandsson-Harris H, Andersson U and Ottosson L. Monoclonal anti-HMGB1 (high mobility group box chromosomal protein 1) antibody protection in two experimental arthritis models. Mol Med 2011; 17: 1039-1044.
[60] Abraham E. Unraveling the role of high mobility group box protein 1 in severe trauma. Crit Care 2009; 13: 1004.

[61] Deng Y, Yang Z, Gao Y, Xu H, Zheng B, Jiang M, Xu J, He Z and Wang X. Toll-like receptor 4 mediates acute lung injury induced by high mobility group box-1. PLoS One 2013; 8: e64375
[62] Lu B, Wang C, Wang M, Li W, Chen F, Tracey KJ and Wang H. Molecular mechanism and therapeutic modulation of high mobility group box 1 release and action: an updated review. Expert Rev Clin Immunol 2014; 10: 713-727.
[63] Zhou TB. Role of high mobility group box 1 and its signaling pathways in renal diseases. J Recept Signal Transduct Res 2014; 34: 348-50.
[64] Kojima M, Tanabe M, Shinoda M, Yamada S, Miyasho T, Suda K, Hibi T, Obara H, Itano O, Kawachi S, Kitajima M, Maruyama I and Kitagawa Y. Role of high mobility group box chromosomal protein 1 in ischemia-reperfusion injury in the rat small intestine. J Surg Res 2012; 178: 466-471.
[65] Oshima G, Shinoda M, Tanabe M, Ebinuma H, Nishiyama R, Takano K, Yamada S, Miyasho T, Masugi Y, Matsuda S, Suda K, Fukunaga K, Matsubara K, Hibi T, Yagi H, Hayashida T, Yamagishi Y, Obara H, Itano O, Takeuchi H, Kawachi S, Saito H, Hibi T, Maruyama I and Kitagawa Y. Increased plasma levels of high mobility group box 1 in patients with acute liver failure. Eur Surg Res 2012; 48: 154-162.
[66] Chen R, Hou W, Zhang Q, Kang R, Fan XG and Tang D. Emerging role of high-mobility group box 1 (HMGB1) in liver diseases. Mol Med 2013; 19: 357-366.
[67] Abraham E, Arcaroli J, Carmody A, Wang H and Tracey KJ. HMG-1 as a mediator of acute lung inflammation. J Immunol 2000; 165: 2950-2954.
[68] Yang M, Antoine DJ, Weemhoff JL, Jenkins RE, Farhood A, Park BK and Jaeschke H. Biomarkers distinguish apoptotic and necrotic cell death during hepatic ischemia-reperfusion injury in mice. Liver Transpl 2014; 20: 1372-1382.
[69] Liu A, Dirsch O, Fang H, Dong W, Jin H, Huang H, Sun J and Dahmen U. HMGB1 translocation and expression is caused by warm ischemia reperfusion injury, but not by partial hepatectomy in rats. Exp Mol Pathol 2011; 91: 502-508.
[70] Liu A, Fang H, Dirsch O, Jin H and Dahmen U. Oxidation of HMGB1 causes attenuation of its pro-inflammatory activity and occurs during liver ischemia and reperfusion. PLoS One 2012; 7: e35379.
[71] Craig DG, Lee P, Pryde EA, Hidalgo E, Hayes PC, Wigmore SJ, Forbes SJ and Simpson KJ. Markedly increased high-mobility group Box 1 protein in a patient with small-for-size syndrome. Case Rep Transplant 2014; 2014: 272498.