HMGB1-associated necroptosis and Kupffer cells M1 polarization underlies remote liver injury induced by intestinal ischemia/reperfusion in rats

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Abbreviations: Abx, antibiotic mix; ALT, alanine aminotransferase; AST, aspartate aminotransferase; DAMP, damage-associated molecular pattern; DAO, diamine oxidase; EP, ethyl pyruvate; FISH, fluorescence in situ hybridization; H&E, hematoxylin-eosin; HMGB1, high-mobility group box-1; I/R, ischemia/reperfusion; IL, interleukin; iNOS, inducible nitric oxide synthase; i-FABP, intestinal fatty acid binding protein; KC, Kupffer cell; LDH, lactate dehydrogenase; LPS, lipopolysaccharide; MCP-1, monocyte chemoattractant protein-1; MLKL, mixed lineage kinase domain-like; Nec-1, necrostatin-1; RAGE, receptor for advanced glycation end products; RIP1/3, receptor-interacting protein kinase 1/3; SMA, superior mesenteric artery; TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labeling; TLR, toll-like receptor.

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Abstract
Reperfusion of the ischemic intestine often leads to drive distant organ injury, especially injuries associated with hepatocellular dysfunction. The precise molecular mechanisms and effective multiple organ protection strategies remain to be developed. In the current study, significant remote liver dysfunction was found after 6 hours of reperfusion according to increased histopathological scores, serum lactate dehydrogenase (LDH), alanine aminotransferase (ALT)/aspartate aminotransferase (AST) levels, as well as enhanced bacterial translocation in a rat intestinal ischemia/reperfusion (I/R) injury model. Moreover, receptor-interacting protein kinase 1/3 (RIP1/3) and phosphorylated-MLKL expressions in tissue were greatly elevated, indicating that necroptosis occurred and resulted in acute remote liver function...
1 INTRODUCTION

Intestinal ischemia/reperfusion (I/R) injury is a common life-threatening complication in clinical situations and is obviously relevant in numerous acute pathological conditions, such as acute mesenteric ischemia, septic shock, small bowel transplantation, and abdominal aortic surgery. Barrier dysfunction aggravates the deleterious complications of intestinal I/R, including systemic inflammatory response syndrome (SIRS) and multiple organ dysfunction syndrome (MODS), which contributes to high morbidity and mortality. However, despite its clinical importance, intestinal I/R and associated injuries to other organ systems remain challenging to treat. In addition, no approved pharmacological interventions have been developed thus far.

The liver is the organ in closest contact with the intestine and is particularly vulnerable to the negative consequences of distant intestinal epithelial barrier disruption because the vasculature of these tissues is coupled in series with the intestinal circulation, such as portal circulation. Moreover, the pathophysiology of remote liver injury after intestinal I/R remains only partially understood; putative mechanisms include sustained pro-inflammatory cytokine challenge and cytokines that are released or activated after enterocyte damage or death. If liver repair and regenerative mechanisms are not activated promptly, especially in patients with chronic liver disease or after liver transplantation, acute liver functional impairment, or poor early graft function will occur. Despite extensive studies, effective multiple organ protective interventions with clinically proven efficacy remain to be developed.

Early inflammatory cytokines are certainly involved in the pathogenesis of intestinal I/R. However, due to the narrow time window, the clinical significance of early inflammatory cytokines is limited. More attention has been focused on the late inflammatory cytokines such as high-mobility group box-1 (HMGB1) and newly recognized histones because these endogenous damage-associated molecular patterns (DAMPs) have a much wider time window for patient treatment and also significantly contribute to multiple organ injuries. Inhibition of extracellular HMGB1 attenuates inflammation and confers protection in various inflammatory-associated diseases, including sepsis, hepatitis, ischemic stroke, and liver I/R. Moreover, HMGB1 reportedly acts as an endogenous DAMP after being released by necrotic cells or actively secreted by macrophage/monocytes into the extracellular environment, thereby triggering and amplifying inflammatory processes. In particular, we previously reported that HMGB1 is the major DAMP released from necroptotic enterocytes during intestinal I/R to induce an inflammatory response and subsequent intestinal damage. Recent advances have revealed the intimate interplay between SIRS or sepsis-induced organ failure and cell death or cell dysfunction resulting from necrosis/necroptosis, apoptosis, and autophagy. Necroptosis is a newly defined lytic type of cell death and a form of regulated necrosis that is inherently associated with inflammation and distinguished from other forms of cell death by the associated activation of the receptor-interacting protein kinase 1 and 3 (RIP1/3) necroosome complex and phosphorylation of mixed lineage kinase domain-like (MLKL). Various studies have indicated that necroptosis is intrinsically involved in the development of kidney and heart transplantation, organ I/R injury, TNF-induced shock, and sepsis. Understanding of precise molecular mechanisms underlying cell death in intestinal I/R-induced hepatic injury will provide the basis for the development of new therapeutic strategies for the prevention of remote organ dysfunction.
injury. Therefore, exploring whether intestinal I/R-induced remote hepatocellular injury and liver dysfunction are associated with hepatocytes necroptosis is meaningful.

Therefore, our present study was undertaken to investigate the remote hepatic injury associated with intestinal I/R and explore the role of cell necroptosis and released HMGB1 in the liver due to cross talk with injured enterocytes.

2 | MATERIALS AND METHODS

2.1 | Animal model

All animal experimental protocols were approved by the National Animal Care and Use Committee of Sun Yat-sen University (Guangzhou, China). Adult male Sprague-Dawley rats (weighing 220-250 g) were obtained from the Animal Center of Sun Yat-sen University (Guangzhou, China). The rats were anesthetized with pentobarbital (30 mg/kg, intraperitoneally) and the intestinal I/R model was established according to our previous research. Briefly, the small intestine was exteriorized with a 2-cm midline laparotomy, and the superior mesenteric artery (SMA) was identified. The SMA was occluded by a noncrushing microvascular clip for 90 minutes. During the experimental period, body temperature was stably maintained at 37°C using a heating pad. After different reperfusion time point, fluid therapy was carried out by intraperitoneally administering 0.5 mL/100 g of normal saline, and the abdominal wall was closed with running monofilament sutures. Then, the rats were allowed free access to water and chow.

2.2 | Animal treatment

According to the random number table, animals were administered with the specific necroptosis inhibitor Nec-1 (necrostatin-1, dissolved in normal saline, 1 mg/kg; BML-AP309-0100, Enzo Life Science, Farmingdale, NY, USA) via intraperitoneal injection 30 minutes before ischemia or at the onset of intestinal reperfusion. Anti-HMGB1 neutralizing antibody (1 mg/kg via intravenous injection; Shino-test Corporation, Japan) and control Ig Y antibody (1 mg/kg via intravenous injection; Shino-test Corporation, Japan) were administered at the onset of intestinal reperfusion. Ethyl pyruvate (EP, 40 mg/kg via intravenous injection; Sigma-Aldrich, St. Louis, MO, USA) was administered 2 hours before ischemia.

For macrophage depletion studies, rats were intravenously injected with liposomal clodronate at 4 μL/g (ClodronateLiposomes.org, Amsterdam, Netherlands) 24 hours prior to intestinal I/R injury. For gut commensal microflora depletion, rats were provided an antibiotic mix (Abx) consisting of ampicillin (1 g/L; Sigma-Aldrich, St. Louis, MO, USA), metronidazole (1 g/L; Sigma-Aldrich, St. Louis, MO, USA), neomycin trisulfate (1 g/L; Sigma-Aldrich, St. Louis, MO, USA), and vancomycin (500 mg/L; Sigma-Aldrich, St. Louis, MO, USA) in drinking water for 4 weeks. Control drinking water was prepared in the same way only without broad-spectrum antibiotics. The depletion of the commensal microflora was based on the protocol of Fagarasan et al and Rakoff-Nahoum et al, and was verified by bacteriological analysis of colonic faces on the MiSeq V3 System (Illumina).

2.3 | Survival analysis

In a separate group of rats (n = 15 per group) receiving the Anti-HMGB1 neutralizing antibody and EP treatment were used to detect the survival time. After 6 hours reperfusion, the survivors were transferred to individual cages and allowed free access to water and food. From the onset of intestinal reperfusion, animals were monitored for 24 hours.

2.4 | Serum biochemical markers and tissue cytokine analysis

Circulating HMGB1 and LPS were measured and gut injury was determined by the portal blood levels of intestinal mucosal damage markers (i-FABP: intestinal fatty acid binding protein; DAO: diamine oxidase). Markers were measured with ELISA kits as described by the manufacturer’s instructions (HMGB1: Arigo Biolaboratories Co., Hsinchu City, Taiwan, China; LPS: i-FABP, DAO: Wuhan USCN Business Co., Ltd, Hubei, China). To assess liver injury, we measure the levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST), and lactate dehydrogenase (LDH) with HITACHI 7080 automated analyzer (Tokyo, Japan).

Tissue cytokine measurements were performed using the Milliplex system (Luminex 200, Millipore, St. Charles, MO, USA). The cytokines analyzed were IL-1β, IL-6, tumor necrosis factor-α (TNF-α), and monocyte chemoattractant protein (MCP)-1 (RECYTMAG-65K, Milliplex, Millipore, St. Charles, MO, USA). Analysis was performed by The ACRED Biological Testing Lab (Guangzhou, China).

2.5 | Morphometric assessment of intestinal and liver damage

Hematoxylin-eosin (H&E) stained sections (4 μm) were evaluated independently by two pathologists blind to the study groups according to the Chiu’s and Eckhoff’s scores for
the intestine and liver, respectively. Briefly, Chiu’s score was used as previously described.\textsuperscript{22} Liver tissues were evaluated at 200x magnification by a point-counting method for the severity of hepatic injury using an ordinal scale as follows: grade 0, minimal or no evidence of injury; grade 1, mild injury consisting of cytoplasmic vacuolation, and focal nuclear pyknosis; grade 2, moderate to severe injury with extensive nuclear pyknosis, cytoplasmic hypereosinophilia, loss of intercellular borders, and mild to moderate neutrophil infiltration; and grade 3, severe injury with disintegration of hepatic cords, hemorrhage, and severe neutrophil infiltration. An average of 100 adjacent points on a 1-mm\(^2\) grid were graded for each specimen.

2.6 | Fluorescence in situ hybridization (FISH)

A specific bacterial probe (Eco1167, 5’-GCA TAA GCG TCG CTG CCG-3’) conjugated to Cy3 (red signal, Sigma-Aldrich, St. Louis, MO, USA) was used to detect bacterial translocation in the liver after intestinal I/R challenge. Cy3 has absorption and emission wavelengths of 552 nm and 570 nm, respectively. The probe binds to a conserved region of bacterial 16S rRNA that is specific for Escherichia coli. Tissue sections were counterstained with 5 μg/mL of 4’6-diamidino-2-phenylindole (DAPI, Sigma-Aldrich, St. Louis, MO, USA) to visualize hepatic cell nuclei. Imaging was performed using an LSM710 confocal microscope (Carl Zeiss, Jena, Germany) at 400x magnification with imaging data analyzed using Image-Pro Plus software.

2.7 | Immunofluorescence and immunohistochemistry

For immunofluorescence, frozen liver tissues were prepared and incubated over night at 4°C with the following antibodies: anti-HMGB1 (1:500, ab18256, Abcam, Cambridge, UK); anti-Toll-like receptor 4 (TLR4, 1:200, 19811-1-AP, Proteintech, Chicago, USA); anti-receptor for advanced glycation end products (RAGE, 1:200, 16346-1-AP, Proteintech, Chicago, USA); anti-inducible nitric oxide synthase (iNOS, 1:200, DMABT-46628MR, Creative Diagnostics, New York, USA); anti-inducible nitric oxide synthase (iNOS, 1:200, ab15323, Abcam, Cambridge, UK) and anti-Arginase-1 (1:200, RT1051, Huabio, Hangzhou, China), followed by a 1 hours incubation at room temperature with secondary FITC-labeled goat anti-rabbit IgG (1:200, GB22303, Servicebio, Wuhan, China) and secondary Cy3-labeled goat anti-mouse antibody (1:200, GB21301, Servicebio, Wuhan, China). Nuclei were counterstained with DAPI (1:10 000). For double immunofluorescence, sections were incubated with a mixture of primary antibodies followed by mixture of secondary antibodies. Next, sections were mounted with neutral balsam. Images were taken with a fluorescence microscope (BX63, Olympus Corporation, Tokyo, Japan) and a confocal microscope (LSM710, Carl Zeiss, Jena, Germany). For immunohistochemical staining, paraffin-embedded liver sections were incubated with a RIP1 antibody (1:200, SAB3500420, Sigma-Aldrich, St. Louis, MO, USA) and RIP3 antibody (1:200, ab62344, Abcam, Cambridge, UK), followed by a secondary HRP-labeled antibody (K5007, Dako, Copenhagen, Denmark) for 1 hours at room temperature. Images were taken using a microscope (BX51, Olympus Corporation, Tokyo, Japan).

2.8 | Flow cytometry analysis of peripheral blood cells

After 6 hours reperfusion, blood was collected from abdominal aorta in tubes with EDTA. Freshly isolated blood mononuclear cells were resuspended in FACS buffer (PBS containing 2% FCS and 0.05% NaN\(_2\)) and then stained with the primary antibodies against the cell surface markers: anti-CD45, anti-CD68, anti-CD86, or anti-CD163 for 30 minutes at 4°C. For intracellular staining to determine CD68 levels, cells were fixed after surface staining and then permeabilized using 0.3% Triton X-100 for 30 minutes, before anti-CD68 staining for 30 minutes. Cell counts were determined using a BD FACS Canto II flow cytometer (BD Biosciences) and analyzed with FlowJo software (FlowJo, LLC, Ashland, OR). The antibodies used for flow cytometry are shown in Supplemental Table 1.

2.9 | Western blot analysis

Liver tissues were harvested and ground in lysis buffer. Cytoplasmic protein extraction was performed with a commercially available kit (Beyotime, Beijing, China). Lysates were centrifuged at 12 000 rpm for 15 minutes, and the supernatant was determined by a bicinchoninic acid assay. Samples (20-40 μg of total protein) were boiled for 5 minutes and loaded on poly acrylamide gels for electrophoresis. Then, we transferred the samples onto polyvinylidene fluoride membranes for immunoblotting. Membranes were incubated with antibodies against HMGB1 (1:1000, ab18256, Abcam, Cambridge, UK); RIP1 (1:1000, SAB3500420, Sigma-Aldrich, St. Louis, MO, USA); RIP3 (1:1000, ab62344, Abcam, Cambridge, UK); p-MLKL (1:1000, AF7419, Affinity Biosciences, Ohio, USA); cleaved caspase 3 (1:1000, 9664, Cell Signaling Technology, Danvers, USA); RAGE (1:1000, 16346-1-AP, Proteintech, Chicago, USA); TLR4 (1:1000, 19811-1-AP, Proteintech, Chicago, USA); and β-actin (1:1000, 4970, Cell Signaling Technology,
Danvers, USA) overnight at 4°C followed by HRP-labeled goat anti-mouse IgG (1:5000, SA00001-1, Proteintech, Chicago, USA) or HRP-labeled goat anti-rabbit IgG (1:5000, SA00001-2, Proteintech, Chicago, USA) for 1 hours at room temperature. The membranes were imaged with a biomolecular imager (ImageQuant LAS 4000 General Electric, Boston, USA). All images were analyzed with Image J software (National Institutes of Health, Bethesda, USA).

2.10 | TUNEL staining

Hepatic cell death was assessed using in situ terminal deoxy-nucleotidyl transferase dUTP nick end labeling (TUNEL) staining (G3250, Promega, Fitchburg, WI, USA) according to the manufacturer’s instructions (Dead-End Fluorometric TUNEL System). TUNEL-positive nuclei were visualized by green FITC fluorescence.

2.11 | Quantitative reverse transcription PCR (RT-qPCR) analysis

RNA was isolated from liver tissues using an RNAeasy plus mini kit (Qiagen, Germany) according to the manufacturer’s instructions. cDNA was generated using the QuantiTect Reverse Transcription Kit (Qiagen, Germany). RT-PCR was performed on cDNA using TaqMan primers and probes in combination with TaqMan PCR Master Mix (Applied Biosystems), and reactions were run on an RT-PCR system (QuantiStudio 6 Flex; Applied Biosystems). Gene expression is displayed as the fold increase and normalized to β-actin. The TaqMan Gene Assay IDs and primer sequences used were as follows: IL-1β (Rn00580432_m1), forward 5′-CTATGGCAACTTTCCCTGAA-3′, reverse 5′-GGCTTTGGAACAAATCTCTTAAT C-3′; iNOS (Rn00561646_m1), forward 5′-TGGAGCGAGTTGTGGATTG-3′, reverse 5′-CCTCTTGTTCTTGAGATTG-3′; Arginase-1 (Rn00691090_m1), forward 5′-GTAGCAGAGACCCAGAAGAATG-3′, reverse 5′-TCCACCCAAATG ACGCAAGCAGGATG-3′; reverse 5′-CTCCACCTCTCTCAGCAGTAATT-3′; IL-10 (Rn01483987_m1), forward 5′-AGTGGACGCAGGATG-3′, reverse 5′-GAGCTCTACAGTGGCAGTCTATG-3′; β-actin (Rn00667869_m1), forward 5′-GCCCATCTATG AGGGTTACGC-3′, reverse 5′-TAA TGTCACGCACG ATTTCCC-3′.

2.12 | Statistical analyses

Statistical analyses were computed using GraphPad Prism 7.0 software (La Jolla, CA, USA). Data are presented as the means ± standard deviation (SD) and analyzed by either one-way ANOVA (Tukey post hoc multiple comparison test for multiple groups) or a Mann-Whitney nonparametric test. Survival time was compared with a Kaplan-Meier curve and log-rank test. For colonic faces bacteriological analysis, the raw data were performed using the Biomedical Genomics Workbench version 4.0 equipped with the Microbial Genomics Module version 2.0 (Qiagen) plugin. A difference of $P < .05$ was considered statistically significant.

3 | RESULTS

3.1 | Intestinal I/R injury caused remote liver dysfunction

Rats that underwent intestinal ischemia at designated (2 hours, 6 hours, 12 hours, and 24 hours) reperfusion times exhibited significant intestinal and liver pathological damage as reperfusion time was extended. Compared to sham animals, rats subjected to 6 hours of reperfusion displayed severe capillary engorgement and intestine villose disruption (Figure 1A), as well as higher Chiu’s score (Figure 1B), levels of i-FABP (Figure 1C) and DAO activity (Figure 1D). Sham-operated animals showed normal-appearing hepatocyte parenchyma. However, acute hepatic injury with marked hepatocyte vacuolization, congestion, sinusoidal, and central vein dilatation were observed at 6 hours reperfusion, following by nearly recovery at 24 hours after reperfusion in the liver (Figure 1A). Furthermore, liver damage peaked at 6 hours after reperfusion according to the changes in Eckhoff’s scores (Figure 1E), serum LDH (Figure 1F), ALT (Figure 1G), and AST (Figure 1H) levels, which almost recovered at 24 hours. These results indicated that liver injury induced by intestinal I/R is a dynamic process, and the critical time point (6 hours of reperfusion) was used for subsequent experiments.

3.2 | Enhanced bacterial translocation was found in remote liver injury after intestinal I/R challenge

A specific FISH-probe detects *E. coli* in liver sections after intestinal I/R challenge. The Gram-negative gut pathobiont *E. coli* translocated as a result of gut barrier breakdown into livers (Figure 2A). Increased bacterial colonization was present in the hepatic sinusoid, which was in close proximity to the endothelium (FISH+ bacteria), especially in the 6 h reperfusion group (Figure 2B). This finding indicated that enhanced bacterial translocation might contribute to hepatic damage after intestinal I/R injury.
To determine whether necroptosis was involved in intestinal I/R-induced hepatocyte cell death, we measured the expression levels of RIP1/3 and MLKL phosphorylation by Western blotting. Clearly, intestinal I/R triggered an upregulation of RIP1/3 and p-MLKL expressions, especially after 6 hours of reperfusion. Next, these proteins gradually declined nearly to baseline levels at 24 hours of reperfusion (Figure 3A-E). Functionally, necroptosis inhibitor Nec-1 pretreatment resulted in significant alleviation of hepatocyte vacuolization.
(Figure 3F), better-preserved liver architecture with lower Eckhoff’s score (Figure 3G), and reduced serum ALT levels (Figure 3H). In addition, the localization of RIP1 and RIP3 was immunohistochemically investigated. RIP1 and RIP3 were strongly expressed in hepatocytes around the central vein area after 6 hours of intestinal reperfusion but were inhibited after Nec-1 administration (Figure 3I). Consistently, RIP1/3 and p-MLKL expressions were markedly downregulated after Nec-1 administration (Figure 3J-M). Moreover, increased hepatocyte cell death after intestinal barrier disruption was further confirmed through TUNEL staining (Figure 3N). However, Nec-1 pretreatment did not reduce the number of TUNEL positive-hepatocytes in the liver (Figure 3O).

Consistently, Nec-1 also did not affect caspase 3 cleavage (Figure 3P-Q).

Notably, the protective effect of Nec-1 was readily detectable even when the compound was administered immediately at the onset of reperfusion, as evidence by significantly alleviated histopathologic changes (Supplemental Figure 1A,B), reduced serum ALT and LDH levels (Supplemental Figure 1C,D). Therefore, in can be reasoned that this extend time window of hepatoprotection by Nec-1 in vivo might reflect a delayed induction of hepatocyte necroptosis during ischemic intestinal injury.

Altogether, these results clearly demonstrated that the necroptosis inhibitor Nec-1 has a protective effect against...

**FIGURE 2** Intestinal ischemia/reperfusion increased the bacterial translocation in remote liver. A, Structures stained with H&E were labeled by FISH using a specific probe for *E. coli* rRNA Eco1167 (red) and nuclei were counterstained with DAPI (blue). Increased bacterial colonization was presented in the hepatic sinusoid (in close proximity to the endothelium) over the different course of reperfusion. Images are representative of at least three individual rats. Scale bars: 50 μm. B, Number of bacterial presences in the hepatic sinusoid. Data are expressed as mean ± SD (n = 6). **P < .01 vs Sham group; ***P < .01 vs R 6 h group**
FIGURE 3  Necroptosis in remote hepatic injury after intestinal barrier disruption. A-E, Expressions of RIP1, RIP3, and p-MLKL in rat liver samples at different time points after intestinal ischemia/reperfusion. F, Histology (H&E staining) change of the liver (400×, scale bar: 100 μm) after necroptosis specific inhibitor Nec-1 treatment before intestinal ischemia. G, Injury scoring of the liver morphology and (H) serum ALT concentration change. I, Localization of RIP1 and RIP3 in the liver after intestinal ischemia/reperfusion with immunohistochemistry staining (400×, scale bar: 100 μm). J-M, Protein blotting of RIP1, RIP3, and p-MLKL after Nec-1 administration with Western blot analysis. N, Hepatocyte cell death accessed by TUNEL staining (green fluorescence, 100×, scale bar: 500 μm) after Nec-1 administration at 6 hours reperfusion. O-Q, Protein blotting of cleaved caspase-3 after Nec-1 administration with Western blot analysis. Data are expressed as mean ± SD (n = 6). *P < .05, **P < .01 vs Sham group; *P < .05, **P < .01 vs R 6 h group or Vehicle group. Vehicle, normal saline
intestinal I/R-induced hepatic injury and that hepatocyte cell death is due, at least partly, to RIP1/3-dependent necroptosis.

### 3.4 HMGB1 cytoplasm translocation mediated hepatic injury

High-mobility group box-1 is a non-histone DNA-binding nuclear protein that is involved in nucleosome stabilization. The translocation of HMGB1 was detected in hepatocytes after intestinal I/R challenge. As shown, the nuclear localization of HMGB1 was observed in the Sham group (Figure 4A, white arrows). However, HMGB1 translocation from the nucleus to the cytoplasm was obvious after 6 hours of reperfusion or Vehicle treatment (Figure 4A, white arrowheads), while Nec-1 pretreatment inhibited ischemia-induced HMGB1 translocation. Total HMGB1 protein levels in hepatocytes were not significantly changed, whereas Nec-1 administration markedly attenuated the increase in cytoplasmic HMGB1 expression (Figure 4B,C). These findings reveal that HMGB1 cytoplasm translocation is involved in remote hepatocyte injury.

### 3.5 HMGB1 neutralization/inhibition attenuated intestinal injury, liver inflammation, and improved survival rate

As shown, there were relative preservation of the intestinal mucosal structure in the anti-HMGB1 and EP treated groups when compared with the R 6 h and Ig Y groups (Figure 5A). In parallel with the morphologic changes, the Chiu’s scores, i-FABP and DAO levels (Figure 5B-D) were also shown decreased after the treatments.

Meanwhile, the protective effects of HMGB1 neutralization and inhibition were further supported by representative liver histologic slides, with alleviated hepatocyte vacuolization, nuclear pyknosis, and better-preserved architecture (Figure 5A). Moreover, HMGB1 neutralization and inhibition were associated with a reduction in liver injury scoring (Figure 5E) and serum ALT levels (Figure 5F), as well as reduced production of pro-inflammatory cytokines IL-1β (Figure 5G), IL-6 (Figure 5H), TNF-α (Figure 5I), and MCP-1 (Figure 5J) in liver tissues. Anti-HMGB1 antibody treatment significantly decrease serum HMGB1 levels, and EP also markedly eliminate high levels of circulating HMGB1 (Figure 5K). Mortality rates at 24 hours after reperfusion were 0%, 66.7%, 46.7%, and 33.3%
FIGURE 5  HMGB1 neutralization or inhibition ameliorated intestinal, hepatic injury and improved survival rate. A, Histology (H&E staining) of the intestinal (100×) and liver tissue (400×). Scale bars: 100 μm. B, Injury scoring of the intestine morphology. C-D, Portal blood levels of intestinal fatty acid binding protein (i-FABP) and diamine oxidase (DAO), E, Injury scoring of the liver morphology and (F) serum ALT concentration change. Concentration of IL-1β (G), IL-6 (H), TNF-α (I) and MCP-1 (J) in liver tissue accessed by Milliplex system. K, Concentration of HMGB1 in circulation. Data are expressed as mean ± SD (n = 6). L, Effect of HMGB1 neutralization or inhibition on survival (n = 15). *P < .05, **P < .01 vs Sham group; #P < .05, ##P < .01 vs R 6 h group or Ig Y group
for the Sham, R 6 h, Anti-HMGB1 and EP groups, respectively. Neutralized HMGB1 activity and release statistically significantly increase survival rates (Figure 5L). Taken together, these findings demonstrated that the administration of anti-HMGB1 and EP reduced liver damage inducing by I/R intestinal lesions as a consequence of intestinal injury improvement. Meanwhile, HMGB1 neutralization/inhibition protects against intestinal I/R-induced mortality.

### 3.6 Macrophage depletion ameliorated the liver injury induced by intestinal ischemia

Liposomal clodronate administration 24 hours prior to intestinal ischemia results in largely depletes resident and infiltrating macrophages in the liver and spleen, as shown by immunohistochemistry for CD68 in comparison with Sham, R 6 h and Lipo-PBS (empty liposomes) groups (Figure 6A,B). Meanwhile, systemic macrophage depletion presented better-preserved liver architecture with lower Eckhoff’s scores (Figure 6C,D) and reduced serum ALT/AST levels (Figure 6E,F). These findings demonstrated that Kupffer cells (KCs) are responsible for liver damage after intestinal ischemia challenge.

### 3.7 HMGB1 neutralization treatment further protected against intestinal ischemia-associated liver damage in microbiota-depleted rats

Extensive depletion of gut microbiota by broad-spectrum antibiotics strategy did not lead to changes in water and food intake, as well as body weight of rats (Supplemental Figure 2A-C). However, the diversity of bacterial communities shrunk after Abx treatment (Supplemental Figure 2D). A comparison of individual bacterial communities, using PERMANOVA principal coordinate analysis (PCoA), showed a distinct clustering by sample cohorts based roughly on treatment (Supplemental Figure 2E). After depletion of microbiota, we found a profound protection against liver injury induced by intestinal I/R as assessed by scoring of liver Eckhoff’s scores (Figure 7A,B), serum ALT, AST, and LDH levels (Figure 7C-E), which were further decreased after HMGB1 neutralizing antibody treatment. As expected,
4 weeks of Abx pretreatment were associated with a significant reduced serum LPS levels, even after intestinal I/R (Figure 7F). Together these findings showed that in the absence of bacterial products (especially LPS), HMGB1 neutralization treatment further help in attenuation of intestinal I/R-associated secondary liver damage.

3.8 | HMGB1 neutralization and inhibition attenuated hepatocyte TLR4/RAGE expression and necroptosis

Using the immunofluorescence technique and colocalization analysis by Image J, we observed increased colocalization of HMGB1 with TLR4 and RAGE in the injury group, which was alleviated after anti-HMGB1 treatment (evidenced by decreased Pearson’s coefficients) (Figure 8A-D). Consistent with the above findings, the protein expression levels of TLR4 and RAGE were significantly downregulated after HMGB1 neutralization and inhibition (Figure 8E,G-H). Furthermore, HMGB1 neutralization and inhibition also documented to inhibit the expressions of RIP1/3 and p-MLKL in hepatocytes (Figure 8E,F,I-K), suggesting an attenuation of hepatocyte necroptosis. Overall, these findings indicated that HMGB1-mediated hepatocyte damage may be responsible for intestinal I/R-induced remote liver injury.

3.9 | HMGB1 neutralization promoted circulating macrophages and hepatic KC toward M2 polarization

Peripheral blood macrophage content was analyzed by selecting cells with dual positivity for CD45 and CD68. Thereafter, CD86 (M1 macrophage marker) and CD163
(M2 macrophage marker) expressions were assessed among the CD45+/CD68+ cells by flow cytometry (Figure 9A). As shown, intestinal I/R resulted in significant increased CD68+/CD86+ M1 macrophages, while an increased number of CD68+/CD163+ M2 macrophages were observed after HMGB1 neutralization treatment (Figure 9B,C).

FIGURE 8 HMGB1 neutralization or inhibition attenuated hepatocyte TLR4/RAGE expression and necroptosis. Dual labeling of (A) HMGB1 (red)/TLR4 (green) and (C) HMGB1 (red)/RAGE (green). Scale bars: 50μm. The Pearson’s correlation and overlap coefficient of HMGB1/TLR4 (B) and HMGB1/RAGE (D) assessed by Image J. E-K, Protein expressions of TLR4, RAGE, RIP1, RIP3, and p-MLKL in liver tissues assessed by Western blot analysis. Data are expressed as mean ± SD (n = 5-6). *P < .05, **P < .01 vs Sham group; #P < .05, ##P < .01 vs R 6 h group or Ig Y group.
Meanwhile, after intestinal I/R injury challenge, the number of hepatic macrophages (CD68+ cells) was significantly increased (Figure 9D,E), indicating an increase in infiltrating KCs in remote hepatic injury. Furthermore, M1 macrophages (CD68+/iNOS+ cells) were markedly increased after 6 hours of reperfusion or Ig Y treatment (Figure 9F), while M2 polarized and presumably beneficial Arginase-1 macrophages (CD68+/Arginase-1+ cells) were significantly higher in the Anti-HMGB1 group than in the R 6 h or Ig Y-treated group (Figure 9H). Macrophage phenotype gene signature transcript levels related to KCs activation and polarization were changed in line with the above data. Thus, in 6 h reperfusion and Ig Y-treated rats, the increased expression of pro-inflammatory iNOS and IL-1β (Figure 9G) was suppressed and that of anti-inflammatory Arginase-1 and IL-10 (Figure 9I) was induced by anti-HMGB1 neutralizing antibody treatment. These results indicated that HMGB1 neutralization-induced M2 type macrophage polarization in the circulating and liver tissue might contribute to protect against remote hepatic injury.

4 | DISCUSSION

Our current study demonstrated the development of acute hepatic injury in rat intestinal I/R model, especially at the
6 hours reperfusion time. Consistently, intestinal ischemia caused barrier disruption and leakage facilitated the passage of bacteria (E. coli) from the intestinal lumen to the portal circulation, which may further trigger inflammatory pathways and contribute to liver injury. More importantly, since the macrophage-depletion study demonstrated that KCs are responsible for liver damage, the progressive increase in HMGB1 cytoplasm translocation, hepatocyte necroptosis, hepatic KCs, and circulating macrophages M1 polarization contributed to remote hepatic injury in the rat intestinal I/R model. In addition, the necroptosis inhibitor Nec-1 mitigated necroptosis-induced HMGB1 translocation to the cytoplasm. HMGB1 neutralizing antibody and its inhibitor EP mitigated the development of hepatocyte necroptosis and subsequent hepatic inflammation. What’s more, HMGB1 neutralization further protects against intestinal I/R-associated liver damage in microbiota-depleted rats. These agents could serve as pharmacological interventions within these pathophysiological processes.

The intestine is an important contributor to MODS, and intestinal I/R injury compromises the whole organism and survival. Unlike other organs, the liver has a dual blood supply system involving the hepatic artery and portal vein that mainly collects blood from the spleen, stomach, intestine, and mesentery. Therefore, the gut-liver axis is crucial for maintaining the normal morphology and function of these two organs. Hepatic microvascular dysfunction, leukocyte accumulation and mediated oxidative stress in non-perfused sinusoids, leukocyte-endothelial cell adhesion, and excessive inflammation are important determinants of liver dysfunction induced by intestinal I/R. Since intestinal leakage could disseminate the gut microflora systemically, activate TLR signaling, and amplify inflammatory cytokine production, the integrity of the gut barrier is essential to prevent the microbiota of a healthy individual from triggering bacteria-derived endotoxemia. In this rat model, intestinal I/R caused the breakdown of the mucosal barrier, as shown in histological HE staining, with an association of increased levels of i-FABP and DAO. According to previous research, translocation of bacterial components in the portal circulation triggers inflammatory pathways and contributes to liver injury through the engagement of various pattern recognizing receptors. As a result of barrier disruption, the Gram-negative gut pathobiont E. coli translocated into livers, and was present in the hepatic sinusoid and in close proximity to the endothelium, suggesting bacterial translocation-induced hepatic damage after intestinal I/R injury. Furthermore, since it could be expected that bacterial products including LPS would activate TLR signaling and amplify inflammatory cytokine production, our current study showed that broad-spectrum antibiotics strategy induced gut microbiome depletion and low serum LPS before intestinal ischemia resulted in protection against liver dysfunction and damage, suggestive of a deleterious effect of “normal” gut microbiome during intestinal I/R. And HMGB1 neutralization treatment further help in attenuation of intestinal I/R-associated secondary liver damage. Interestingly, a previous study using similar strategy also showed that a microbiota-depleted gut was associated with a reduced cellular inflammation within ischemic kidneys and with a protection against renal I/R injury. These data imply that antibiotic prophylaxis before intestinal I/R may also have a role in mitigating liver injury in such circumstances.

Effective therapies in treating intestinal I/R-induced remote hepatic injury are lacking because of an incomplete understanding of its pathophysiology. Necroptosis is morphologically characterized as necrotic cell death and mediated by the kinases of RIP1 and RIP3, which form a necroptosis inducing protein complex (Necrosome). Meanwhile, the necrosome phosphorylate the MLKL, which subsequently results in the rapid, active and dynamic release of cell DAMPs following the loss of plasma membrane integrity and promotes ongoing inflammation and secondary tissue injury. Growing evidence suggests that instead of blocking the inflammatory cascade directly, novel strategies focusing on cell necroptosis may hold greater promise for improving outcomes in organ injury. We and others previously reported that necroptosis can promote inflammatory injury and acts as a key modulator in the pathogenesis of intestinal I/R injury and transplant survival in donor kidney, lung, and heart. In addition, RIP1-dependent necrosis is involved in the pathological manifestation of acetaminophen-induced hepatotoxicity. Moreover, RIP1 inhibition conferred resistance to oxidative stress in hepatocytes and may be beneficial for acetaminophen-induced fulminant hepatic failure. Notably, in our current study, intestinal I/R triggered an upregulation of RIP1/3 and p-MLKL expressions in remote liver injury, especially after 6 hours of reperfusion. Moreover, RIP1/3-positive hepatocytes were located around the central vein and portal triad area. As expected, Nec-1 pretreatment suppressed the RIPK pathway expression in hepatocyte after intestinal I/R stimulation and significantly improved remote hepatocellular function. We previously described that Nec-1 administration before intestinal ischemia significantly protects against intestinal mucosal injury after 6 hours and 24 hours reperfusion. Although administration of Nec-1 given as a pretreatment was protective in our model, strategies that use this compound after the onset of injury would be clinically relevant. As with pretreatment, the delayed administration of Nec-1 at the onset of reperfusion also conferred significant protection against intestinal ischemic-associated hepatic injury, which reflect a delayed induction of hepatocyte necroptosis in this context. Taken together, it is plausible that the beneficial effects of Nec-1 treatment on reducing hepatocyte necroptosis is more likely depends on the pro-inflammatory cytokine challenge alleviation and intestinal damage amelioration. However,


treatment with Nec-1 alone showed no significant effect on reducing the number of TUNEL-positive apoptotic hepatocytes, as well as caspase 3 cleavage. Accordingly, it could be speculated that apoptosis may become a predominant form of hepatocytes after intestinal I/R when the necroptotic pathway is inhibited.

Necroptosis causes the release of DAMPs (including HMGB1, heat-shock proteins, and IL-33) and activates both innate and adaptive immunity, further promoting many of the harmful immunologic responses observed in organ I/R injury. Necroptosis inhibitor Nec-1 decreases concanavalin-A-induced acute hepatitis, which is mainly characterized by hepatocyte necrotic cell death resulting in LDH and HMGB1 release. Similarly, our current study revealed that necroptotic hepatocyte induced HMGB1 translocation from the nucleus to cytoplasm was inhibited by Nec-1. Once in the extracellular milieu, HMGB1 triggers cellular and biological inflammatory responses via interacting with multiple cell surface receptors, including RAGE, and TLR2, TLR4, and TLR9. As expected, blocking HMGB1 with a neutralizing antibody, as well as the HMGBl inhibitor EP, significantly attenuated liver histopathological damage, tissue inflammatory cytokine levels, and hepatocyte necroptosis accompanied with the intestinal mucosal injury improvement. Therefore, the prominent effects of therapeutic HMGB1 neutralization/inhibition in our study may be explained by effects on both inflammation and necroptotic cell death. HMGB1 neutralizing antibody and EP also inhibited the expression of TLR4 and RAGE. HMGB1 binds to RAGE soon after injury, thereby activating pro-inflammatory pathways and exacerbating myocardial injury in a mouse heart I/R injury model. Further evidence indicates that TLR4 ligands are increased during liver I/R and that TLR4 activation can promote liver injury. The present results implied that the HMGB1/TLR4/RAGE axis may help to regulate remote hepatic injury after intestinal I/R.

Macrophages can quickly respond to endogenous stimulating factors after tissue injury. KCs are resident macrophages and phagocytes serving as sentinels for liver homeostasis and inflammatory disease. Activated KCs are usually defined as classically and alternatively activated macrophages (M1/M2), and their population may be critical for protection or promotion of liver injury and diseases. Functions of activated KCs, however, are still controversial. Some research has claimed that KCs play a pathogenic role through their secretion of pro-inflammatory factors, such as TNF-α, IL-1β, and NO. In contrast, other studies have demonstrated that KCs can exert protective effects through the secretion of regulatory factors (such as IL-10), or orchestrate with nonparenchymal cells in the liver (including monocytes, neutrophils, and natural killer T cells) for a counteraction against pathogens. Previous study showed that in a mouse liver I/R model, hyperglycemia-induced

**FIGURE 10** The proposed mechanisms of necroptosis induced by HMGB1 in remote liver injury after intestinal I/R. In the current rat model, HMGB1 was first released from necroptotic enterocytes after intestinal I/R challenge. Intestinal barrier disruption and leakage, as a result, facilitates the HMGB1 release and *E. coli* translocation from the lumen to the portal circulation, which further triggers inflammatory pathways. HMGB1/TLR4/RAGE signaling activation promotes hepatic Kupffer cell M1 polarization and hepatocyte necroptotic cell death, thereby contributes to acute liver injury. Nec-1 administration alleviates hepatocyte necroptosis via inhibiting the RIP1/3 signaling pathway, MLKL phosphorylation, and reducing the HMGB1 cytoplasm translocation. Meanwhile, macrophage depletion by liposomal clodronate ameliorates the liver injury induced by intestinal ischemia, which demonstrated that Kupffer cells are responsible for liver damage. Inhibition of HMGB1 with EP and blockade of HMGB1 with anti-HMGB1 neutralizing antibody significantly suppressed hepatocyte necroptosis, tissue inflammation, hepatic Kupffer cell M1 polarization and liver function was partially restored. What’s more, HMGB1 neutralization treatment further protects against intestinal ischemia-associated liver damage in antibiotic induced microbiota-depleted rats.
CHOP over-activation inhibited M2 KCs polarization, leading to excessive intrahepatic inflammation and exacerbation of liver I/R injury. Another research also found that spermine-mediated autophagy inhibits M1 polarization but promoting M2 polarization of KCs leading to attenuated thioacetamide-induced liver injury.

Previous researches already demonstrated that systemic depletion of macrophages prevents hippocampal neuroinflammation and memory dysfunction after experimental tibial fracture, improves renal I/R injury, and decreases inflammatory mediators in endotoxin-treated rats. Similarly, we found that liposomal clodronate led to effective depletion of the mature macrophage population in the liver and spleen, as a consequence, presented better-preserved liver architecture with lower injury scores and reduced serum ALT/AST levels after intestinal I/R. These findings demonstrated that KCs are responsible for liver damage after intestinal ischemia challenge.

Moreover, we further performed the dual presence of CD45 (leukocytes common antigen) and CD68 (pan macrophage marker) in peripheral blood, which was followed by surface staining of CD86 (M1 marker) and CD163 (M2 marker). We identified an M1-dominant macrophage phenotype after intestinal I/R, while an increased M2 phenotype were observed after HMGB1 neutralization treatment. Likewise, our results found that the number of infiltrating KCs (CD68+ cells) was significantly increased in remote liver injury after intestinal I/R challenge, and most were M1-type macrophages (CD68+/iNOS+ cells). Furthermore, the M1 gene signatures (iNOS and IL-1β) were markedly upregulated markedly in injured livers. However, HMGB1 neutralization significantly attenuated KC polarization toward the M1 phenotype but increased the number of M2-type macrophages (CD68+/Arginase-1+ cells), as well as M2 gene signatures (Arginase-1 and IL-10). Since the inflammation driven by M1 macrophages is counterbalanced by the anti-inflammatory M2-polarized macrophages, which can promote inflammation resolution and tissue repair, HMGB1 neutralizing antibody administration after intestinal ischemia could have inhibited M1, but the promotion of M2 macrophage polarization in circulation and hepatic might contribute to protection against remote hepatic injury in this model.

Our current study has some limitations. First, it appears that the intestinal damage is by far more severe than the liver injury in the acute time-frame, which can be reasoned that we just observed the gut-liver axis effects in nondiseased livers of healthy animals but not in diseased or aged animals and tried to provide evidences of the time window and therapeutic potential for distant organ protection strategies after severe intestinal I/R injury. Systemic diseases such as acute hepatitis and liver cirrhosis markedly interfere with gut-liver crosstalk and require further investigation. Second, multiple doses of agents should be evaluated to confirm the optimal dose and therapeutic window for maximal protection against remote liver injury. Moreover, it would be more clinically relevant if the neutralization strategies would have similar effects of hepatoprotection in other inflammatory disease models. Third, we did not determine the redox states of circulating HMGB1. This information is critical for a better understanding of the role of HMGB1 signaling after intestinal I/R as posttranslational modifications regulate receptor usage and functionality.

Fourth, the mechanisms of macrophage reprogramming toward M2-like phenotype after HMGB1 neutralization still warrants further investigation.

In summary, our data highlight the functional importance of the HMGB1 signaling pathway in the mediation of intestinal I/R-provoked remote liver injury. Furthermore, we provide evidence that necroptosis plays an important role in hepatocyte cell death after intestinal I/R challenge, and that neutralization/inhibition of HMGB1 exerts potent hepatoprotective effects in vivo (Figure 10). Hence, necroptosis inhibition and neutralization/inhibition of HMGB1 can serve as effective targeted therapies to attenuate the intestinal I/R-induced acute liver dysfunction, and might be appropriate for a more global clinical application, such as small bowel transplantation and sepsis.

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CONFLICT OF INTEREST
The authors of this manuscript have no conflicts of interest to disclose as described by The FASEB Journal.

AUTHOR CONTRIBUTION
S. Wen, Z. Ke, and W. Huang designed the research; S. Wen, X. Li, Y. Ling, L. Yang, J. Shen, and H. Lai performed the research; Y. Li contributed to the bacteriological analysis of colonic faces; S. Chen and Q. Deng contributed new reagents or analytic tools; Y. Qiu, Y. Zhan and X. Zhang analyzed the data; S. Wen and X. Liang wrote the paper; and Z. Ke and W. Huang performed critical revisions of the manuscript.

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SUPPORTING INFORMATION
Additional supporting information may be found online in the Supporting Information section.

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