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S100A9 aggravates hepatic ischemia–reperfusion injury associated with hepatocyte CCL2 expression and macrophage recruitment

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Abstract

Hepatic ischemia-reperfusion injury (HIRI) is characterized by sterile inflammation and innate immune activation, yet the mechanisms linking early inflammatory signals to subsequent macrophage recruitment remain incompletely understood. Here, integrative transcriptomic and proteomic profiling identified S100 calcium-binding protein A9 (S100A9) as a prominent HIRI-associated inflammatory mediator. Pharmacological neutralization of S100A9 and genetic loss of *S100a9* attenuated liver injury, proinflammatory cytokine expression, and apoptosis in a mouse model of 70% partial warm HIRI. In AML12 cells subjected to oxygen-glucose deprivation/reoxygenation, recombinant S100A9 further increased apoptosis, and bulk RNA sequencing identified *Ccl2*, encoding C-C motif chemokine ligand 2 (CCL2), as a major S100A9-responsive transcript. Consistently, *S100a9* deficiency reduced hepatic CCL2 expression and the hepatic accumulation of macrophage-like cells after HIRI, whereas conditioned medium from S100A9-treated injured AML12 cells enhanced RAW264.7 macrophage migration. Serum S100A9 concentrations were significantly higher after partial hepatectomy than before surgery in paired patient samples. Together, these findings identify S100A9 as a functional inflammatory mediator in HIRI and support a potential S100A9–

hepatocyte CCL2–macrophage recruitment axis. S100A9 may therefore represent a therapeutic target for ischemia-reperfusion-associated liver injury.

Keywords: Hepatic ischemia-reperfusion injury; S100A9; CCL2; sterile inflammation; hepatocytes; macrophage recruitment.

1. Introduction

Hepatic ischemia-reperfusion injury (HIRI) is a major complication of liver resection and transplantation and contributes to postoperative hepatocellular dysfunction, early allograft dysfunction, and graft failure[1-4]. HIRI is initiated by metabolic and mitochondrial disturbances during ischemia and is markedly amplified upon reperfusion, when oxidative stress, hepatocellular injury, damage-associated molecular pattern release, and innate immune activation collectively drive sterile inflammation[5-8]. Although numerous inflammatory mediators have been implicated in this process, the molecular signals that couple injured parenchymal cells to infiltrating immune populations and sustain inflammatory amplification remain incompletely understood.

Neutrophils and monocyte/macrophage populations are major innate immune effectors during HIRI. Neutrophils accumulate rapidly in the postischemic liver and promote tissue injury through the release of reactive oxygen species, granule proteases, neutrophil extracellular traps, and other inflammatory mediators[9-11]. Recruited monocytes and macrophage

populations further shape the hepatic inflammatory microenvironment and contribute to sustained tissue injury[12,13]. However, how early neutrophil-rich inflammatory responses are coupled to hepatocyte-derived chemokine signals and subsequent macrophage accumulation remains insufficiently defined. Identifying upstream mediators that connect these cellular responses may therefore provide new insight into the mechanisms underlying inflammatory amplification during HIRI.

S100 calcium-binding protein A9 (S100A9), also known as myeloid-related protein 14, is an EF-hand calcium-binding protein of the S100 family and commonly associates with S100A8 to form the heterodimeric complex calprotectin[14]. S100A9 is highly enriched in neutrophils and monocytes and, after extracellular release, can function as an alarmin with damage-associated molecular pattern-like activity, activating pattern-recognition receptor signaling and amplifying inflammatory responses[14-16]. Experimental studies have implicated S100A9 in diabetes-associated nonalcoholic fatty liver disease and sepsis-associated acute liver injury, whereas elevated circulating S100A9 has been associated with disease severity and adverse outcomes in hepatitis B virus-related acute-on-chronic liver failure[17-19]. More directly relevant to hepatic ischemia-reperfusion injury, recent studies showed that *S100a9* deficiency or knockdown attenuated inflammatory injury in steatotic liver ischemia-reperfusion models, with effects involving macrophage polarization and

inflammasome activation[20,21]. These findings establish S100A9 as an important inflammatory mediator in liver injury but have largely focused on immune cells as its downstream effectors. Whether extracellular S100A9 directly alters the inflammatory response of stressed hepatocytes and thereby contributes to subsequent immune-cell recruitment during non-steatotic HIRI remains insufficiently understood.

C-C motif chemokine ligand 2 (CCL2), also known as monocyte chemoattractant protein-1, is a major chemokine involved in the mobilization and tissue recruitment of monocytes expressing C-C motif chemokine receptor 2 (CCR2) [22,23]. Recruited CCR2-expressing monocytes can subsequently differentiate into monocyte-derived macrophage populations within injured tissues. In experimental liver injury, CCL2–CCR2 signaling is an important regulator of inflammatory monocyte and monocyte-derived macrophage accumulation[24]. Consistent with this role, genetic disruption of *Ccl2* or *Ccr2* and pharmacological inhibition of CCR2 have been reported to reduce inflammatory monocyte recruitment and attenuate HIRI[22]. Importantly, hepatocytes are not merely passive targets of inflammatory injury. Under stress, they can acquire an immunoregulatory phenotype and produce cytokines and chemokines that influence the local immune microenvironment. Hepatocyte-derived CCL2 has been implicated in macrophage recruitment in experimental endotoxemia and

steatohepatitis[25,26]. These observations raise the possibility that extracellular inflammatory mediators generated during HIRI may enhance hepatocyte chemokine responses and thereby contribute to hepatocyte–myeloid cell communication.

In the present study, we investigated whether S100A9 functions as an upstream inflammatory mediator linking hepatocyte injury to chemokine responses and macrophage recruitment during HIRI. We first used integrative transcriptomic and proteomic profiling to identify HIRI-associated inflammatory candidates and then evaluated the functional contribution of S100A9 using pharmacological neutralization and *S100a9* deficiency. Downstream responses of stressed hepatocytes to extracellular S100A9 were characterized using an oxygen-glucose deprivation/reoxygenation model and bulk RNA sequencing, followed by assessment of macrophage migration and hepatic macrophage-like cell accumulation. Paired perioperative serum samples from patients undergoing partial hepatectomy were additionally examined to assess the clinical relevance of S100A9 induction. This integrated approach was designed to define the pathogenic role of S100A9 in HIRI and to determine whether S100A9-responsive hepatocyte chemokine signaling is associated with macrophage recruitment during sterile hepatic inflammation.

2. Methods

2.1 Ethics approval

The clinical study was approved by the Ethics Committee of the Third Xiangya Hospital of Central South University (Approval No. 22181). Written informed consent was obtained from all participants before sample collection. All procedures involving human participants were conducted in accordance with the ethical principles of the Declaration of Helsinki.

The animal experimental protocol was approved by the Animal Ethics Committee of Central South University (Approval No. CSU-2024-0056). All animal procedures were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and relevant institutional guidelines.

2.2 Animals

Eight-week-old male specific pathogen-free (SPF) C57BL/6 mice were used in this study. Global *S100a9* knockout (KO) mice on a C57BL/6 background were generated by Cyagen Biosciences Inc. through homologous recombination-mediated disruption of the *S100a9* gene. Age- and sex-matched wild-type C57BL/6 mice were used as controls. All mice were housed under SPF conditions with free access to food and water and maintained on a 12-h light/dark cycle in a temperature- and humidity-controlled environment at 18–21°C and 55 ± 5% relative humidity. Mice were allowed to acclimatize for 1 week before experimentation.

2.3 Mouse model of 70% partial warm HIRI

Mice were subjected to a 70% partial warm HIRI model as previously

described[27]. Briefly, mice were anesthetized by intraperitoneal injection of pentobarbital sodium at a dose of 50 mg/kg using a 0.6% solution. After a midline abdominal incision, an atraumatic microvascular clip was applied to occlude the portal venous and hepatic arterial inflow to the cephalad liver lobes, resulting in approximately 70% hepatic ischemia without mesenteric congestion. After 60 min of partial warm ischemia, the clip was removed to initiate hepatic reperfusion. Successful reperfusion was confirmed by restoration of the color of the ischemic liver lobes. Mice were euthanized 4 h after reperfusion, and serum and liver tissue samples were collected for subsequent analyses.

Sham-operated mice underwent the same anesthesia, laparotomy, and hepatic hilar dissection without vascular occlusion. WT-HIRI and *S100a9* KO-HIRI mice underwent the same ischemia and reperfusion procedures.

2.4 Pharmacological neutralization of S100A9

An anti-S100A9 neutralizing antibody and the corresponding isotype control IgG were obtained from Beijing Protein Innovation (Beijing, China). With modifications to previously reported in vivo S100A9-neutralization protocols[28,29], mice were randomly assigned to two groups: the control group (CTL), which received isotype control IgG, and the anti-S100A9 group, which received the anti-S100A9 neutralizing antibody. Antibodies were administered by retro-orbital intravenous injection, an established route for systemic administration in mice[30], at

a dose of 100 µg per mouse per injection every other day for 7 days. The final dose was administered 24 h before induction of HIRI. After completion of the antibody treatment regimen, mice in both groups were subjected to 60 min of partial warm hepatic ischemia followed by 4 h of reperfusion, as described above. Serum and liver tissue samples were subsequently collected for biochemical, histological, and molecular analyses.

2.5 Measurement of serum alanine aminotransferase activity

Serum alanine aminotransferase (ALT) activity was measured as an indicators of hepatocellular injury using a commercial assay kit (Cat. No. C009-2-1; Nanjing Jiancheng Bioengineering Institute, Nanjing, China) according to the manufacturer's instructions.

2.6 Hematoxylin and eosin (H&E) Staining

Liver tissues were fixed in 10% neutral-buffered formalin at 4°C for 48 h, embedded in paraffin, and sectioned at a thickness of 4 µm. Paraffin-embedded sections were deparaffinized in xylene and rehydrated through a graded ethanol series to deionized water. The sections were then stained with hematoxylin and eosin, dehydrated through a graded ethanol series, cleared in xylene, and mounted with Cytoseal mounting medium (Cat. No. 8310; Thermo Scientific, USA). Histopathological changes were observed under a light microscope.

2.7 Immunofluorescence staining

199 Paraffin-embedded liver sections were deparaffinized in xylene and
200 rehydrated through a graded ethanol series to deionized water. After
201 washing with phosphate-buffered saline (PBS), antigen retrieval was
202 performed by heating the sections in 10 mM Tris-EDTA buffer (pH 9.0) at
203 approximately 100°C for 20 min. After cooling to room temperature, the
204 sections were washed with PBS and blocked for 60 min at room
205 temperature using the blocking buffer provided in the Tyramide
206 SuperBoost™ kit with Alexa Fluor™ 488 or Alexa Fluor™ 555 tyramide
207 (Cat. No. B40932 or B40933; Invitrogen, USA).

208 For immunofluorescence staining, sections were incubated overnight at
209 4°C with the following primary antibodies diluted in 5% donkey serum
210 containing 0.3% Triton X-100: anti-S100A9 (Cat. No. 26992-1-AP;
211 Proteintech; 1:100), rat anti-Ly6G (Cat. No. 88876; Cell Signaling
212 Technology; 1:100), anti-MPO (Cat. No. ab208670; Abcam; 1:100), anti-
213 CCL2 (Cat. No. 26161-1-AP; Proteintech; 1:100), anti-CCR2 (Cat. No.
214 16153-1-AP; Proteintech; 1:200), and anti-CD68 (Cat. No. ab125212;
215 Abcam; 1:100). After washing three times with PBS, sections were
216 incubated with species-appropriate horseradish peroxidase-conjugated
217 secondary antibodies for 60 min at room temperature. Specifically, Ly6G
218 staining was detected using an HRP-conjugated anti-rat IgG secondary
219 antibody, whereas the other primary antibodies were detected using
220 species-appropriate HRP-conjugated secondary antibodies. Fluorescence

signals were then developed using Alexa Fluor™ 488 or Alexa Fluor™ 555 tyramide working solution according to the manufacturer's instructions. The reaction was stopped using the reaction stop reagent, and the sections were washed with PBS.

For sequential double immunofluorescence staining, including S100A9/Ly6G, S100A9/MPO, and CD68/CCR2 staining, the first round of staining was completed as described above. The sections were then subjected to heat-mediated antibody stripping in citrate buffer (pH 6.0) before incubation with the second primary antibody overnight at 4°C. The second signal was developed using a different Alexa Fluor™ tyramide fluorophore. For S100A9/Ly6G co-staining, the rat anti-Ly6G primary antibody was detected using HRP-conjugated goat anti-rat IgG (H+L), preadsorbed (Cat. No. E-AB-1195), followed by signal development with Alexa Fluor™ 488 tyramide working solution according to the manufacturer's instructions. Nuclei were counterstained with mounting medium containing 4',6-diamidino-2-phenylindole (DAPI; Cat. No. S2110; Solarbio, Beijing, China). Fluorescence images were acquired using a fluorescence microscope.

2.8 Image acquisition and quantification

Fluorescence images were acquired using a Zeiss Axio Observer microscope under identical imaging settings within each staining experiment. For each section, at least five non-overlapping fields were

captured at 100× magnification. Image quantification was performed on the original fluorescence images using ZEN software (Zeiss, Germany). For single-marker staining, S100A9-positive and CCL2-positive cells were counted and expressed as the number of positive cells per high-power field. For double immunofluorescence staining, Ly6G⁺S100A9⁺, MPO⁺S100A9⁺, and CCR2⁺CD68⁺ cells were counted and expressed as the number of double-positive cells per high-power field (HPF). Representative images were exported for figure preparation, and brightness and contrast were adjusted uniformly when necessary without altering the original data interpretation.

2.9 The integrative genomic analysis of transcriptome and proteome

Transcriptome profiling

Total RNA was extracted from fresh mice liver tissue by using the TRIzol reagent (Invitrogen, CA, USA) according to the manufacturer's protocol. RNA purity and quantification were evaluated using the NanoDrop 2000 spectrophotometer (Thermo Scientific, USA). RNA integrity was assessed using the Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). Then the libraries were constructed using VAHTS Universal V6 RNA-seq Library Prep Kit according to the manufacturer's instructions. The transcriptome sequencing and analysis were conducted by OE Biotech Co., Ltd. (Shanghai, China).

Proteome profiling

Total protein was extracted from fresh mice liver tissue. The total protein concentrations were quantified by bicinchoninic acid assay. According to the measured protein concentration, we took the same quantity protein from each sample, and diluted different groups of samples to the same concentration and volume. The samples were labeled with iTRAQ labeling reagents (4381663, ABSCIEX) and then subjected to Liquid Chromatography and tandem MS analysis. Protein analysis and relative iTRAQ quantification services were undertaken by Oebiotech (Shanghai, China).

The integrative genomic analysis of transcriptome and proteome

All expressed genes with quantifiable proteins were matched, and the relationship between genes and proteins were exhibited by quadrant diagram and venn diagram. Based on the differentially expressed genes (DEGs) and proteins (DEPs) expression data, pearson/spearman/kendall correlation coefficients were calculated by R, then histograms of the correlation coefficient distributions and cluster analysis heat map were drawn.

2.10 Reverse transcription quantitative PCR(RT-qPCR)

Total RNA was extracted from liver tissues or AML12 cells using an RNA extraction kit (Cat. No. AG21023; Accurate Biology, China) according to the manufacturer's instructions. cDNA was synthesized from the extracted RNA using the Evo M-MLV RT Kit (Cat. No. AG11728; Accurate Biology,

China). RT-qPCR was performed using SYBR Green Master Mix (Cat. No. RK21203; ABclonal Technology, China) on a Roche LightCycler 480 II system. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the internal reference gene. Relative mRNA expression levels were calculated using the $2^{-\Delta\Delta C_t}$ method. The primer sequences are listed in Supplementary Table S1.

2.11 Western blot analysis

Liver tissues were homogenized in radioimmunoprecipitation assay (RIPA) lysis buffer (Cat. No. MA0151; Meilunbio, Wuhan, China) supplemented with protease inhibitor cocktail, phosphatase inhibitor cocktail (Roche, Switzerland), and 1 mM phenylmethylsulfonyl fluoride (PMSF) using a MagNA Lyser homogenizer (Servicebio, Wuhan, China). All procedures were performed on ice or at 4°C. Tissue lysates were centrifuged, and the supernatants were collected for protein analysis. Protein concentrations were determined using a bicinchoninic acid (BCA) protein assay kit according to the manufacturer's instructions. Equal amounts of protein were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto polyvinylidene fluoride membranes (Millipore, CA, USA). After blocking with 5% bovine serum albumin for 1 h at room temperature, the membranes were incubated with primary antibodies overnight at 4°C, followed by incubation with appropriate secondary antibodies for 1 h at room temperature. Protein bands were

visualized using an enhanced chemiluminescence kit (Biosharp, Anhui, China), and images were captured using the VisionWorks system (Analytik Jena AG, Germany).

The following primary antibodies were used: anti- β -actin (Cat. No. R380624; ZEN BIO, China; 1:5000), anti-S100A9 (Cat. No. R25648; ZEN BIO, China; 1:1000), and anti-cleaved caspase-3 (Cat. No. 341034; ZEN BIO, China; 1:1000).

2.12 AML12 cell culture and treatment

Mouse hepatocyte AML12 cells were purchased from Procell Life Science & Technology Co., Ltd. (CL-0602; Wuhan, China). Cells were cultured in AML12 complete medium (CM-0602; Procell, Wuhan, China) at 37°C in a humidified atmosphere containing 5% CO₂. For experiments, AML12 cells were seeded into 6-well plates and subjected to OGD/R treatment when they reached approximately 60–70% confluence.

Recombinant S100A9 protein was obtained from Beijing Protein Innovation and dissolved in PBS to prepare a stock solution at 0.5 mg/mL.

To establish an in vitro ischemia-reperfusion-like model, AML12 cells were subjected to oxygen-glucose deprivation/reoxygenation (OGD/R).

Briefly, the culture medium was replaced with glucose-free DMEM/F12 medium (PM150322; Procell, Wuhan, China) supplemented with 1% penicillin/streptomycin, and cells were placed in an anaerobic environment generated using an AnaeroPack system (C01; Mitsubishi Gas Chemical,

Japan) for 6 h to induce OGD. After OGD, cells were returned to normoxic culture conditions with complete medium for 6 h of reoxygenation.

During the reoxygenation phase, AML12 cells were treated with recombinant S100A9 at final concentrations of 0, 1, 2, and 5 µg/mL. The dose-response experiment was used to evaluate the effect of recombinant S100A9 on OGD/R-induced AML12 cell apoptosis. For bulk RNA sequencing, RT-qPCR validation, and conditioned medium collection, recombinant S100A9 was used at 5 µg/mL during reoxygenation unless otherwise indicated.

2.13 Flow cytometry analysis of apoptosis

AML12 cells were harvested using EDTA-free trypsin and washed three times with cold PBS. The cells were resuspended in binding buffer and stained with 5 µL Annexin V conjugated to fluorescein isothiocyanate (Annexin V-FITC) and 10 µL propidium iodide (PI; Meilunbio, China) according to the manufacturer's instructions. After incubation for 15 min at room temperature in the dark, the cells were analyzed using a BD FACSCanto II flow cytometer. The percentages of cells in each quadrant were quantified using FlowJo v10.4 software (Tree Star, Ashland, OR, USA). Total apoptotic cells were defined as the sum of early apoptotic cells (Annexin V⁺/PI⁻) and late apoptotic cells (Annexin V⁺/PI⁺). The apoptotic rate was expressed as the percentage of total apoptotic cells among all analyzed cells.

2.14 Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) staining

TUNEL staining of paraffin-embedded liver sections was performed using the TransDetect In Situ Fluorescein TUNEL Apoptosis Detection Kit (Cat. No. FA301; TransGen Biotech, Beijing, China) according to the manufacturer's instructions. Briefly, paraffin-embedded sections were deparaffinized in xylene and rehydrated through a graded ethanol series to deionized water, followed by washing with PBS. The sections were then incubated with the TUNEL reaction mixture for 1 h at 37°C in the dark. Nuclei were counterstained with Hoechst staining solution provided in the kit. Fluorescence images were acquired using a Zeiss Axio Observer microscope. The percentage of TUNEL-positive cells was calculated as the number of TUNEL-positive nuclei divided by the total number of Hoechst-positive nuclei.

2.15 Enzyme-linked immunosorbent assay (ELISA)

Serum S100A9 levels in patients undergoing partial hepatectomy were measured using a commercially available human S100A9 ELISA kit (Cat. No. JL19159-96T; Jianglai Biotechnology, Shanghai, China) according to the manufacturer's instructions. All serum samples were assayed in duplicate, and the mean value of duplicate measurements was used for subsequent analysis.

2.16 Statistical analysis

Data are presented as mean $\pm$ standard error of the mean (SEM). Data distribution was assessed before statistical testing. For normally distributed data, comparisons between two independent groups were performed using an unpaired Student's *t*-test, whereas paired preoperative and postoperative serum S100A9 levels were compared using a paired Student's *t*-test. Comparisons among multiple groups were performed using one-way analysis of variance (ANOVA), followed by Tukey's multiple comparisons test when appropriate. A two-sided *P* value < 0.05 was considered statistically significant. Statistical analyses were performed using GraphPad Prism software.

3. Results

3.1 Integrative transcriptomic and proteomic analysis identifies S100A9 as a candidate HIRI-associated inflammatory mediator

Mice were subjected to 70% partial hepatic ischemia followed by reperfusion to establish a mouse model of HIRI. Compared with the sham group, mice in the HIRI group exhibited marked histopathological liver injury, characterized by hepatocyte swelling, sinusoidal congestion, and inflammatory cell infiltration on H&E-stained liver sections (Figure 1A). Consistently, Suzuki's injury scores were significantly higher in the HIRI group than in the sham group (Figure 1B). Serum ALT levels were also significantly increased after HIRI (Figure 1C). In parallel, the expression levels of proinflammatory mediators, including *IL-1 β* , *IL-6*, *TNF- α* and

iNOS were markedly elevated in the HIRI group compared with the sham group (Figure 1D). These findings confirmed the successful establishment of liver injury and inflammatory activation after HIRI.

To explore the molecular alterations associated with HIRI, we next performed transcriptomic and proteomic profiling of liver tissues from the sham and HIRI groups. Integrative analysis of the transcriptome and proteome was then conducted to identify candidate molecules potentially involved in HIRI progression. A total of 1,479 differentially expressed genes (DEGs; $P < 0.05$ and $|\log_2 \text{fold change}| \geq 1.0$) and 489 differentially expressed proteins (DEPs; $P < 0.05$ and $|\log_2 \text{fold change}| \geq 0.5$) were identified between the sham and HIRI groups. Hierarchical clustering analysis showed distinct transcriptomic and proteomic expression patterns between the two groups (Figure 1E). Spearman correlation analysis further revealed a positive correlation between transcriptomic and proteomic changes, with 42.8% of matched mRNA-protein pairs showing concordant regulation (Figure 1F). The distribution of corresponding mRNA and protein changes was visualized using a scatterplot based on log₂-transformed expression ratios, in which red and green dots represented concordantly upregulated and downregulated mRNA-protein pairs, respectively (Figure 1G).

Further integration of the transcriptomic and proteomic datasets identified 54 molecules that were consistently upregulated at both the mRNA and

protein levels and 51 molecules that were consistently downregulated (Figure 1H). The top 10 concordantly upregulated mRNA-protein pairs are summarized in Supplementary Table S2. Among these candidates, *S100a9*/S100A9 showed the most prominent upregulation at both the transcript and protein levels, suggesting that S100A9 may represent a prominent HIRI-associated inflammatory candidate.

3.2 HIRI-induced S100A9 upregulation is spatially associated with neutrophil infiltration

To validate the multi-omics findings, we first examined S100A9 expression in liver tissues after HIRI. Immunofluorescence staining showed that hepatic S100A9-positive cells were markedly increased in the HIRI group compared with the sham group (Figure 2A and 2B). Consistently, RT-qPCR analysis showed a significant increase in hepatic *S100a9* mRNA expression after HIRI (Figure 2C). Western blotting further confirmed that S100A9 protein expression was markedly elevated in ischemia-reperfused liver tissues compared with sham liver tissues (Figure 2D and 2E). These results confirmed robust S100A9 upregulation after HIRI at both the mRNA and protein levels.

Given that S100A9 is a myeloid-enriched inflammatory mediator closely linked to neutrophil-associated inflammation, we next examined whether S100A9 upregulation occurred in parallel with neutrophil infiltration. Double immunofluorescence staining showed increased Ly6G-positive

cells in ischemia-reperfused liver tissues compared with sham tissues, indicating enhanced neutrophil infiltration after HIRI (Figure 2F and 2H). Notably, the number of Ly6G⁺S100A9⁺ double-positive cells was markedly increased after HIRI (Figure 2F and 2I). Similarly, MPO immunofluorescence staining showed increased MPO-positive cells after HIRI (Figure 2G and 2J), and MPO⁺S100A9⁺ double-positive cells were also significantly increased in ischemia-reperfused liver tissues (Figure 2G and 2K). These findings demonstrate a close spatial association between HIRI-induced S100A9 upregulation and neutrophil infiltration and suggest that infiltrating neutrophils may contribute to the increased hepatic S100A9 signal during HIRI.

3.3 Pharmacological blockade of S100A9 attenuates liver injury and inflammatory responses during HIRI

Having identified S100A9 as a neutrophil-associated candidate mediator upregulated after HIRI, we next investigated whether S100A9 functionally contributes to hepatic injury. To this end, an anti-S100A9 neutralizing antibody was administered in the mouse HIRI model, and the treatment scheme is shown in Figure 3A. Compared with isotype control IgG-treated HIRI mice, mice receiving the anti-S100A9 neutralizing antibody exhibited markedly alleviated hepatic histopathological injury, as reflected by reduced hepatocyte swelling, sinusoidal congestion, and inflammatory cell infiltration on H&E staining (Figure 3B). Consistently, histological

injury scores were significantly lower in the anti-S100A9-treated group than in the control IgG-treated group (Figure 3C).

In parallel with the histological improvement, pharmacological blockade of S100A9 significantly reduced serum ALT levels after HIRI (Figure 3D), indicating attenuated hepatocellular injury. Moreover, anti-S100A9 antibody treatment markedly decreased the levels of proinflammatory cytokines, including *IL-1 β* , *IL-6*, and *TNF- α* , compared with control IgG treatment (Figure 3E–G). Conversely, the anti-inflammatory cytokine *IL-10* was significantly increased following S100A9 blockade (Figure 3H), suggesting a shift toward a less proinflammatory hepatic microenvironment. Collectively, these results demonstrate that pharmacological neutralization of S100A9 attenuates liver injury and dampens inflammatory responses during HIRI.

3.4 Genetic deficiency of *S100a9* protects against HIRI

To further validate the pathogenic role of S100A9 in HIRI, we subjected *S100a9*-deficient mice and wild-type mice to the same 70% partial warm HIRI procedure and compared the severity of liver injury between the two groups[31]. WT and *S100a9* KO mice were subjected to 60 min of partial hepatic ischemia followed by reperfusion. Immunofluorescence staining, Western blotting, and RT-qPCR confirmed that S100A9 protein and mRNA expression were markedly diminished in liver tissues from *S100a9* KO mice compared with WT mice after HIRI (Figure 4A-D). We next

evaluated whether *S100a9* deficiency affected the severity of HIRI. H&E staining showed that *S100a9* KO mice exhibited markedly attenuated liver injury compared with WT mice after HIRI, as evidenced by reduced hepatocyte swelling, sinusoidal congestion, and inflammatory cell infiltration (Figure 4E). Consistently, Suzuki's injury scores were significantly lower in the *S100a9* KO group than in the WT group (Figure 4F). Serum ALT levels were also significantly lower in *S100a9* KO mice than in WT mice after HIRI (Figure 4G). In addition, the levels of proinflammatory cytokines were markedly reduced in *S100a9* KO mice compared with WT mice (Figure 4H). Together with the neutralizing antibody experiments, these genetic data provide complementary evidence that S100A9 functionally contributes to liver injury and inflammatory responses during HIRI.

3.5 S100A9 contributes to HIRI-associated apoptosis and aggravates OGD/R-induced AML12 cell apoptosis in vitro

Apoptosis is an important pathological component of HIRI and contributes to parenchymal cell loss and subsequent inflammatory amplification[32]. We therefore examined whether S100A9 contributes to apoptosis during HIRI.

TUNEL staining showed that apoptotic cells were markedly increased in liver tissues after HIRI (Figure 5A and 5B). Consistently, Western blot analysis showed elevated cleaved caspase-3 protein expression in the HIRI

group compared with the sham group (Figure 5C and 5D), indicating enhanced apoptotic injury in ischemia-reperfused liver tissues. Notably, compared with WT mice, *S100a9* KO mice exhibited reduced apoptosis after HIRI, as reflected by fewer TUNEL-positive cells and decreased cleaved caspase-3 expression (Figure 5E-H). These findings suggest that *S100a9* deficiency attenuates HIRI-associated apoptosis in vivo.

We next investigated whether S100A9 could aggravate hepatocyte apoptosis under ischemia-reperfusion-like conditions in vitro. AML12 cells were first subjected to oxygen-glucose deprivation (OGD) and then treated with recombinant S100A9 protein at 0, 1, 2, and 5 $\mu\text{g/mL}$ during the reoxygenation phase. Apoptosis was subsequently assessed by flow cytometry. Recombinant S100A9 tended to further increase OGD/R-induced AML12 cell apoptosis in a concentration-related manner; however, a statistically significant increase was observed only at 5 $\mu\text{g/mL}$ compared with the OGD/R group without S100A9 treatment, whereas 1 and 2 $\mu\text{g/mL}$ did not reach statistical significance (Figure 5I and 5J). These in vitro findings suggest that extracellular S100A9 can aggravate hepatocyte apoptosis under OGD/R conditions, particularly at the highest concentration tested.

3.6 S100A9 induces a chemokine-related transcriptional response and *Ccl2* upregulation in injured hepatocytes

To define the transcriptional response of injured hepatocytes to S100A9,

AML12 cells were first subjected to oxygen-glucose deprivation and then treated with recombinant S100A9 protein at 5 µg/mL during the reoxygenation phase, followed by bulk RNA sequencing. Transcriptomic analysis identified a limited set of differentially expressed genes between OGD/R-treated AML12 cells and S100A9-treated OGD/R AML12 cells. Among these genes, *Ccl2* was one of the most prominently upregulated transcripts after S100A9 stimulation (Figure 6A). Heatmap visualization further showed that several chemokine-related genes, including *Ccl2*, *Cxcl1*, and *Cxcl2*, were increased in S100A9-treated cells compared with control cells (Figure 6B).

The RNA-seq finding was further validated by RT-qPCR, which confirmed significantly increased *Ccl2* mRNA expression in AML12 cells following S100A9 treatment during reoxygenation (Figure 6C). Consistently, immunofluorescence staining of ischemia-reperfused liver tissues showed that S100a9-deficient mice had fewer hepatic CCL2-positive cells than WT mice after HIRI (Figure 6D and 6E), supporting the in vivo relevance of S100A9-associated CCL2 induction. Collectively, these findings indicate that S100A9 elicits a chemokine-related transcriptional response in OGD/R-stressed hepatocytes, with *Ccl2* representing a prominent S100A9-responsive transcript.

3.7 S100A9-stimulated hepatocytes enhance macrophage migration during HIRI

Given that S100A9 induced CCL2 expression in injured hepatocytes, we next examined whether S100A9-stimulated hepatocytes could promote macrophage recruitment. AML12 cells were first subjected to oxygen-glucose deprivation and then treated with recombinant S100A9 protein at 5 μ g/mL during the reoxygenation phase. Conditioned medium was subsequently collected and applied to RAW264.7 macrophage Transwell migration assays. Compared with conditioned medium from OGD/R-treated AML12 cells, conditioned medium from S100A9-treated OGD/R AML12 cells markedly enhanced RAW264.7 macrophage migration (Figure 7A and 7B), indicating that S100A9 treatment increases the macrophage-directed chemotactic activity of conditioned medium from OGD/R-stressed hepatocytes.

To further evaluate whether S100A9 was associated with macrophage accumulation in vivo, we performed double immunofluorescence staining for CCR2 and CD68 in ischemia-reperfused liver tissues. Compared with WT mice, S100a9-deficient mice exhibited markedly reduced accumulation of CCR2⁺CD68⁺ macrophage-like cells after HIRI (Figure 7C and 7D). Together with the observed hepatocyte *Ccl2* response, these findings support an association between S100A9-responsive hepatocyte chemokine signaling and macrophage migration/accumulation during HIRI and are consistent with a potential involvement of CCL2–CCR2 signaling.

3.8 Perioperative serum S100A9 levels increase in patients undergoing partial hepatectomy

To explore the clinical relevance of S100A9 in liver surgery, we measured perioperative serum S100A9 levels in patients undergoing partial hepatectomy. A total of 60 patients were enrolled, and paired serum samples were collected before and after surgery for S100A9 detection. Compared with preoperative levels, serum S100A9 levels were significantly increased after partial hepatectomy (Figure 8). These findings are consistent with the experimental observation of S100A9 upregulation after HIRI and suggest that S100A9 may also be involved in human hepatic injury responses associated with liver surgery.

4. Discussion

In this study, integrative transcriptomic and proteomic profiling identified S100A9 as a prominent inflammatory mediator associated with HIRI, and its upregulation was further validated at both the transcript and protein levels. Hepatic S100A9 signals were spatially associated with Ly6G-positive and MPO-positive infiltrating cells after HIRI. Functionally, both pharmacological neutralization of S100A9 and genetic loss of *S100a9* attenuated liver injury and inflammatory responses, supporting a pathogenic role for S100A9 in experimental HIRI. S100A9 also aggravated apoptosis in OGD/R-stressed AML12 cells and induced a chemokine-related transcriptional response characterized by marked *Ccl2* upregulation.

Consistent with these in vitro findings, *S100a9* deficiency reduced hepatic CCL2 expression and the accumulation of CCR2⁺CD68⁺ macrophage-like cells after HIRI, whereas conditioned medium from S100A9-treated injured AML12 cells enhanced RAW264.7 macrophage migration. Furthermore, serum S100A9 concentrations increased significantly after partial hepatectomy in paired patient samples. Collectively, these findings support S100A9 as a functional inflammatory mediator in HIRI and suggest a potential link between S100A9-responsive hepatocyte CCL2 expression and macrophage recruitment.

An important strength of the present study is that S100A9 was prioritized through an integrative transcriptomic and proteomic strategy rather than selected solely on the basis of prior knowledge. Transcriptomic profiling captures broad gene-expression responses to HIRI, whereas proteomic profiling provides complementary information on changes at the protein level. Because mRNA abundance does not necessarily parallel protein abundance owing to post-transcriptional regulation, translational control, and protein turnover[33-35], the concordant upregulation of S100A9 at both levels strengthened its biological relevance as a HIRI-associated candidate. This multi-omics signal was subsequently confirmed by RT-qPCR, immunoblotting, and immunofluorescence and was functionally supported by both anti-S100A9 neutralizing antibody treatment and *S100a9* deficiency. Although previous studies have established S100A9 as

an important inflammatory mediator in liver injury and steatotic liver ischemia-reperfusion settings, our integrative analysis independently identified S100A9 as a prominent inflammatory signal in the present HIRI model. S100A9, either alone or as part of the S100A8/A9 complex, is widely recognized as a myeloid-enriched alarmin capable of amplifying sterile inflammation, promoting leukocyte recruitment, and stimulating cytokine production[36,18]. More directly related to liver I/R injury, recent studies have shown that S100A9 is increased in steatotic livers after I/R insult and that *S100a9* deletion reduces liver inflammatory injury, monocyte/macrophage and neutrophil infiltration, proinflammatory mediator expression, and macrophage M1 polarization, potentially through TLR4/NF- κ B signaling[20]. Another recent study linked S100A9–TLR2 signaling to macrophage NLRP3 inflammasome activation in fatty liver ischemia-reperfusion injury[21]. Thus, the principal contribution of the present study is not simply the demonstration that S100A9 is increased during HIRI, but the integration of multi-omics discovery with pharmacological and genetic functional validation and, importantly, the identification of injured hepatocytes as downstream responders to S100A9. Our findings further suggest that S100A9-responsive hepatocyte CCL2 expression may contribute to the subsequent macrophage recruitment response, thereby extending previous work on the proinflammatory role of S100A9 in hepatic ischemia-reperfusion injury.

S100A9 is highly enriched in neutrophils and monocytes and can be released extracellularly as an inflammatory alarmin during sterile injury [36,37]. This property is particularly relevant to HIRI, in which reperfusion rapidly triggers innate immune activation and leukocyte infiltration, with neutrophils acting as early effector cells that amplify hepatocellular injury through the release of reactive oxygen species, proteases, neutrophil extracellular traps, and other inflammatory mediators[9,10,38]. In the present study, hepatic S100A9 expression was markedly increased after HIRI, and S100A9 signals showed substantial spatial overlap with both Ly6G-positive and MPO-positive infiltrating cells. Ly6G is commonly used to identify infiltrating neutrophils in mouse tissues, whereas MPO is a neutrophil-enriched granule enzyme that provides complementary evidence of neutrophil-associated localization. The concordant increase and spatial overlap of S100A9 with both markers support a close association between HIRI-induced S100A9 upregulation and neutrophil infiltration. Beyond this spatial association, extracellular S100A9 may contribute to inflammatory amplification by acting on injured hepatocytes and enhancing downstream chemokine responses. Nevertheless, because S100A9 is also expressed by monocytes and other myeloid populations, our findings support a neutrophil-associated pattern of S100A9 expression but do not establish neutrophils as its exclusive or predominant cellular source during HIRI.

Apoptotic cell death is a well-recognized pathological component of HIRI and contributes to parenchymal cell loss and subsequent inflammatory amplification[39-41]. In the present study, HIRI was accompanied by increased TUNEL positivity and elevated cleaved caspase-3 expression, whereas *S100a9* deficiency attenuated these apoptotic changes. Consistently, recombinant S100A9 further increased apoptosis in OGD/R-stressed AML12 cells, with the most pronounced effect observed at the highest concentration tested. Because S100A9 was administered during the reoxygenation phase, these in vitro findings suggest that extracellular S100A9 may act as a secondary inflammatory stimulus that exacerbates apoptotic injury in hepatocytes already primed by ischemia-reperfusion-like stress. This interpretation is biologically plausible because reperfusion is characterized by oxidative stress, innate immune activation, and increased exposure of injured parenchymal cells to extracellular inflammatory mediators[39,42]. However, the pathogenic contribution of S100A9 to HIRI is unlikely to be limited to apoptotic injury alone. Stressed or dying hepatocytes can actively shape the local immune microenvironment by releasing damage-associated signals and producing cytokines and chemokines that influence immune-cell recruitment and activation[43,44]. Thus, S100A9-enhanced apoptotic injury may represent one component of a broader inflammatory amplification process in which extracellular S100A9 not only aggravates hepatocellular injury but also

alters the inflammatory phenotype of stressed hepatocytes.

Beyond direct hepatocellular injury, our data suggest that S100A9 may shift stressed hepatocytes toward a chemokine-producing phenotype that favors macrophage recruitment. Although hepatocytes are traditionally regarded as parenchymal targets of ischemia-reperfusion injury, increasing evidence indicates that stressed hepatocytes can actively participate in hepatic immune regulation by producing cytokines and chemokines that shape the local inflammatory microenvironment [45,43,25,26]. In this context, the bulk RNA-seq analysis was particularly informative because it provided transcriptome-wide evidence that *Ccl2* was among the most prominent S100A9-responsive chemokine transcripts in OGD/R-stressed hepatocytes. Importantly, *Ccl2* was not selected solely as a predefined candidate but emerged from an unbiased transcriptomic comparison as one of the most strongly upregulated transcripts following recombinant S100A9 treatment during reoxygenation. This finding is biologically relevant because CCL2, also known as monocyte chemoattractant protein-1, is a major ligand for CCR2 and plays a central role in the recruitment of CCR2-expressing inflammatory monocytes and macrophage populations into injured tissues, including the liver[43,46,47]. Thus, S100A9-responsive *Ccl2* induction provides a plausible chemokine-based link through which injured hepatocytes may influence CCR2-associated myeloid-cell recruitment. The biological relevance of this transcriptomic

705 signal was further supported by RT-qPCR validation in AML12 cells and
706 by the reduced hepatic CCL2 expression observed in *S100a9*-deficient
707 mice after HIRI. Functionally, conditioned medium from S100A9-treated
708 OGD/R-stressed AML12 cells markedly enhanced RAW264.7
709 macrophage migration, whereas *S100a9* deficiency reduced the hepatic
710 accumulation of CCR2⁺CD68⁺ macrophage-like cells after HIRI. Previous
711 experimental studies have further shown that disruption of CCL2–CCR2
712 signaling or inhibition of CCR2-dependent inflammatory monocyte
713 recruitment attenuates HIRI [46,22]. Taken together, these findings support
714 the positioning of hepatocyte *Ccl2*/CCL2 responses downstream of
715 S100A9 and suggest that S100A9 may amplify HIRI not only by
716 exacerbating hepatocellular injury but also by promoting a chemokine-
717 associated macrophage recruitment response. These findings extend
718 previous S100A9-HIRI studies by identifying injured hepatocytes as active
719 downstream responders to S100A9 and as potential intermediates linking
720 early myeloid inflammatory signals to subsequent CCR2-associated
721 macrophage accumulation during HIRI.

722 The paired clinical serum data provide additional translational support for
723 the relevance of S100A9 to human liver injury associated with partial
724 hepatectomy. As an extracellular alarmin released during inflammatory
725 tissue injury, S100A9 has been investigated as a circulating marker of
726 inflammatory activity in several human diseases[36]. In chronic hepatitis

B, circulating S100A9 levels have been reported to correlate with viral load and histological necroinflammatory activity, suggesting that serum S100A9 may reflect ongoing hepatic inflammatory injury[48]. Consistent with its proinflammatory role, experimental studies have further shown that genetic deficiency or pharmacological inhibition of S100A9 attenuates hepatic inflammation and tissue injury in models of sepsis-associated acute liver injury and steatotic liver ischemia-reperfusion injury[18,20]. In the present study, serum S100A9 concentrations were significantly higher after partial hepatectomy than before surgery, paralleling the marked increase in hepatic S100A9 observed in the experimental HIRI model. This postoperative elevation suggests that S100A9 induction is not restricted to experimental HIRI and may also accompany inflammatory liver injury associated with hepatic surgery. However, because partial hepatectomy elicits complex surgical and systemic inflammatory responses, these clinical findings should be interpreted as supportive rather than as evidence that circulating S100A9 specifically reflects human HIRI.

Several limitations should be acknowledged. First, although hepatic S100A9 signals were closely associated with Ly6G-positive and MPO-positive infiltrating cells, the current data do not establish neutrophils as the exclusive or predominant cellular source of S100A9 during HIRI. Second, although recombinant S100A9 increased *Ccl2* expression in stressed hepatocytes, conditioned medium from S100A9-treated

hepatocytes enhanced macrophage migration, and *S100a9* deficiency reduced hepatic CCL2 expression and CCR2⁺CD68⁺ macrophage-like cell accumulation, direct functional interruption of the CCL2–CCR2 axis was not performed. CCL2 neutralization, CCR2 blockade, or hepatocyte-specific *Ccl2* manipulation will therefore be required to establish the causal requirement of this pathway. Finally, the clinical analysis was limited to paired perioperative serum S100A9 measurements in patients undergoing partial hepatectomy. Larger and better-characterized clinical cohorts will be needed to determine whether circulating S100A9 is associated with hepatic ischemic burden, the severity of postoperative liver injury, or clinical outcomes.

5. Conclusions

In summary, this study identifies S100A9 as a functional inflammatory mediator that contributes to experimental HIRI. Integrative multi-omics analysis identified S100A9 as a prominent HIRI-associated inflammatory signal, whereas pharmacological neutralization and genetic deficiency provided functional evidence supporting its pathogenic role in inflammatory liver injury. Additional in vivo and in vitro findings implicate S100A9 in HIRI-associated apoptotic injury. Furthermore, the coordinated changes in S100A9-responsive hepatocyte *Ccl2* expression, hepatic CCL2 abundance, macrophage migration, and hepatic CCR2⁺CD68⁺ macrophage-like cell accumulation support a potential S100A9–

hepatocyte CCL2–macrophage recruitment axis during HIRI. Although the causal requirement of CCL2–CCR2 signaling remains to be established, these findings extend the current understanding of S100A9-mediated inflammatory amplification and identify S100A9 as a candidate therapeutic target for ischemia-reperfusion-associated liver injury.

Abbreviations

ALT

Alanine aminotransferase

ANOVA Analysis of variance

BCA

bicinchoninic acid

CCL2

C-C motif chemokine ligand 2

CCR2

C-C motif chemokine receptor 2

CTL

Control

DAPI

4',6-diamidino-2-phenylindole

DEGs Differentially expressed genes

DEPs

Differentially expressed proteins

793	ECL
794	Enhanced chemiluminescence
795	ELISA
796	Enzyme-linked immunosorbent assay
797	FITC
798	Fluorescein isothiocyanate
799	GAPDH
800	Glyceraldehyde-3-phosphate dehydrogenase
801	H&E
802	Hematoxylin and eosin
803	HIRI
804	Hepatic ischemia-reperfusion injury
805	HPF
806	High-power field
807	KO
808	Knockout
809	MPO
810	Myeloperoxidase
811	OGD/R
812	Oxygen-glucose deprivation/reoxygenation
813	PBS
814	Phosphate-buffered saline

815 **PI**
816 Propidium iodide
817 **PMSF**
818 Phenylmethylsulfonyl fluoride
819 **RIPA**
820 Radioimmunoprecipitation assay
821 **RT-qPCR**
822 Reverse transcription quantitative polymerase chain reaction
823 **S100A9**
824 S100 calcium-binding protein A9
825 **SEM**
826 Standard error of the mean
827 **SPF**
828 Specific pathogen-free
829 **TUNEL**
830 Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling;
831 **WT**
832 Wild-type
833
834 **Statements and Declarations**
835 **Ethics approval**
836 The clinical study was approved by the Ethics Committee of the Third

Xiangya Hospital of Central South University (Approval No. 22181).

Written informed consent was obtained from all participants before sample collection. All procedures involving human participants were conducted in accordance with the ethical principles of the Declaration of Helsinki.

The animal experimental protocol was approved by the Animal Ethics Committee of Central South University (Approval No. CSU-2024-0056).

All animal procedures were performed in accordance with the institutional guidelines for animal care and use.

Consent to participate

Written informed consent was obtained from all participants before sample collection.

Consent to publish

Not applicable.

Data Availability

All data included in this study are available upon request by contacting the corresponding author.

Competing interests

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Author contributions

Shudong Xie and Weitai Yan contributed equally to the study; Shudong Xie, Weitai Yan, Yuhang Wu and Chen Zhou performed experiments and analyzed the data; Shudong Xie and Pengpeng Zhang designed the research and wrote the manuscript; Qifa Ye, Yingzi Ming, Pengpeng Zhang and Ying Niu supervised the whole project; All authors provided critical revisions and took responsibility for the work; All authors have read and approved the final manuscript.

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Footnotes

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References

1. de Rougemont, O., Lehmann, K., Clavien, P.A. 2009. Preconditioning, organ preservation, and postconditioning to prevent ischemia-reperfusion injury to the liver. *Liver Transplantation* 15(10):1172-1182.
2. Boros, P., Bromberg, J. 2006. New cellular and molecular immune pathways in

ischemia/reperfusion injury. *American Journal of Transplantation* 6(4):652-658.

3. Hirao, H., Nakamura, K., Kupiec-Weglinski, J.W. 2021. Liver ischaemia-reperfusion injury: a new understanding of the role of innate immunity. *Nat Rev Gastroenterol Hepatol* 19(4):239-256. 10.1038/s41575-021-00549-8

4. Liu, J., Man, K. 2023. Mechanistic Insight and Clinical Implications of Ischemia/Reperfusion Injury Post Liver Transplantation. *Cell Mol Gastroenterol Hepatol* 15(6):1463-1474. 10.1016/j.jcmgh.2023.03.003

5. Hirao, H., Nakamura, K., Kupiec-Weglinski, J.W. 2022. Liver ischaemia-reperfusion injury: a new understanding of the role of innate immunity. *Nature reviews Gastroenterology & hepatology* 19(4):239-256. 10.1038/s41575-021-00549-8

6. Huang, M., Cai, H., Han, B., Xia, Y., Kong, X., Gu, J. 2022. Natural Killer Cells in Hepatic Ischemia-Reperfusion Injury. *Frontiers in immunology* 13(870038). 10.3389/fimmu.2022.870038

7. van Golen, R.F., Reiniers, M.J., Olthof, P.B., van Gulik, T.M., Heger, M. 2013. Sterile inflammation in hepatic ischemia/reperfusion injury: present concepts and potential therapeutics. *J Gastroenterol Hepatol* 28(3):394-400. 10.1111/jgh.12072

8. George, J., Lu, Y., Tsuchishima, M., Tsutsumi, M. 2024. Cellular and molecular mechanisms of hepatic ischemia-reperfusion injury: The role of oxidative stress and therapeutic approaches. *Redox Biol* 75(103258). 10.1016/j.redox.2024.103258

9. Oliveira, T.H.C.d., Marques, P.E., Proost, P., Teixeira, M.M.M. 2017. Neutrophils: a cornerstone of liver ischemia and reperfusion injury. *Lab Invest* 98(1):51-62. 10.1038/labinvest.2017.90

10. Kaltenmeier, C., Yazdani, H.O., Handu, S., Popp, B., Geller, D., Tohme, S. 2022. The Role of Neutrophils as a Driver in Hepatic Ischemia-Reperfusion Injury and Cancer Growth. *Frontiers In Immunology* 13(887565). 10.3389/fimmu.2022.887565

11. Huang, H., Tohme, S., Al-Khafaji, A.B., Tai, S., Loughran, P., Chen, L., . . . Tsung, A. 2015. Damage-associated molecular pattern-activated neutrophil extracellular trap exacerbates sterile inflammatory liver injury. *Hepatology* 62(2):600-614. 10.1002/hep.27841

12. Ju, C., Tacke, F. 2016. Hepatic macrophages in homeostasis and liver diseases: from pathogenesis to novel therapeutic strategies. *Cell Mol Immunol* 13(3):316-327. 10.1038/cmi.2015.104

13. Ocuin, L.M., Zeng, S., Cavnar, M.J., Sorenson, E.C., Bamboat, Z.M., Greer, J.B., . . . DeMatteo, R.P. 2012. Nilotinib protects the murine liver from ischemia/reperfusion injury. *J Hepatol* 57(4):766-773. 10.1016/j.jhep.2012.05.012

14. Vogl, T., Gharibyan, A.L., Morozova-Roche, L.A. 2012. Pro-inflammatory S100A8 and S100A9 proteins: self-assembly into multifunctional native and amyloid complexes. *Int J Mol Sci* 13(3):2893-2917. 10.3390/ijms13032893

15. Vogl, T., Tenbrock, K., Ludwig, S., Leukert, N., Ehrhardt, C., van Zoelen, M.A.D., . . . Roth, J. 2007. Mrp8 and Mrp14 are endogenous activators of Toll-like receptor 4, promoting lethal, endotoxin-induced shock. *Nat Med* 13(9):1042-1049.

16. Gebhardt, C., Németh, J., Angel, P., Hess, J. 2006. S100A8 and S100A9 in inflammation and cancer. *Biochem Pharmacol* 72(11):1622-1631.

17. Cai, Q., Zhu, J., Cui, X., Xia, Y., Gao, H., Wang, X., Cheng, M. 2022. S100A9 promotes inflammatory response in diabetic nonalcoholic fatty liver disease. *Biochem Biophys Res Commun* 618(127-132). 10.1016/j.bbrc.2022.06.026

18. Zhang, Y., Wu, F., Teng, F., Guo, S., Li, H. 2023. Deficiency of S100A9 Alleviates Sepsis-

927 Induced Acute Liver Injury through Regulating AKT-AMPK-Dependent Mitochondrial Energy
 928 Metabolism. *Int J Mol Sci* 24(3):10.3390/ijms24032112
 929 19. Zhang, Y., Zhang, X., Han, J., Guo, Y., He, J., Yang, F., . . . Zhang, J. 2023. Plasma S100A8 and
 930 S100A9 Are Strong Prognostic Factors for Hepatitis B Virus-Related Acute-on-Chronic Liver
 931 Failure. *Can J Gastroenterol Hepatol* 2023(6164611). 10.1155/2023/6164611
 932 20. Sheng, M., Liu, W., Lu, Z., Lin, Y., Yu, W. 2024. S100A9 regulates M1 macrophage polarization
 933 and exacerbates steatotic liver ischemia-reperfusion injury. *Transpl Immunol* 87(102134).
 934 10.1016/j.trim.2024.102134
 935 21. Sheng, M., Liu, W., Cao, Y., Wang, S., Lin, Y., Yu, W. 2024. TARGETING S100A9-TLR2 AXIS
 936 CONTROLS MACROPHAGE NLRP3 INFLAMMASOME ACTIVATION IN FATTY LIVER ISCHEMIA
 937 REPERFUSION INJURY. *Shock* 63(2):292-298. 10.1097/SHK.0000000000002470
 938 22. Song, P., Zhang, J., Zhang, Y., Shu, Z., Xu, P., He, L., . . . Li, Q. 2017. Hepatic recruitment of
 939 CD11b+Ly6C+ inflammatory monocytes promotes hepatic ischemia/reperfusion injury. *Int J Mol*
 940 *Med* 41(2):935-945. 10.3892/ijmm.2017.3315
 941 23. Fantuzzi, L., Tagliamonte, M., Gauzzi, M.C., Lopalco, L. 2019. Dual CCR5/CCR2 targeting:
 942 opportunities for the cure of complex disorders. *Cell Mol Life Sci* 76(24):4869-4886.
 943 10.1007/s00018-019-03255-6
 944 24. Karlmark, K.R., Weiskirchen, R., Zimmermann, H.W., Gassler, N., Ginhoux, F., Weber, C., . . .
 945 Tacke, F. 2009. Hepatic recruitment of the inflammatory Gr1+ monocyte subset upon liver injury
 946 promotes hepatic fibrosis. *Hepatology* 50(1):261-274. 10.1002/hep.22950
 947 25. Sun, X., Wu, J., Liu, L., Chen, Y., Tang, Y., Liu, S., . . . Cai, J. 2022. Transcriptional switch of
 948 hepatocytes initiates macrophage recruitment and T-cell suppression in endotoxemia. *J Hepatol*
 949 77(2):436-452. 10.1016/j.jhep.2022.02.028
 950 26. Kang, J., Postigo-Fernandez, J., Kim, K., Zhu, C., Yu, J., Meroni, M., . . . Pajvani, U.B. 2023.
 951 Notch-mediated hepatocyte MCP-1 secretion causes liver fibrosis. *JCI Insight*
 952 8(3):10.1172/jci.insight.165369
 953 27. Shen, X.-D., Ke, B., Zhai, Y., Gao, F., Anselmo, D., Lassman, C.R., . . . Kupiec-Weglinski, J.W.
 954 2003. Stat4 and Stat6 signaling in hepatic ischemia/reperfusion injury in mice: HO-1 dependence
 955 of Stat4 disruption-mediated cytoprotection. *Hepatology* 37(2):296-303.
 956 28. Zhang, X., Wei, L., Wang, J., Qin, Z., Wang, J., Lu, Y., . . . Ma, J. 2017. Suppression Colitis and
 957 Colitis-Associated Colon Cancer by Anti-S100a9 Antibody in Mice. *Frontiers In Immunology*
 958 8(1774). 10.3389/fimmu.2017.01774
 959 29. Cesaro, A., Anceriz, N., Plante, A., Pagé, N., Tardif, M.R., Tessier, P.A. 2012. An inflammation
 960 loop orchestrated by S100A9 and calprotectin is critical for development of arthritis. *PLoS One*
 961 7(9):e45478. 10.1371/journal.pone.0045478
 962 30. Yardeni, T., Eckhaus, M., Morris, H.D., Huizing, M., Hoogstraten-Miller, S. 2011. Retro-orbital
 963 injections in mice. *Lab Anim (NY)* 40(5):155-160. 10.1038/labani0511-155
 964 31. Li, Y., Chen, B., Yang, X., Zhang, C., Jiao, Y., Li, P., . . . Du, J. 2019. S100a8/a9 Signaling Causes
 965 Mitochondrial Dysfunction and Cardiomyocyte Death in Response to Ischemic/Reperfusion
 966 Injury. *Circulation* 140(9):751-764. 10.1161/circulationaha.118.039262
 967 32. Wang, P.-P., Huang, X., Yang, M.-W., Fang, S.-Y., Hong, F.-F., Yang, S.-L. 2021. Effects of
 968 non-drug treatment on liver cells apoptosis during hepatic ischemia-reperfusion injury. *Life Sci*
 969 275(119321). 10.1016/j.lfs.2021.119321
 970 33. Vogel, C., Marcotte, E.M. 2012. Insights into the regulation of protein abundance from

proteomic and transcriptomic analyses. *Nat Rev Genet* 13(4):227-232. 10.1038/nrg3185

34. Payne, S.H. 2014. The utility of protein and mRNA correlation. *Trends Biochem Sci* 40(1):1-3. 10.1016/j.tibs.2014.10.010

35. Buccitelli, C., Selbach, M. 2020. mRNAs, proteins and the emerging principles of gene expression control. *Nat Rev Genet* 21(10):630-644. 10.1038/s41576-020-0258-4

36. Wang, S., Song, R., Wang, Z., Jing, Z., Wang, S., Ma, J. 2018. S100A8/A9 in Inflammation. *Frontiers In Immunology* 9(1298). 10.3389/fimmu.2018.01298

37. Pruenster, M., Vogl, T., Roth, J., Sperandio, M. 2016. S100A8/A9: From basic science to clinical application. *Pharmacol Ther* 167(120-131). 10.1016/j.pharmthera.2016.07.015

38. Jaeschke, H. 2006. Mechanisms of Liver Injury. II. Mechanisms of neutrophil-induced liver cell injury during hepatic ischemia-reperfusion and other acute inflammatory conditions. *Am J Physiol Gastrointest Liver Physiol* 290(6):G1083-G1088.

39. Jaeschke, H., Lemasters, J.J. 2003. Apoptosis versus oncotic necrosis in hepatic ischemia/reperfusion injury. *Gastroenterology* 125(4):1246-1257.

40. Zhang, S., Rao, S., Yang, M., Ma, C., Hong, F., Yang, S. 2022. Role of Mitochondrial Pathways in Cell Apoptosis during He-Patic Ischemia/Reperfusion Injury. *Int J Mol Sci* 23(4):10.3390/ijms23042357

41. Gujral, J.S., Bucci, T.J., Farhood, A., Jaeschke, H. 2001. Mechanism of cell death during warm hepatic ischemia-reperfusion in rats: apoptosis or necrosis? *Hepatology* 33(2):397-405.

42. Lu, L., Zhou, H., Ni, M., Wang, X., Busuttil, R., Kupiec-Weglinski, J., Zhai, Y. 2016. Innate Immune Regulations and Liver Ischemia-Reperfusion Injury. *Transplantation* 100(12):2601-2610.

43. Saiman, Y., Friedman, S.L. 2012. The role of chemokines in acute liver injury. *Front Physiol* 3(213). 10.3389/fphys.2012.00213

44. Mihm, S. 2018. Danger-Associated Molecular Patterns (DAMPs): Molecular Triggers for Sterile Inflammation in the Liver. *Int J Mol Sci* 19(10):10.3390/ijms19103104

45. Zhou, Z., Xu, M.-J., Gao, B. 2015. Hepatocytes: a key cell type for innate immunity. *Cell Mol Immunol* 13(3):301-315. 10.1038/cmi.2015.97

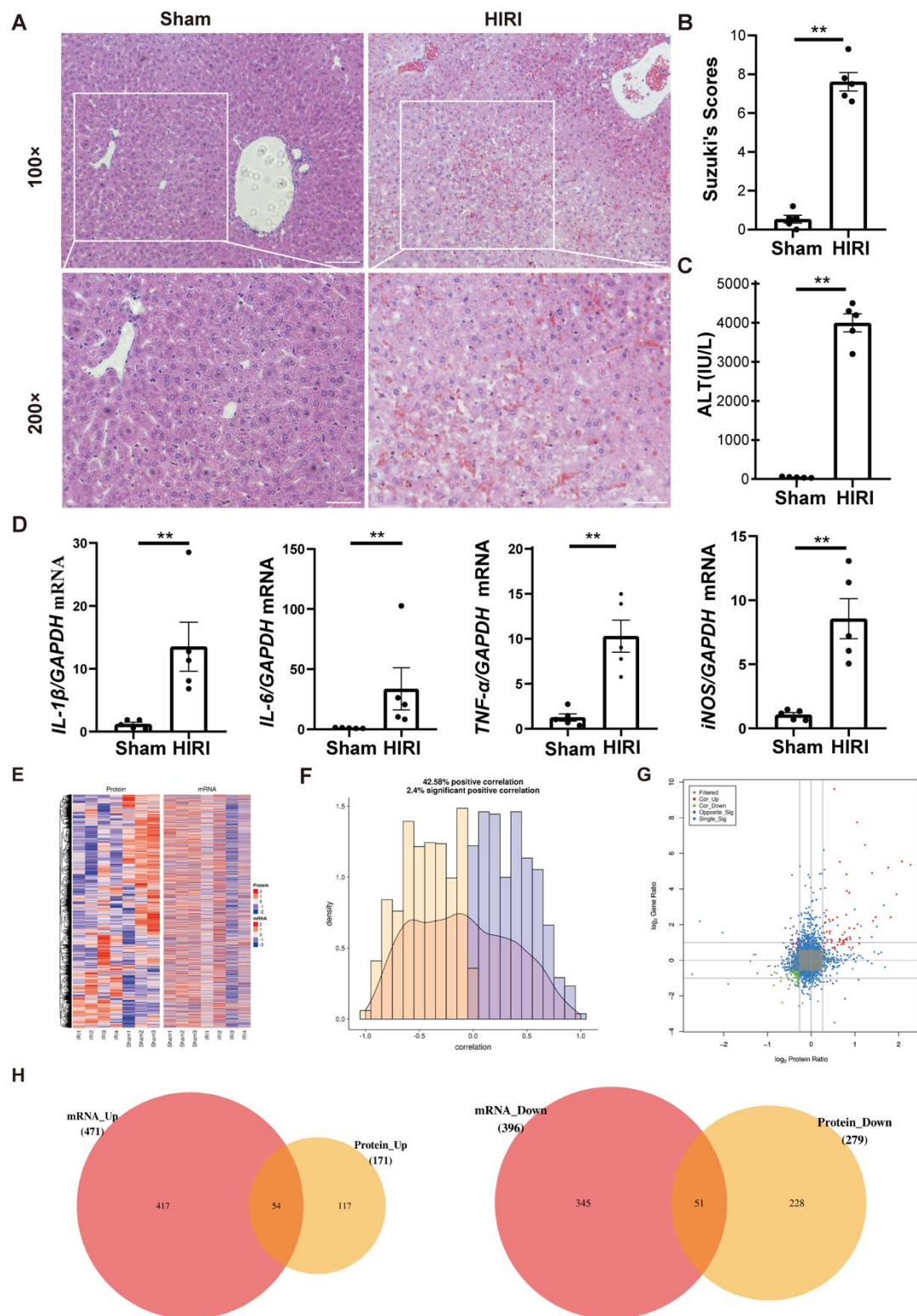
46. Zhang, J., Xu, P., Song, P., Wang, H., Zhang, Y., Hu, Q., . . . Zhang, J. 2016. CCL2-CCR2 signaling promotes hepatic ischemia/reperfusion injury. *J Surg Res* 202(2):352-362. 10.1016/j.jss.2016.02.029

47. Zimmermann, H.W., Trautwein, C., Tacke, F. 2012. Functional role of monocytes and macrophages for the inflammatory response in acute liver injury. *Front Physiol* 3(56). 10.3389/fphys.2012.00056

48. Wu, R., Zhang, Y., Xiang, Y., Tang, Y., Cui, F., Cao, J., . . . Duan, L. 2018. Association between serum S100A9 levels and liver necroinflammation in chronic hepatitis B. *J Transl Med* 16(1):83. 10.1186/s12967-018-1462-2

Figure titles and legends

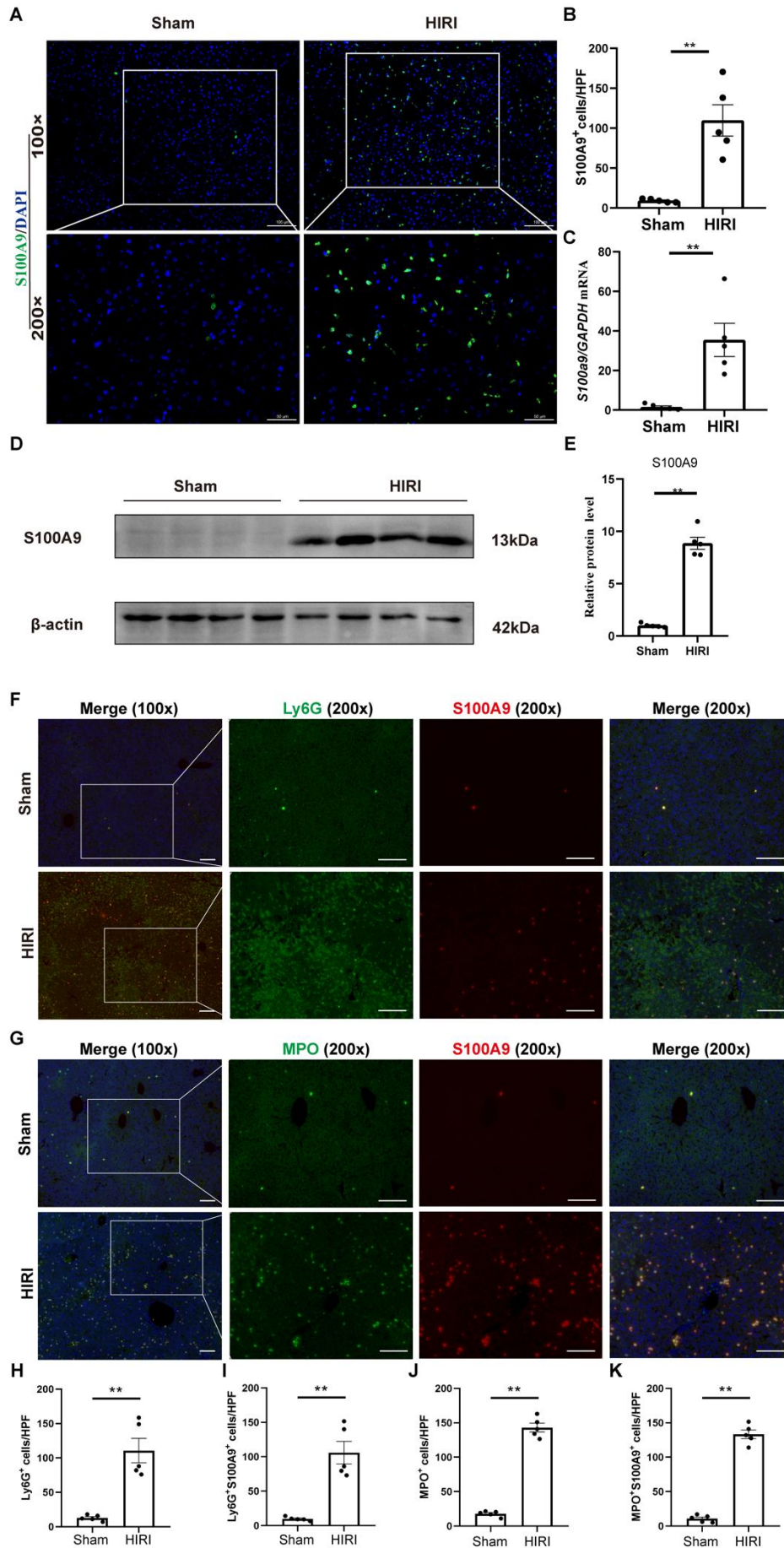
Fig. 1. Establishment of the HIRI model and multi-omics profiling.



(A) Representative H&E-stained liver sections from sham and HIRI mice at original magnifications of 100× and 200×. Scale bars, 100 μm for the 100× images and 50 μm for the 200× images. n = 5 mice per group. (B)

Quantification of Suzuki's injury scores. (C) Serum ALT levels in sham and HIRI mice. (D) RT-qPCR analysis of hepatic *IL-1 β* , *IL-6*, *TNF- α* , and *iNOS* mRNA expression. (E) Heatmap showing differentially expressed proteins and genes in liver tissues from sham and HIRI mice. (F) Correlation distribution of matched mRNA-protein pairs between the transcriptomic and proteomic datasets. (G) Scatterplot showing the distribution of matched mRNA and protein expression changes based on log2-transformed expression ratios. Concordantly upregulated and downregulated mRNA-protein pairs are highlighted. (H) Venn diagrams showing concordantly upregulated and downregulated molecules identified by integrative transcriptomic and proteomic analysis. Data in quantitative panels are presented as mean $\pm$ SEM. Statistical comparisons were performed using unpaired Student's t-test. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

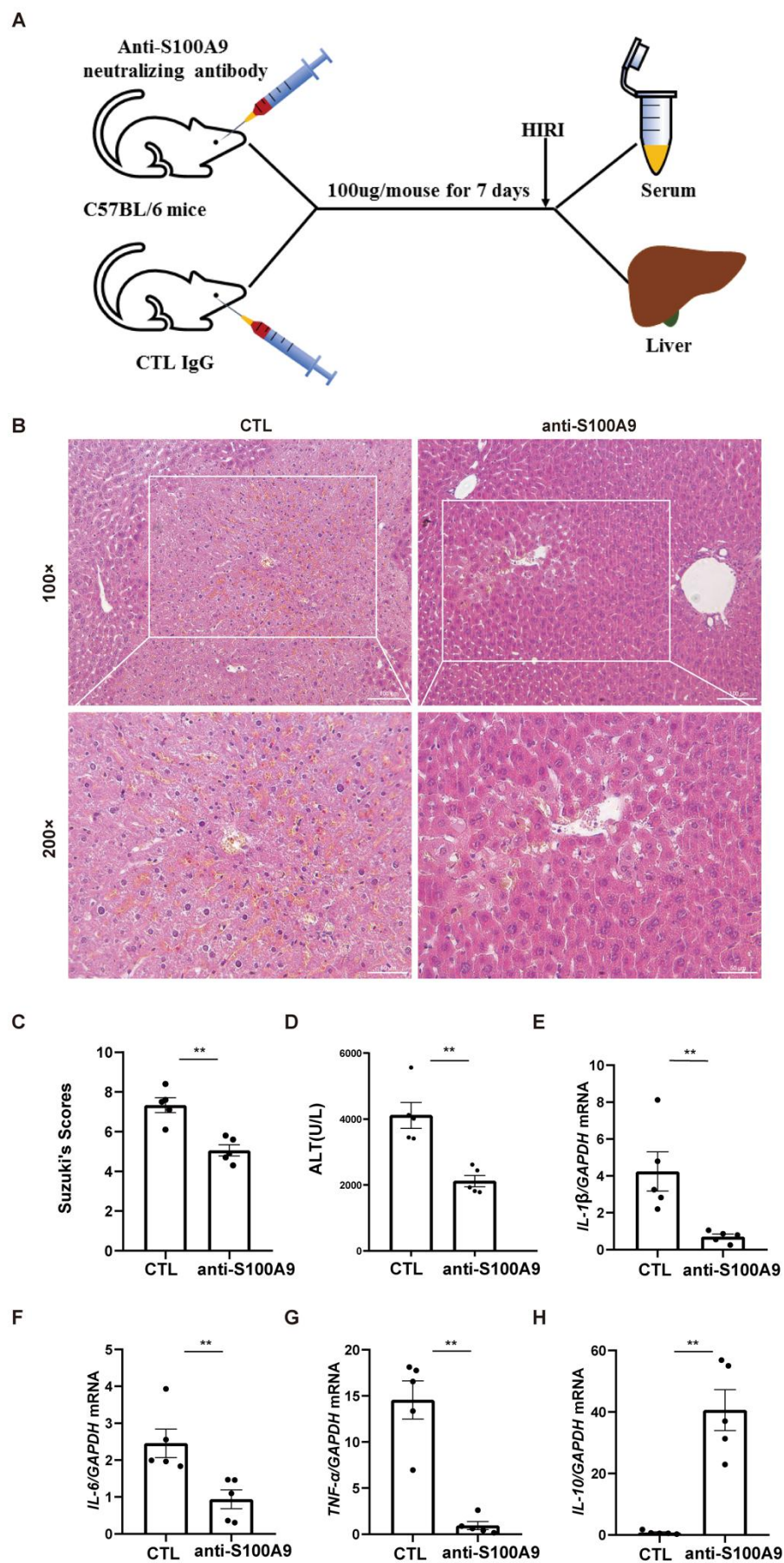
Fig. 2. S100A9 upregulation is associated with neutrophil infiltration during HIRI.



(A) Representative immunofluorescence images showing S100A9 expression in liver sections from sham and HIRI mice. S100A9 is shown in green, and nuclei are counterstained with DAPI in blue. Original magnifications, 100× and 200×. Scale bars, 100 μm for the 100× images and 50 μm for the 200× images. n = 5 mice per group. (B) Quantification of S100A9-positive cells per high-power field. (C) RT-qPCR analysis of hepatic *S100a9* mRNA expression. (D) Western blot analysis of S100A9 protein expression in liver tissues. (E) Quantification of S100A9 protein expression normalized to β-actin. (F) Representative double-immunofluorescence images showing Ly6G and S100A9 staining in liver sections from sham and HIRI mice. Ly6G is shown in green, S100A9 in red, and nuclei are counterstained with DAPI in blue. Original magnifications, 100× and 200×. Scale bars, 100 μm. n = 5 mice per group. (G) Representative double-immunofluorescence images showing MPO and S100A9 staining in liver sections from sham and HIRI mice. MPO is shown in green, S100A9 in red, and nuclei are counterstained with DAPI in blue. Original magnifications, 100× and 200×. Scale bars, 100 μm. n = 5 mice per group. (H) Quantification of Ly6G-positive cells. (I) Quantification of Ly6G⁺S100A9⁺ double-positive cells. (J) Quantification of MPO-positive cells. (K) Quantification of MPO⁺S100A9⁺ double-positive cells. Data are presented as mean ± SEM. Statistical comparisons were performed using unpaired Student's t-test. **P* < 0.05, ***P* < 0.01,

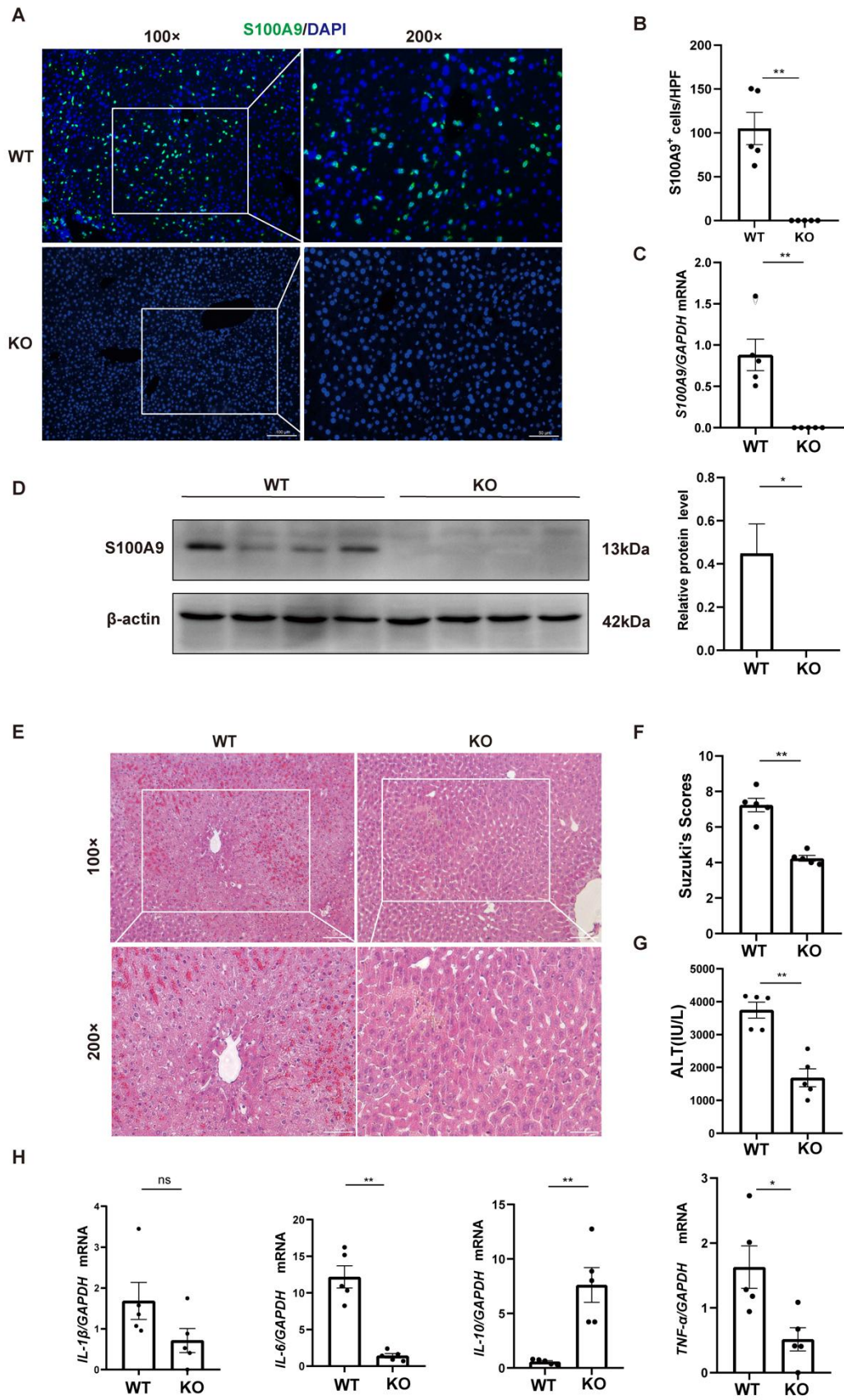
1052 *** $P < 0.001$.

1053 **Fig. 3. S100A9 blockade attenuates HIRI.**



(A) Schematic illustration of anti-S100A9 neutralizing antibody administration in the mouse HIRI model. C57BL/6 mice received anti-S100A9 neutralizing antibody or control IgG before HIRI induction, followed by serum and liver tissue collection after reperfusion. (B) Representative H&E-stained liver sections from control IgG-treated and anti-S100A9-treated HIRI mice at original magnifications of 100× and 200×. Scale bars, 100 μm for the 100× images and 50 μm for the 200× images. n = 5 mice per group. (C) Quantification of Suzuki's injury scores. (D) Serum ALT levels. (E–H) RT-qPCR analysis of hepatic *IL-1β*, *IL-6*, *TNF-α* and *IL-10* mRNA expression. Data are presented as mean ± SEM. Statistical comparisons were performed using unpaired Student's *t*-test. **P* < 0.05, ***P* < 0.01, ****P* < 0.001.

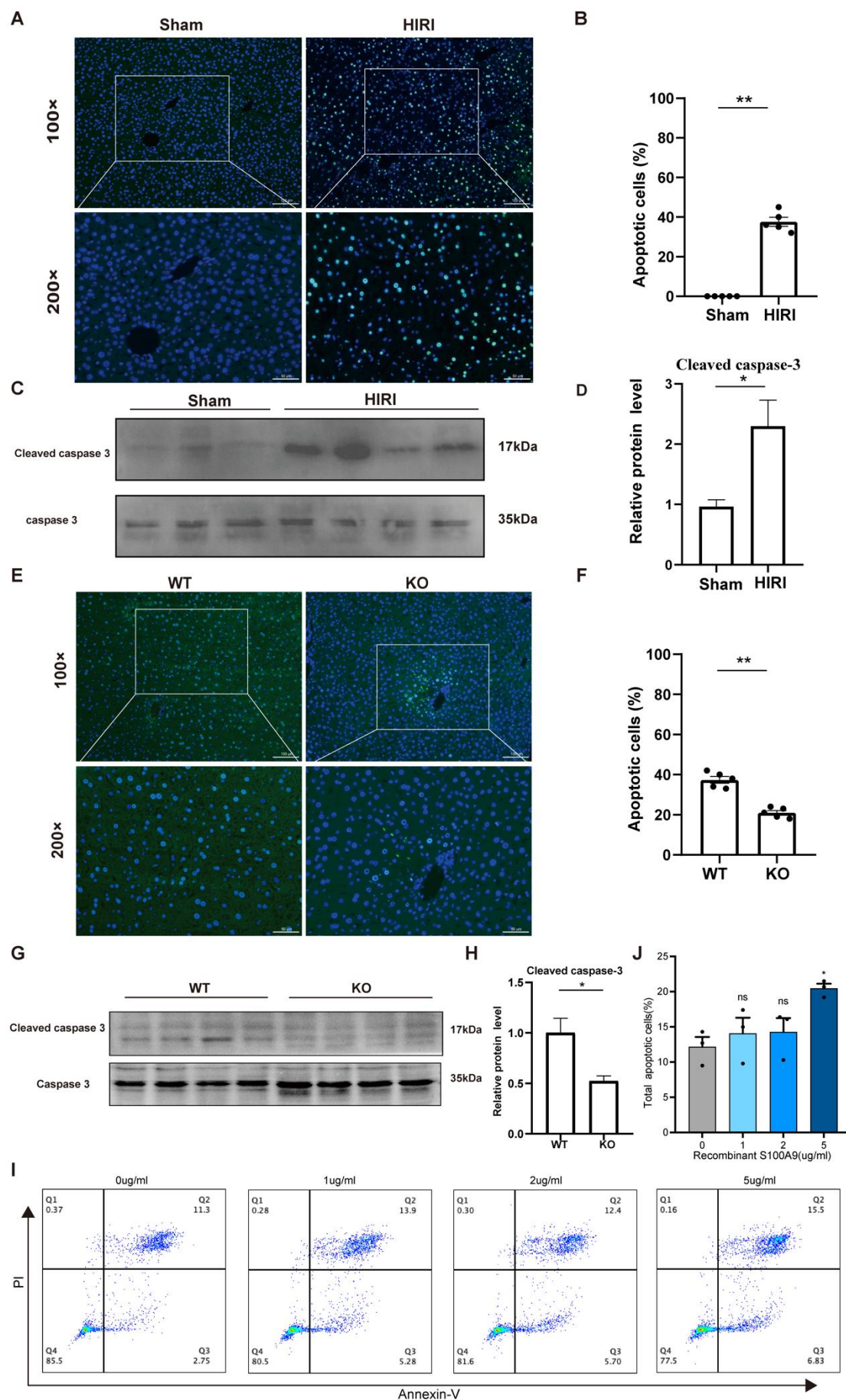
Fig. 4. S100A9 deficiency protects against HIRI.



(A) Representative immunofluorescence images showing S100A9 staining

in liver sections from WT and *S100a9* KO mice after HIRI. S100A9 is shown in green, and nuclei are counterstained with DAPI in blue. Original magnifications, 100× and 200×. Scale bars, 100 μm for the 100× images and 50 μm for the 200× images. n = 5 mice per group. (B) Quantification of S100A9-positive cells per high-power field. (C) RT-qPCR analysis of hepatic *S100a9* mRNA expression. (D) Western blot analysis of S100A9 protein expression and densitometric quantification normalized to β-actin. (E) Representative H&E-stained liver sections from WT and *S100a9* KO mice after HIRI. Original magnifications, 100× and 200×. Scale bars, 100 μm for the 100× images and 50 μm for the 200× images. n = 5 mice per group. (F-G) Quantification of Suzuki's injury scores and serum ALT levels. (H) RT-qPCR analysis of hepatic *IL-1β*, *IL-6*, *IL-10*, and *TNF-α* mRNA expression. Data in quantitative panels are presented as mean ± SEM. Statistical comparisons were performed using unpaired Student's *t*-test. ns, not significant; $P < 0.05$, $P < 0.01$ *** $P < 0.001$.

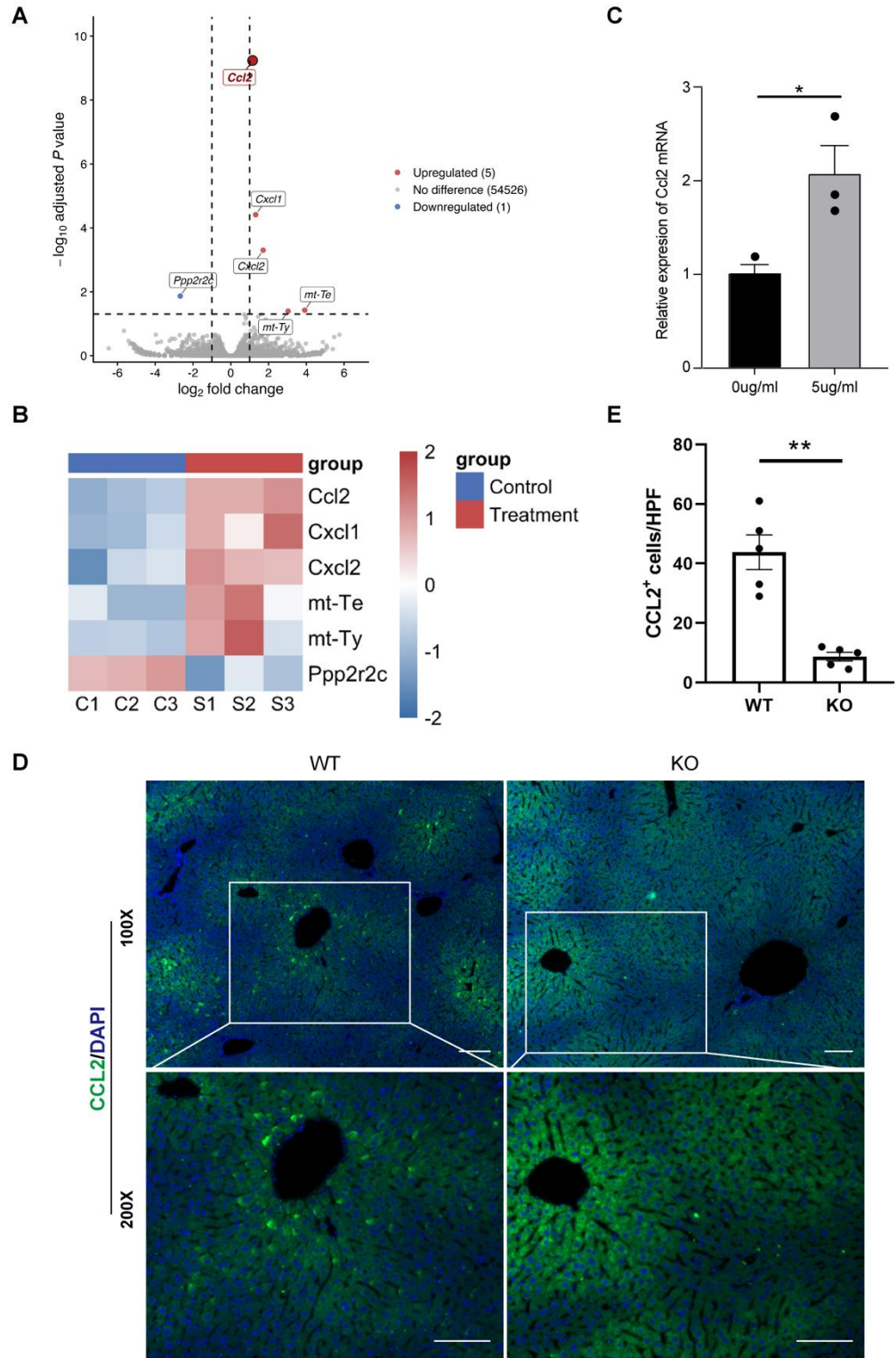
Fig. 5. S100A9 contributes to HIRI-associated apoptosis.



(A) Representative TUNEL-stained liver sections from CTL and HIRI mice. Original magnifications, 100× and 200×. Scale bars, 100 μm for the 100× images and 50 μm for the 200× images. n = 5 mice per group. (B) Quantification of apoptotic cell rates in liver tissues. (C) Western blot analysis of cleaved caspase-3 and total caspase-3 expression in control and HIRI liver tissues. (D) Densitometric quantification of cleaved caspase-3 protein expression normalized to total caspase-3. (E) Representative TUNEL staining images of liver tissues from WT and *S100a9* KO mice after HIRI. Images are shown at 100× and 200× magnification. (F) Quantification of apoptotic cell rates in WT and *S100a9* KO mice after HIRI. (G) Western blot analysis of cleaved caspase-3 and total caspase-3 expression in liver tissues from WT and *S100a9* KO mice after HIRI. (H) Densitometric quantification of cleaved caspase-3 protein expression normalized to total caspase-3. (I) Representative Annexin V/PI flow cytometry plots of OGD/R-treated AML12 cells treated with recombinant S100A9 at 0, 1, 2, and 5 μg/mL during reoxygenation. (J) Quantification of AML12 cell apoptosis rates. Data are presented as mean ± SEM. Statistical comparisons were performed using unpaired Student's *t*-test for two-group comparisons and one-way ANOVA for multiple-group comparisons. ns, not significant; *P* < 0.05, *P* < 0.01. HIRI, hepatic ischemia-reperfusion injury; KO, knockout; OGD/R, oxygen-glucose deprivation/reoxygenation; PI, propidium iodide; WT, wild-type. Scale

bars are indicated in the images.

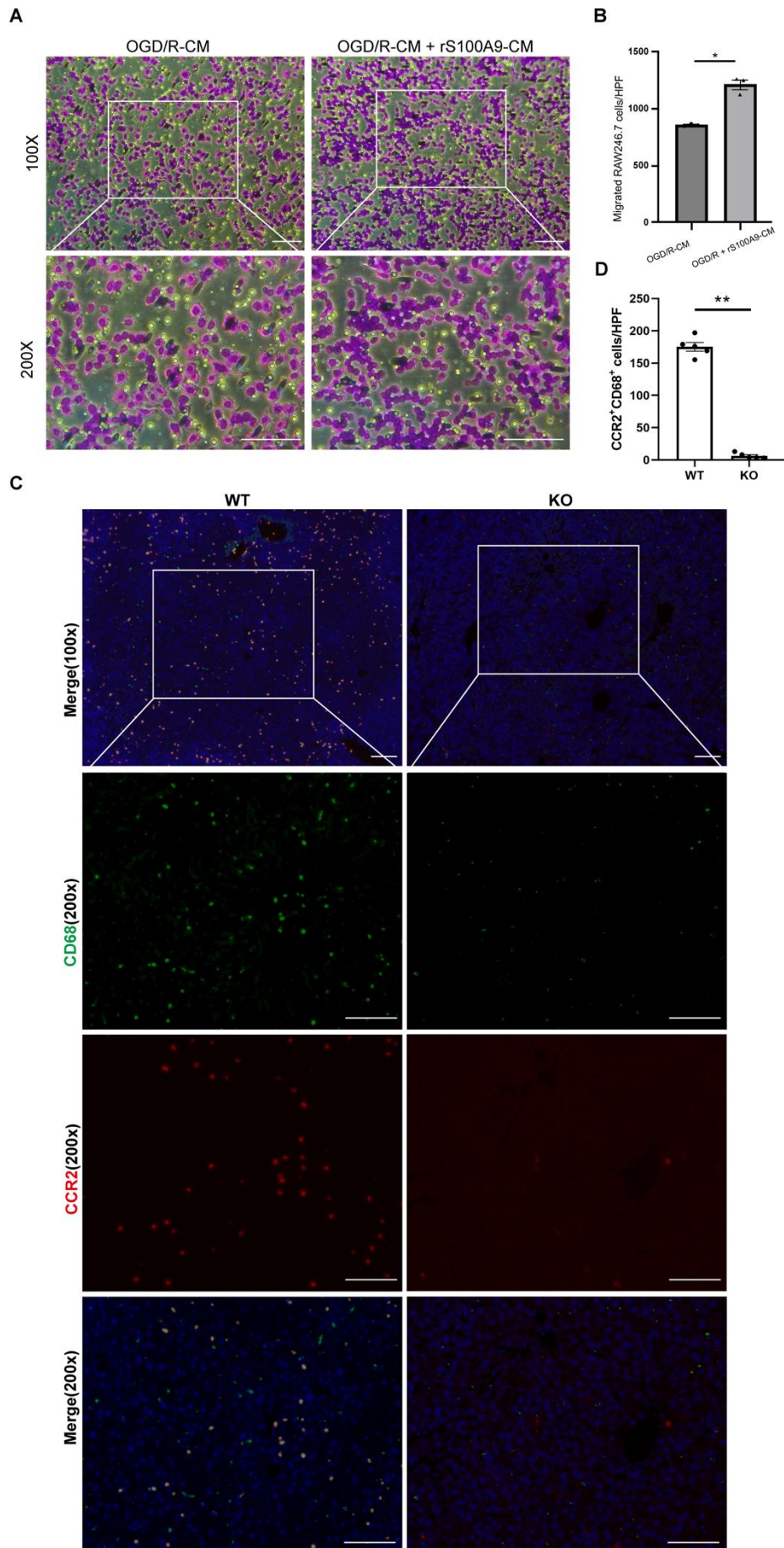
Fig. 6. S100A9 induces CCL2 expression in injured hepatocytes.



(A) Volcano plot showing differentially expressed genes between OGD/R-

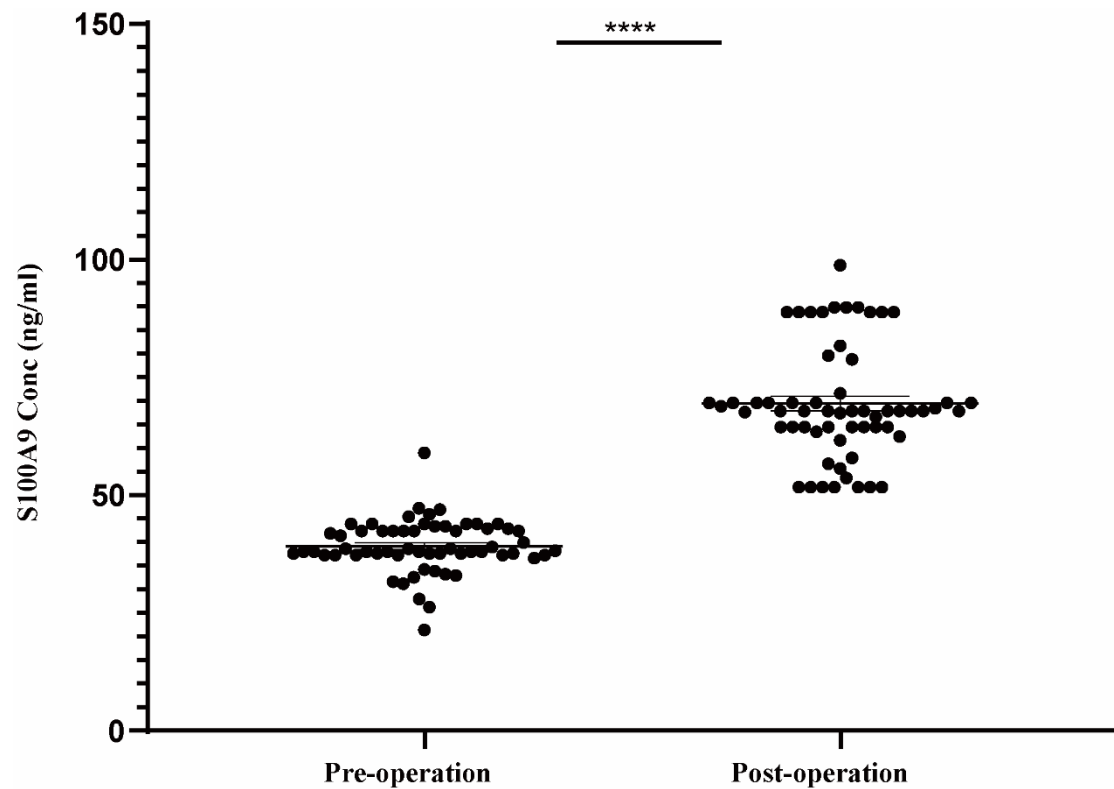
treated AML12 cells and S100A9-treated OGD/R AML12 cells. AML12 cells were subjected to oxygen-glucose deprivation followed by reoxygenation, during which recombinant S100A9 protein was administered at 5 µg/mL. *Ccl2* was highlighted as one of the most prominently upregulated genes after S100A9 treatment. (B) Heatmap showing the expression patterns of selected differentially expressed genes, including chemokine-related genes, in control and S100A9-treated AML12 cells. C1–C3 indicate control samples, and S1–S3 indicate S100A9-treated samples. (C) RT-qPCR validation of *Ccl2* mRNA expression in AML12 cells after S100A9 treatment during reoxygenation. (D) Representative immunofluorescence images showing hepatic CCL2 expression in ischemia-reperfused liver tissues from WT and S100a9-deficient mice. CCL2 is shown in green, and nuclei are counterstained with DAPI in blue. Scale bars are indicated in the images. (E) Quantification of hepatic CCL2-positive cells in WT and S100a9-deficient mice after HIRI. Data are presented as mean ± SEM. Statistical comparisons were performed using unpaired Student's t-test. *P < 0.05, **P < 0.01. OGD/R, oxygen-glucose deprivation/reoxygenation; HIRI, hepatic ischemia-reperfusion injury; HPF, high-power field.

Fig. 7. S100A9-stimulated hepatocytes enhance macrophage migration and recruitment.



(A) Representative images of RAW264.7 macrophage migration after incubation with conditioned medium collected from OGD/R-treated AML12 cells or S100A9-treated OGD/R AML12 cells. AML12 cells were subjected to oxygen-glucose deprivation followed by reoxygenation, during which recombinant S100A9 protein was administered at 5 µg/mL. Images are shown at 100× and 200× magnification. (B) Quantification of migrated RAW264.7 cells per field. (C) Representative immunofluorescence images showing CCR2 and CD68 expression in ischemia-reperfused liver tissues from WT and S100a9-deficient mice. CD68 is shown in green, CCR2 in red, and nuclei are counterstained with DAPI in blue. Images are shown at 100× and 200× magnification. (D) Quantification of CCR2⁺CD68⁺ cells per high-power field. Data are presented as mean ± SEM. Statistical comparisons were performed using unpaired Student's *t*-test. **P* < 0.05, ***P* < 0.01. OGD/R, oxygen-glucose deprivation/reoxygenation; CM, conditioned medium; rS100A9, recombinant S100A9; HIRI, hepatic ischemia-reperfusion injury; HPF, high-power field. Scale bars are indicated in the images.

Fig. 8. Serum S100A9 levels are elevated in patients with HIRI after partial hepatectomy.



Serum S100A9 levels were measured in paired preoperative and postoperative serum samples from 60 patients undergoing partial hepatectomy. Compared with preoperative levels, serum S100A9 concentrations were significantly increased after surgery. Data are presented as mean $\pm$ SEM. Statistical comparison was performed using paired Student's t-test. ****P < 0.0001.

Supplementary Files

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