Supplementary materials

Human Antigen R (HuR): A New Regulator of Heme Oxygenase-1 Cytoprotection in Mouse and Human Liver Transplant Injury

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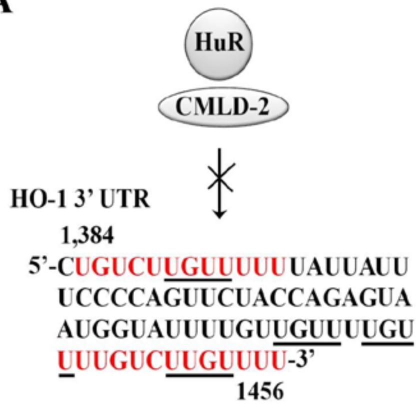
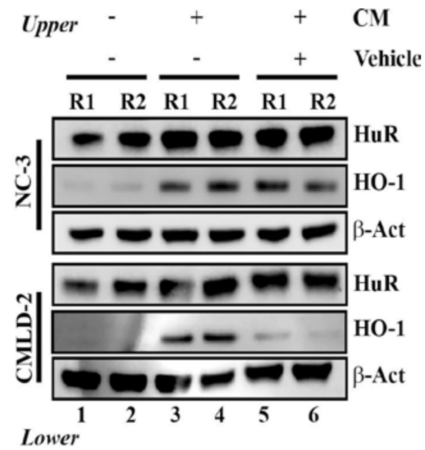
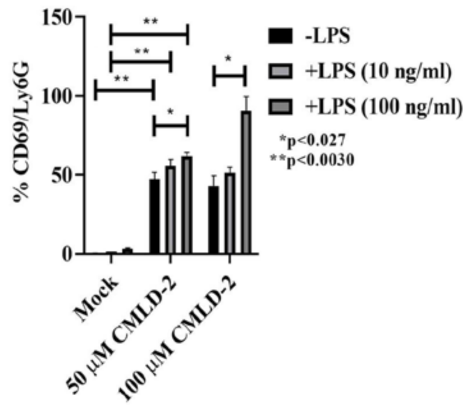
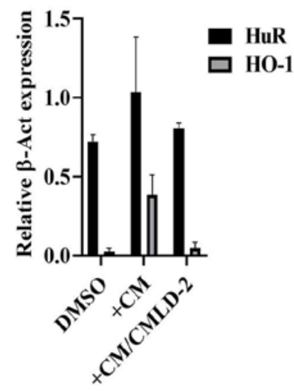
Fig. S1









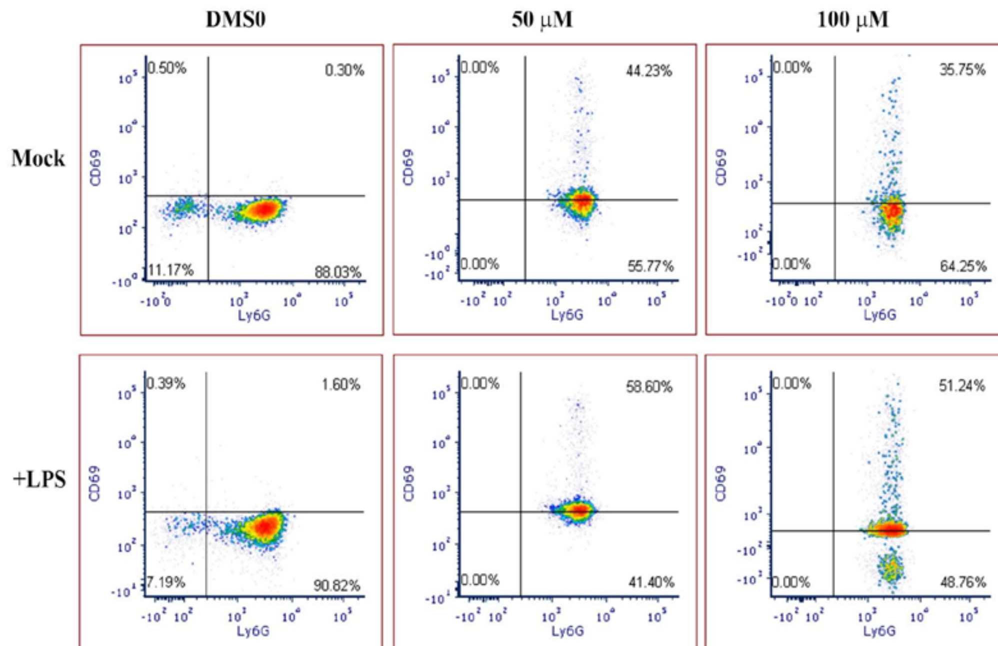


Figure S1: Inhibition of HuR destabilizes the 3'UTR of HO-1 mRNA and leads to activation of primary cultured neutrophils (CD69+Ly6G+).

(A) Small molecule inhibitor of HuR, CMLD-2 competitively binds to HuR directly disrupting HuR–ARE interactions. The 3' UTR of HO-1 contains multiple HuR seed binding sites (underline) where at least two are predicted consensus sites (red). (B) *Upper*, total lysates from CM-conditioned hepatocyte cultures were analyzed for differences between HuR, HO-1, and β-Act after treatment with DMSO, NC-3 (noncompetitive control) or CMLD-2. *Bottom*, data shown are mean ± SD of representative samples n =2 (repeated at least three independent times). (C) Quantitation of CD69+Ly6G+ neutrophil population preconditioned with CMLD-2 at indicated concentrations by flow cytometry as presented in (D). Most significant data are mean ± SD, $^{**}p < 0.0030$, 50 μM CMLD-2 versus Mock and $^{**}p < 0.0042$, 50 μM CMLD-2 +LPS (10 ng/ml LPS) versus Mock +LPS. n=3 of representative samples.

Fig. S2

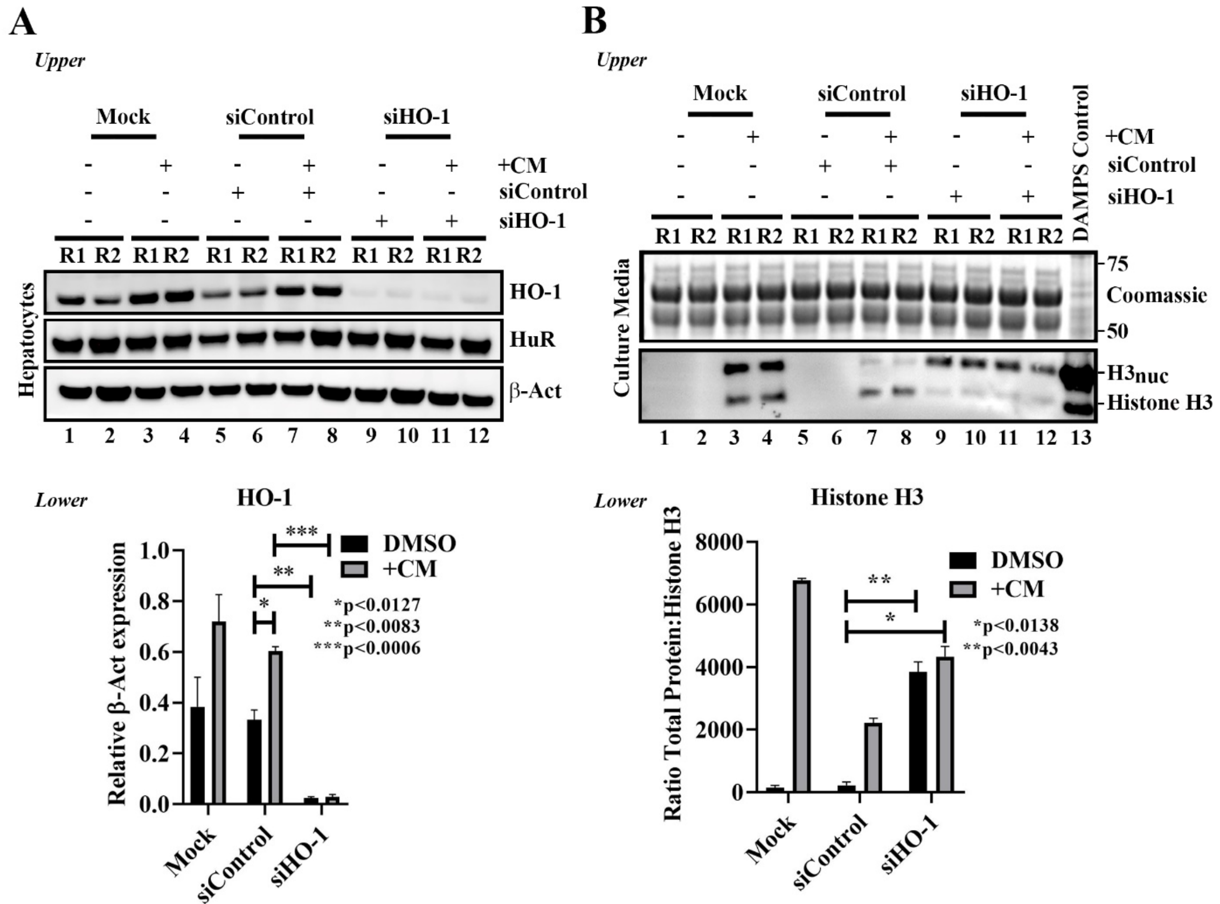


Figure S2: Silencing of HO-1 exacerbates hepatocellular toxicity during *in vitro* warm IRI but does not involve HuR signaling.

(A) *Upper,* Total lysates from CM-conditioned primary derived hepatocyte cultures treated with siControl or siHO-1 siRNAs were probed by western blot for differences between HuR, HO-1, and β-Act, as a loading control. *Bottom,* Unpaired two-tailed student t-test of representative samples HO-1 as presented were calculated relative β-Act expression. (B) Hepatocellular toxicity was assessed as in Figure 2 D. **p< 0.0043, siHuR versus siControl, in absence of CM, for n=2 (repeated at least three independent times).

Fig. S3

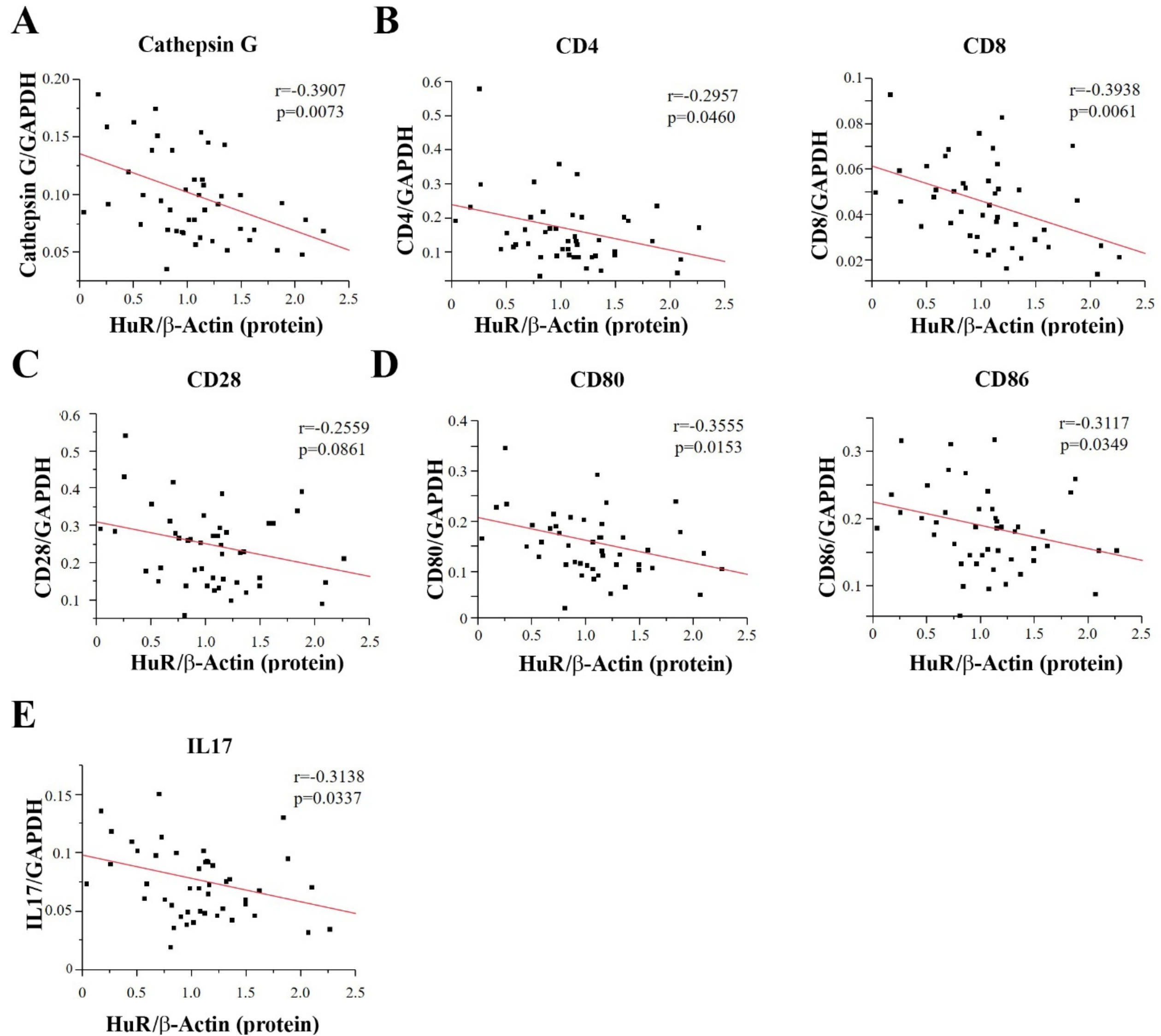


Figure S3: Liver graft HuR is negatively correlated with innate/adaptive immune activation in LT patients.

Hepatic Bx samples were collected from fifty-one human LT cases approximately 2h after portal reperfusion under IRB protocol. Relationship between HuR and (A) neutrophil marker Cathepsin G; (B) T-cell markers CD4, CD8 and (C) CD28; and macrophage markers (D) CD80 and CD86; and (E) Th17-derived IL17 is negatively regulated. r: Spearman's correlation coefficient.

Fig. S4

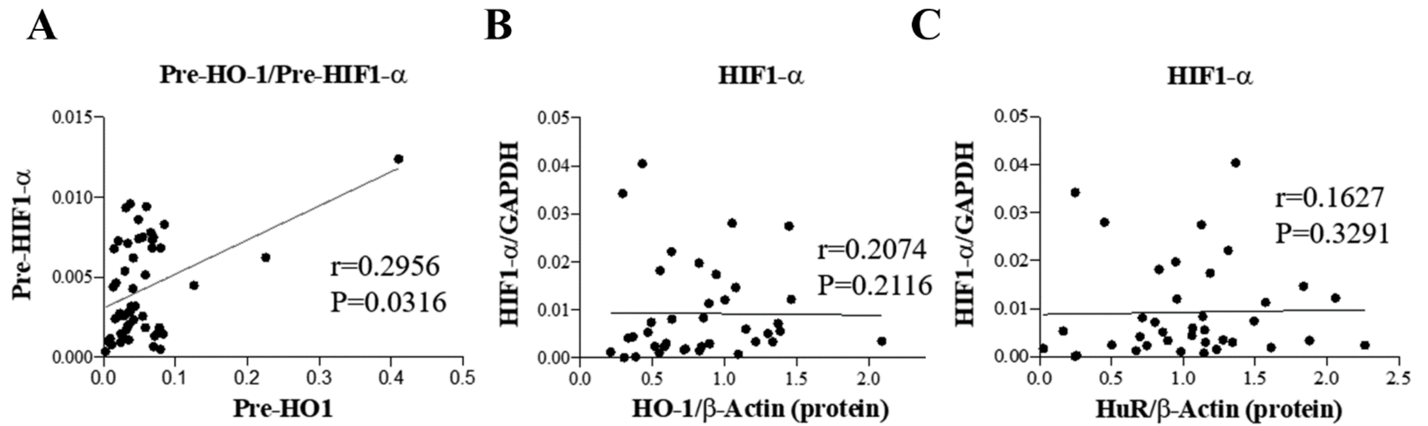


Figure S4: HIF-1-α expression in pre-transplant cold-stored human liver Bx samples is positively correlated with HO-1 expression.

Fifty-one pre-transplant human liver Bx samples were analyzed for (A) HIF-1-α and HO-1 levels with β-actin normalization. Relationship between HIF-1-α and HO-1 showed statistical correlation at 18 h after cold storage. By contrast, LT samples obtained approximately 2h after portal reperfusion showed no statistical correlation between HIF-1-α and (B) HO-1 or (C) HuR. r: Spearman's correlation coefficient.

Fig. S5

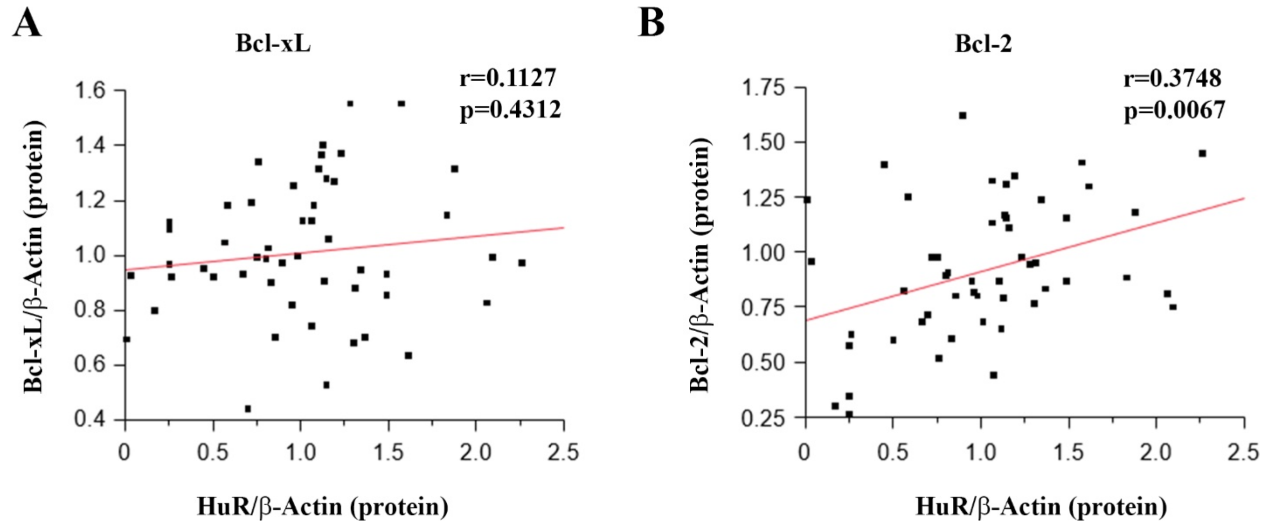


Figure S5: Liver graft BcL-2 is positively correlated with HuR expression in LT patients.

Fifty-one human LT hepatic Bx samples (approximately 2h after portal reperfusion) were analyzed for Bcl-xL and HuR protein levels with β-actin normalization. (A) Bcl-xL/HuR showed no statistical correlation whereas (B) Bcl-2/HuR showed significant correlation. r: Spearman's correlation coefficient.

Table S1. Demographic data and clinical parameters of recipients and donors used in this study

A. Recipient's preoperative variables and surgical parameters

Variables	Low HuR (n=25)	High HuR (n=26)	p value
Age (years)	59 (23-73)	59 (30-75)	0.836
Gender (M/F)	18 (72.0) / 7 (28.0)	19 (73.1) / 7 (26.9)	>0.999
Race			0.723
White	11 (44.0)	11 (42.3)	
Hispanic	7 (28.0)	11 (42.3)	
Black	3 (12.0)	1 (3.8)	
Asian	3 (12.0)	2 (7.7)	
Others	1 (4.0)	1 (3.8)	
BMI (kg/m^2)	24.8 (14.5-40.0)	27.4 (19.2-47.5)	0.221
Past History			
Diabetes	7 (28.0)	10 (38.5)	0.555
Hypertension	10 (40.0)	10 (38.5)	0.91
Heart disease	5 (20.0)	1 (3.8)	0.073
Abdominal surgery	8 (32.0)	9 (34.6)	0.843
Disease etiology			0.185
Viral hepatitis	14 (56.0)	13 (50.0)	
EtOH	1 (4.0)	7 (26.9)	
Cryptogenic cirrhosis / NASH	4 (16.0)	4 (15.4)	
ALF	2 (8.0)	0	
PBC	1 (4.0)	1 (3.8)	
Others	3 (12.0)	1 (3.8)	
ABO			
identical	25 (100)	26 (100)	NA
MELD score	29 (6-47)	34 (7-44)	0.181
Pre-transplant AST (IU/L)	56 (24-1918)	77 (23-274)	0.356
Pre-transplant ALT (IU/L)	51 (14-3705)	35.5 (11-158)	0.16
T-Bil (g/dl)	49 (13-207)	55 (22-157)	>0.999
PT-INR	1.9 (1.0-4.1)	1.95 (1.1-2.9)	0.77
CIT (min)	439 (163-749)	416.5 (150-762)	0.756
WIT (min)	49 (29-71)	54.5 (25-79)	0.239

* Continuous values are shown as median (range), and categorized values are shown as number (percent).

B. Donor's parameters

Variables	Low HuR (n=25)	High HuR (n=26)	p value
Age (years)	45 (16-74)	41 (7-67)	0.777
Gender (M/F)	13 (52.0) / 12 (48.0)	11 (42.3) / 15 (57.7)	0.488
Race			0.348
White	16 (64.0)	15 (57.7)	
Hispanic	6 (24.0)	8 (30.8)	
Black	0	2 (7.7)	
Asian	3 (12.0)	1 (3.8)	
BMI (kg/m^2)	24.3 (19.7-42.6)	26.3 (13.4-38.4)	0.498
Pre-transplant AST (IU/L)	51 (12-306)	35.5 (10-314)	0.742
Pre-transplant ALT (IU/L)	28 (11-669)	20.5 (8-191)	0.16
T-Bil (g/dl)	0.7 (0.3-4.9)	0.5 (0.2-2.1)	0.207
PT-INR	1.2 (1.0-1.6)	1.25 (1.0-2.0)	0.745
DCD	1 (4.0)	2 (7.7)	0.575

* Continuous values are shown as median (range), and categorized values are shown as number (percent).

Table S2. Primer sequences used in this study

Experimental Arm:

β2-microglobulin	Mm00437762
CXCL1	Mm4207460
CXCL10	Mm00445235
CXCL2	Mm00436450
CD68	Mm03047343
CD80	Mm00711660
CD86	Mm00444540
HMOX1	Mm00516005
HuR	Mm00516011
MCP1	Mm00441242
NF-kB	Mm00476361

Clinical Arm:

Cathepsin G	5’-GAGTCAGACGGAATCGAAACG-3’ 5’-CGGAGTGTATCTGTTCCCCTC-3’
CD4	QuantiTect Primer Assay (QT02401812)
CD28	QuantiTect Primer Assay (QT00001267)
CD80	5’-AAACTCGCATCTACTGGCAAA-3’ 5’-GGTTCTTGTACTCGGGCCATA-3’
CD86	5’-CTGCTCATCTATACACGGTTACC-3’ 5’-GGAAACGTCGTACAGTTCTGTG-3’
GAPDH	5’-GGAGCGAGATCCCTCCAAAAT-3’ 5’-GGCTGTTGTCATACTTCTCATGG-3’
HMOX1	5’-AAGACTGCGTTCCTGCTCAAC-3’ 5’-AAAGCCCTACAGCAACTGTCG-3’
IL17	5’-TCCCACGAAATCCAGGATGC-3’ 5’-GGATGTTCAGGTTGACCATCAC-3’

Supplementary Experimental Procedures

Hepatocytes, bone marrow-derived macrophages, neutrophils isolation

Primary mouse hepatocytes were isolated by a two-stage collagenase perfusion method (1). Bone marrow cells were isolated/cultured (5×10^6/well) with 10% L929 cell medium (GM-CSF) for 6 days to yield the bone marrow-derived macrophages (BMM). Neutrophils were isolated using Easysep Mouse Neutrophil Enrichment kit (StemCell Technologies, Cambridge, MA). Expression of CD69+/Ly6G+ cells was measured by flow cytometry using antibodies CD11B (FITC, clone M1/70), Ly6G (PerCP/Cy5.5, clone 1A8), CD69 (Brilliant Violet 510, clone H1.2F3) from BioLegend (San Diego, CA).

TUNEL/LDH assay, serum biochemistry, LT histology/IRI grading

Cell death in formalin-fixed paraffin-embedded liver sections (5µm) was detected by the ApopTag Plus Peroxidase In Situ Apoptosis Kit (Millipore, Temecula, CA). Results were scored semiquantitatively by blindly counting the number of positive cells in 10 HPF/section. Indicators of hepatocellular injury were analyzed using Pierce LDH Cytotoxicity Assay Kit (Thermo Fisher Scientifics) and Infinity AST/ALT (GOT) reagent using serum after sampling (ThermoFisher Scientific). Hepatocellular damage was also evaluated by analyzing cell culture supernatants for Histone H3 (Ab#4499, Cell Signaling). The positive DAMPs control was prepared by culturing hepatocytes in 1x HBSS for 48h followed by supernatant concentration. Formalin-fixed paraffin-embedded liver sections (5µm) were stained with hematoxylin and eosin (H&E). The severity of IRI was graded using Suzuki's criteria (2).

RNAi interference/reagents

Silencer Select HuR (s201497), HMOX-1 (s67607) and Negative control (4390843) siRNAs were purchased (Thermo Fisher Scientific). Hepatocytes were transfected with these siRNAs using RNAiMAX Transfection Reagent (Invitrogen, Waltham, MA) in advance of CMLD-2 or CM treatment (3). A total of 1.0µg of RNA in most cases was reverse-transcribed into cDNA using High-Capacity RNA-to-cDNA (ThermoFisher Scientific). Quantitative PCR was performed using QuantStudio 3 (Applied Biosystems, Foster City, CA). The primers sequences are listed (Table S2). The expression of the target gene was normalized to housekeeping genes β2-microglobulin, GAPDH or β-Actin for Hepatocytes, BMM or LT biopsies. LPS (Invivogen, San Diego, CA) derived from E. coli 0111:B4 was used at a final concentration of 0.5µg/ml for hepatocytes or 0.1µg/ml for macrophages/neutrophils. HuR inhibitor CMLD-2 (Calbiochem, Burlington, MA) and NC-3, from the laboratory of Jeff Aubé (UNC Eshelman School of Pharmacy), were used (30µM for 12-24h). NE-PER (Thermo Fisher) was used for nuclear/cytoplasmic of proteins from liver homogenates.

Hepatocyte hypoxia/reoxygenation

For simulating the hypoxic-ischemic condition, serum-free DMEM was exchanged before cells were challenged (<0.1% O_2) in an Anaerobic Gas Generator chamber (Thermo Fisher Scientific) at 37°C or 4°C. After 90min, cells were brought back to normoxic conditions (air/5% CO_2) for indicated times at 37°C.

Western blots

Cells were lysed in M-PER Mammalian Protein Extraction Reagent (ThermoFisher Scientific) supplemented with Halt Protease and Phosphatase Inhibitor Cocktail (ThermoFisher Scientific). Total lysate/liver biopsy proteins (20-30μg/sample) were resolved by SDS-PAGE gel electrophoresis and transferred to nitrocellulose membrane. Antibodies for HuR (sc-5261, Santa Cruz Biotechnology), HO-1 (ab13243, Abcam) and β-Act (8457S, Cell Signaling) were used as probes. StarBright Blue 700 goat anti-rabbit IgG or DyLight 800 goat anti-Mouse IgG was used as secondary Ab and high-end imaging was performed (ChemiDoc MP, BioRad).

Immunofluorescence

BMMs were fixed with 4% paraformaldehyde for 10min, followed by the incubation with 0.1% Triton X-100 in PBS. Cells were blocked with Protein Block, Serum-Free (X0909, Agilent, Santa Clara, CA) for 1h. Then chamber slides were stained with mouse anti-HuR Ab (Santa Cruz Biotechnology, Inc, Dallas, TX) or rabbit anti-HO-1 Ab (Enzo Life Sciences, Farmingdale, NY) respectively. Signals were visualized with secondary Alexa Fluor Abs.

RNA sequencing

Libraries for RNA-Seq were prepared with Kapa Stranded mRNA. The workflow consisted of mRNA enrichment, cDNA generation, and end repair to generate blunt ends, A-tailing, adaptor ligation, and PCR amplification. Different adaptors were used for multiplexing samples. The data was sequenced on HiSeq3000 using a single-read 50bp read run. The differential expression analyses between samples (siHuR +CM vs siControl +CM) were performed by Basepair: Next Generation Sequencing (NGS). The significantly differentially expressed genes were found with parameters P-value <= 0.001, and Fold change < -3 or >3. Using the list of significantly differentially expressed genes, the Canonical pathway analysis, Disease & Function analysis, and Networks analysis were performed by IPA.

Warm liver IRI models

We used an established mouse model of warm hepatic ischemia followed by reperfusion (4). Groups of mice were injected with heparin (100U/kg) and an atraumatic clip was used to interrupt the arterial and portal venous blood supply to the cephalad liver lobes. After 60min on a heating pad set to 30°C, the clip

was removed, and mice were sacrificed at 6h of reperfusion. The *in vitro* “warm” inflammation model depended on preconditioning primary cultured mouse hepatocytes for 12h with a cytokine mixture that included TNF-α (500U/ml), IFN-γ (100U/ml), IL1-β (10U/ml) and endotoxin LPS (5μg/ml) exposure.

Statistical Analysis

GraphPad Prism 8.0.1 was used for statistical analyses where SEM represents the mean value SD quotient relative the square root of N. Group comparisons were performed using Mann-Whitney U test for continuous values and Fisher’s exact test for categorical variables, respectively. Spearman's correlation coefficient (r) was used to evaluate the strength of linear relationship between variables. The cumulative graft survival rate was analyzed by Kaplan-Meier method, and differences between groups were compared using a log-rank test. A *p*-value of <0.05 was considered statistically significant.

References

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