Supplementary Materials

Macrophage heme oxygenase-1-SIRT1-p53 axis regulates sterile inflammation in liver ischemia-reperfusion injury

Kojiro Nakamura[1], Min Zhang[2], Shoichi Kageyama[1], Bibo Ke[1], Takehiro Fujii[1], Rebecca A. Sosa[3], Elaine F. Reed[3], Nakul Datta[1], Ali Zarrinpar[1], Ronald W. Busuttil[1], Jesus A. Araujo[2], Jerzy W. Kupiec-Weglinski[1]

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Methods

Clinical liver transplant study/sample collection

Twenty-one adult primary orthotropic liver transplant (OLT) recipients were recruited under IRB protocol (13-000143) between May 10, 2013 and April 6, 2015. Patients provided informed consent prior to their participation in the study. The demographic data and clinical parameters of recipients and donors are shown in Table S1. Routine standard of care and immunosuppressive therapy was administered as specified by UCLA liver transplant protocols. Study data were collected and managed using REDCap electronic data capture tools hosted at UCLA [1]. All donor organs, procured from donation after brain death with standardized techniques, were perfused with and stored in cold University of Wisconsin solution (ViaSpan; Bristol-Meyers Squibb Pharma, Garden City, NY). Cold ischemia time was defined as the time from the perfusion of the donor with preservation solution to the removal of the liver from cold storage. Recipient venous blood was collected prior to the transplant and at post-operative day 1-14 (POD1-14). Liver function was evaluated with standard of care tests, including, serum alanine aminotransferase (ALT), aspartate aminotransferase (AST) and total bilirubin (T-Bil). Protocol Tru-Cut needle biopsies (Bx) were obtained intra-operatively from the left lobe approximately 2h after portal reperfusion (prior to surgical closing of abdomen) and snap-frozen.

Animals

Male wild type (WT) mice (C57BL/6; 6-8 weeks of age) were obtained from The Jackson Laboratory (Bar Harbor, ME). Myeloid-specific HO-1 deficient (mHO-1 KO) and HO-1 transgenic (mHO-1 Tg) mice were produced at UCLA. Briefly, mHO-1 KO mice were generated by crossing floxed HO-1 KO mice [2] with lysM Cre transgenic mice [3] at the C57BL/6 background. Homozygous mice for both floxed and Cre transgenic alleles (HO-$1^{fl/fl}$, lysM $Cre^{+/+}$) were used as mHO-1 KO mice, while HO-$1^{fl/fl}$, lysM $Cre^{-/-}$ were employed as controls. The mHO-1 Tg mice were generated by crossing floxed HO-1 Tg mice [4] with lysM Cre transgenic mice [3] at the C57BL/6 background. Homozygous mice for the floxed and Cre transgene alleles (HO-$1^{fl/fl}$, lysM $Cre^{+/+}$) were used as mHO-1 Tg mice. Floxed HO-1 KO mice were generated by Mamiya et al [2] and obtained from RIKEN (stock number: RBRC03163). Floxed HO-1 Tg mice were generated and obtained from Dr. Agarwal [4]. LysM and Cre Tg mice were generated by Clausen et al [5] and obtained from Dr. Reddy (UCLA). Details on mouse characterization will be reported elsewhere (Zhang et. al., manuscript in preparation). Baseline HO-1 and SIRT1 expression in BMDM from WT, mHO-1 KO and mHO-1 Tg mice were comparable with BMDM + LPS setting (data not shown). Mice were housed in the UCLA facility under specific pathogen-free conditions. All animals received human care according to the criteria outlined in the Guide for the Care and Use of Laboratory Animals (NIH publication 86-23, revised 1985).

Reagents

Resveratrol (Res), lipopolysaccharide (LPS) were purchased (Sigma Aldrich, St. Louis, MO). Res, a naturally occurring polyphenol, activates SIRT1 to regulate cell stress response, metabolism and survival by deacetylating target proteins [6]. We used siRNA silencing to confirm Res effects were SIRT1-specific. Gene-specific siRNA against SIRT1, p19Arf, and p53 were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). The scrambled (control) siRNA were designed and synthesized (Qiagen, Valencia, CA), as described [7].

Liver IRI model

We used an established mouse model of partial warm hepatic IRI [8, 9]. Animals were anesthetized, injected with heparin (100U/kg), and an atraumatic clip was used to interrupt artery/portal venous blood supply to the left/middle liver lobes. After 90min of in situ ischemia, the clamp was removed and mice were sacrificed at 6h of reperfusion. Res (25mg/kg) or vehicle (15% ethanol, VHC) was administrated i.p. at 1h prior to ischemia. Sham-operated mice underwent the same procedure without vascular occlusion.

Serum biochemistry

Serum ALT and AST levels in mice, an indicator of hepatocellular injury, were measured by IDEXX Laboratories (Westbrook, ME).

Liver histology

Formalin-fixed, paraffin-embedded liver sections (5μm) were stained with hematoxylin and eosin (HE). The severity of liver IRI was assessed blindly and graded according to the Suzuki's criteria on a scale from 0 to 4. No necrosis, congestion/centrilobular ballooning is given a score of 0, while severe congestion and >60% lobular necrosis is given a value of 4 [10].

TdT-mediated dUTP nick end labeling (TUNEL) assay

Apoptosis in formalin-fixed paraffin-embedded murine liver sections was detected by TUNEL assay using ApopTag Plus Peroxidase in Situ Apoptosis Kit (Millipore, Temecula, CA) following the manufacturer's protocol. Results were scored semi-quantitatively by blindly counting the number of TUNEL-positive cells in 10 HPF/section (×400).

Immunohistochemistry

Immunofluorescence staining of HO-1 and hepatic macrophages in murine livers was performed using rabbit anti-HO-1 Ab (Enzo Life Sciences, Farmingdale, NY) and rat anti-CD68 Ab (AbD Serotec). Signals were visualized with secondary Ab: Alexa Fluor 488 anti-rabbit IgG and Alexa Fluor 594 anti-rat IgG (Molecular Probes, Eugene, OR). Immunofluorescent staining of HO-1, CD68 and TUNEL in human liver Bx samples was conducted using rabbit anti-HO-1 Ab (Enzo Life Sciences), mouse anti-CD68 Ab (BD Biosciences) and TUNEL Detection Kit (Sigma Aldrich), followed by visualizing with secondary Ab: Alexa Fluor 594 anti-rabbit IgG and Alexa Fluor 350 anti-mouse IgG. Immunofluorescence staining of CD11b, Ly6G and 4 Hydroxynonenal (4HN) was performed using rat anti-CD11b Ab (BD Biosciences), rat anti-Ly6G Ab (AbD Serotec) and rabbit anti-4HN Ab (Abcam, Cambridge, MA).

Cell isolations and in vitro cultures

Femurs and tibias were removed from WT, mHO-1 KO and mHO-1 Tg mice, and bone marrow-derived macrophages (BMDM) were generated, as described [9, 11]. The siRNA transfection for BMDM was conducted using Lipofectamine reagent (Invitrogen, San Diego, CA) according to the manufacturer's protocol. After 24h, cells were supplemented with LPS (100ng/ml) for 6h. In some experiments, cells were pretreated for 12h with Res (100μM) or vehicle (15% ethanol, VHC).

Western blot analysis

Proteins were extracted from frozen liver tissue samples, BMDM. Protein concentration was measured using BCA Protein Assay Kit (Thermo Fisher Scientific, Rockford, IL). Equal amount of protein extracts was electrophoresed, blotted, and incubated with primary Ab, secondary HRP-conjugated Ab, and developed. Primary Ab detecting HO-1 (Enzo Life Sciences), SIRT1, p53, p14, cleaved caspase 3 (Cell Signaling Technology, Danvers, MA), MDM2, β-actin (Santa Cruz Biotechnology) were used for human studies. Primary Ab against SIRT1, p-Stat1 (Tyr701), p-IkBα (Ser32), IkBα. iNOS, Ac-p53 (Lys379), PUMA, cleaved caspase 3 (Cell Signaling Technology), p19, p53, MDM2, HO-1 (H-105), β-actin (Santa Cruz Biotechnology), CD206 (R&D systems, Minneapolis, MN), IL10 (Abcam) were used for murine studies.

To compare target protein expression in multiple human OLT samples, densitometry quantification was conducted as follows. In a preliminary study, one of the Bx samples expressing all target proteins was chosen and assigned as a "reference" sample. Equal amount of protein lysate from each sample was applied to each well/gel, and the target band intensity was normalized by reference sample in the same gel, followed by normalization with β-actin.

Analysis of p53 ubiquitination

Immunoprecipitation of ubiquitinylated proteins was performed using the UbiQapture-Q Kit

(Enzo Life Sciences), following the manufacturer's instructions. Briefly, protein samples were harvested from BMDM and protein concentration was measured by BCA assay. Ubiquitinylated proteins were captured from 25µg of total proteins lysates using 40µl UbiQapture-Q matrix. Captured and uncaptured fraction samples were analyzed by Western blotting, as detailed above.

Quantitative RT-PCR analysis

Total RNA was extracted from frozen liver tissue samples or BMDM using RNAse Mini Kit (Qiagen). A total of 5.0µg of RNA was reverse-transcribed into cDNA. Quantitative PCR was performed using DNA Engine with Chromo 4 Detector (MJ Research, Waltham, MA) [12]. The primers sequences are listed in Supplementary Table 2. The expression of the target gene was normalized to the housekeeping gene HPRT or B2M.

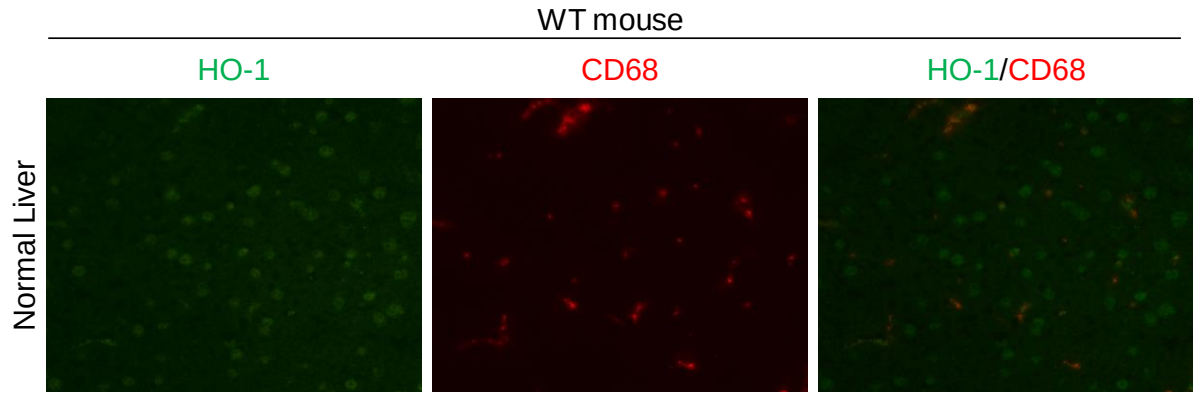
ELISA

ELISA assay to detect MCP1 was performed using mouse MCP-1 ELISA Kit (Thermo Fisher Scientific).

Statistics

In mouse experiments, differences between two groups or multiple groups were assessed using a Student *t*-test and 1-way ANOVA followed by Tukey's HSD test, respectively. For human data, continuous values were analyzed by Mann–Whitney U test and categorical variables by Fisher's exact test. $P<0.05$ was considered statistically significant. JMP for Windows 8.0 (SAS Institute, Cary, NC) was used for statistical analyses.

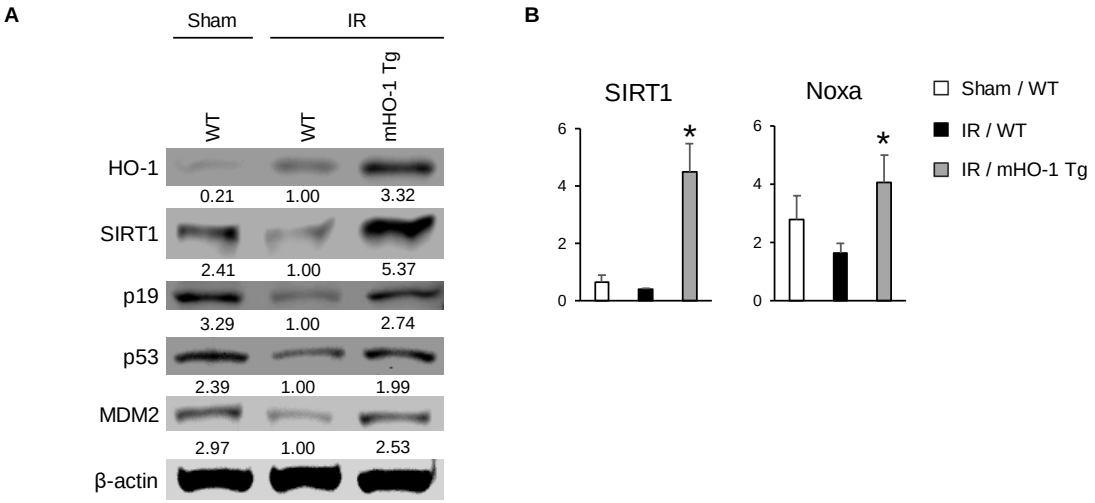
Supplementary Fig. 1



Supplementary Fig. 1: Localization of HO-1 in naive liver.

Immunofluorescence staining of HO-1 (left panel; green), CD68 hepatic macrophages (middle panel; red) and merged images (right panel). Representative of three experiments is shown.

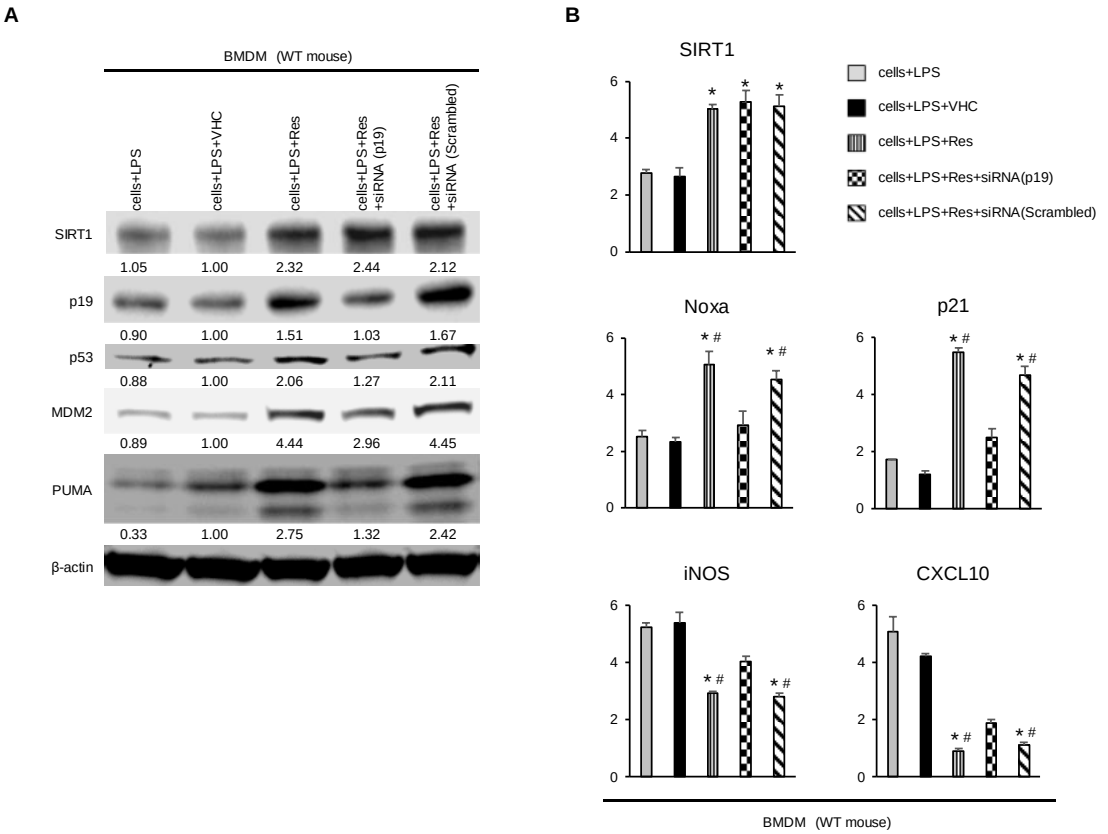
Supplementary Fig. 2



Supplementary Fig. 2: Myeloid-specific HO-1 overexpression upregulates SIRT1/p53 in vivo.

Groups of WT and myeloid-specific HO-1 transgenic (mHO-1 Tg) mice were subjected to 90min of liver partial warm ischemia followed by 6h reperfusion. (**A**) Western blot-assisted detection of HO-1, SIRT1, p19, p53 and MDM2. β-actin expression serves as an internal control for normalization. The values under the bands represent the relative ratio of normalized intensity compared to IR/WT. Representative of three experiments is shown. (**B**) Quantitative RT-PCR-assisted detection of mRNA coding for SIRT1 and Noxa. Data were normalized to HPRT gene expression (n=4/group) and presented as the means ± SD. * $p<0.05$ vs. IR/WT (1-way ANOVA).

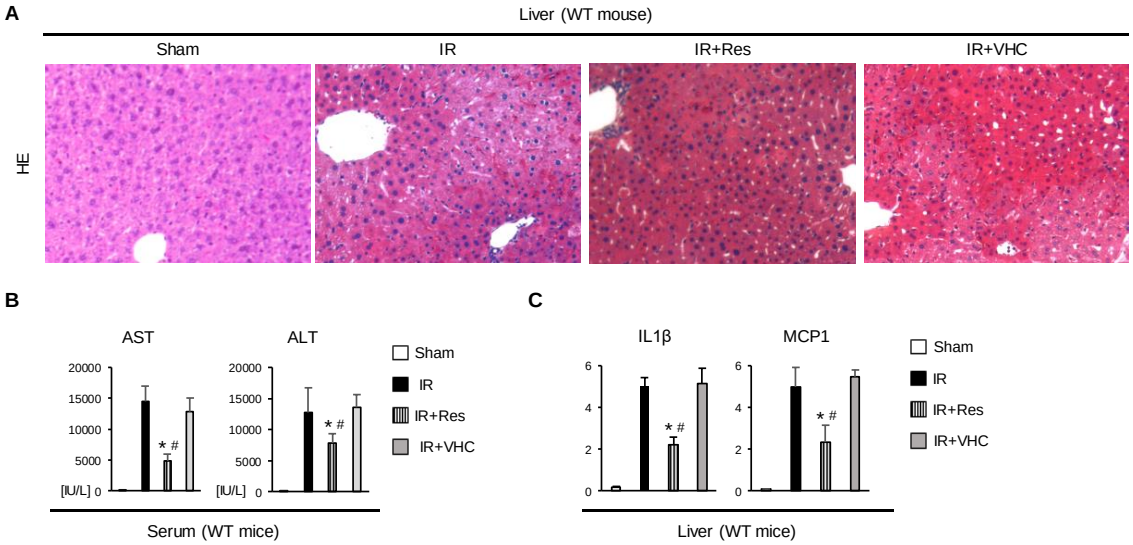
Supplementary Fig. 3



Supplementary Fig. 3: SIRT1 induces p53 and suppresses macrophage activation through p19.

LPS-stimulated BMDM (100ng/ml, 6h) were treated with Res (SIRT1 inducer, 100μM, 12h), vehicle (15% ethanol, VHC), p19-siRNA or scrambled-siRNA. (**A**) Western blot-assisted detection of SIRT1, p19, p53, MDM2 and PUMA. The intensity of the bands was normalized by dividing the target band intensity by that of housekeeping β-actin. The values under the bands represent the relative ratio of normalized intensity compared to that of cells + LPS + VHC. Representative of three experiments is shown. (**B**) Quantitative RT-PCR-assisted detection of mRNA coding for SIRT1, Noxa, p21, iNOS and CXCL10. Data were normalized to B2M gene expression (n=4/group). Data are presented as the means ± SD. * $p<0.05$ vs. cells + LPS + VHC, #$p<0.05$ vs. cells + LPS + Res + siRNA (p19) (1-way ANOVA).

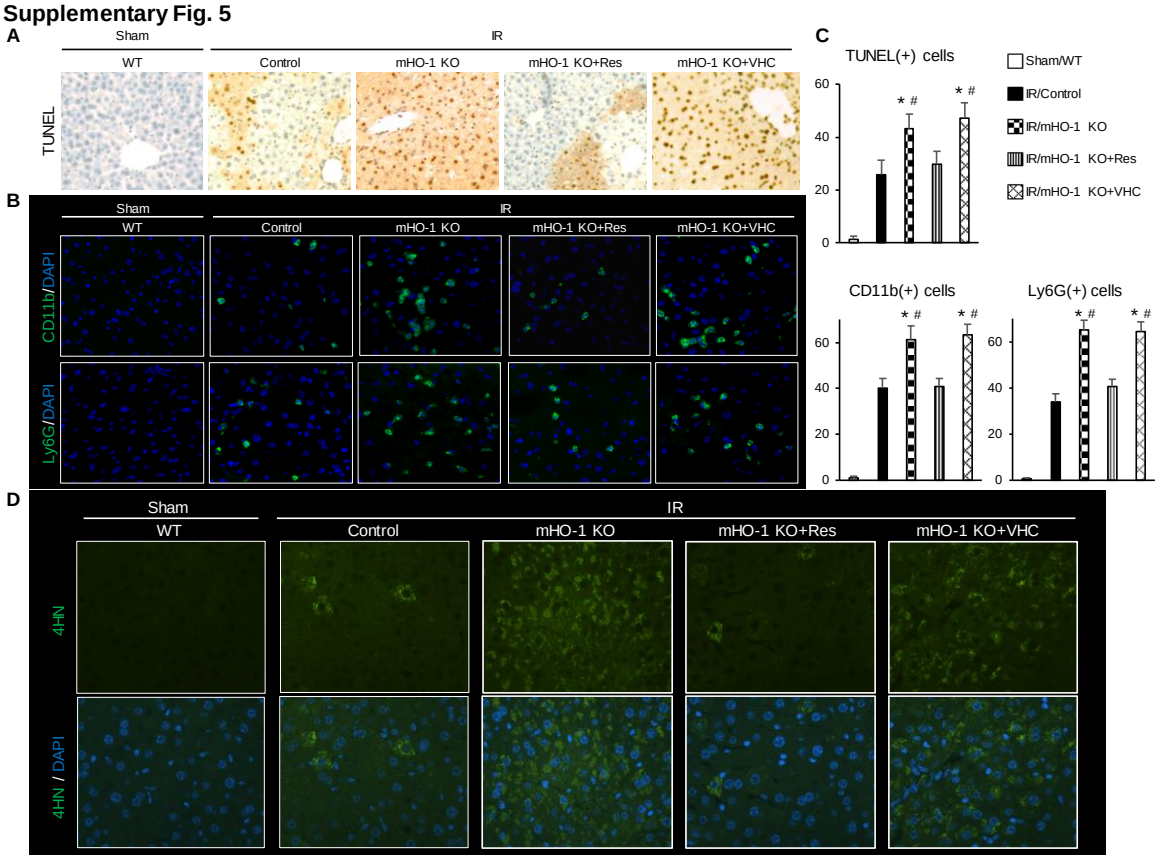
Supplementary Fig. 4



Supplementary Fig. 4: SIRT1 ameliorates hepatocellular damage and inflammation in IR-stressed livers.

Mice (C57/BL6; n=4-5/group) were subjected to 90min of liver partial warm ischemia followed by 6h reperfusion. Some animals were pretreated with SIRT1 inducer, Resveratrol (Res, 25mg/kg i.p.) or vehicle (VHC, 15% ethanol) at 1h prior to the ischemia insult. (**A**) Representative liver histology (original magnification, ×100) (**B**) Hepatocellular function evaluated by serum ALT and AST levels [IU/L] (n=4-5/group). (**C**) Quantitative RT-PCR-assisted detection of IL1β and MCP1. Data normalized to HPRT gene expression (n=4/group). Data are presented as the means ± SD. * $p<0.05$ vs. IR, # $p<0.05$ vs. IR+VHC (1-way ANOVA).

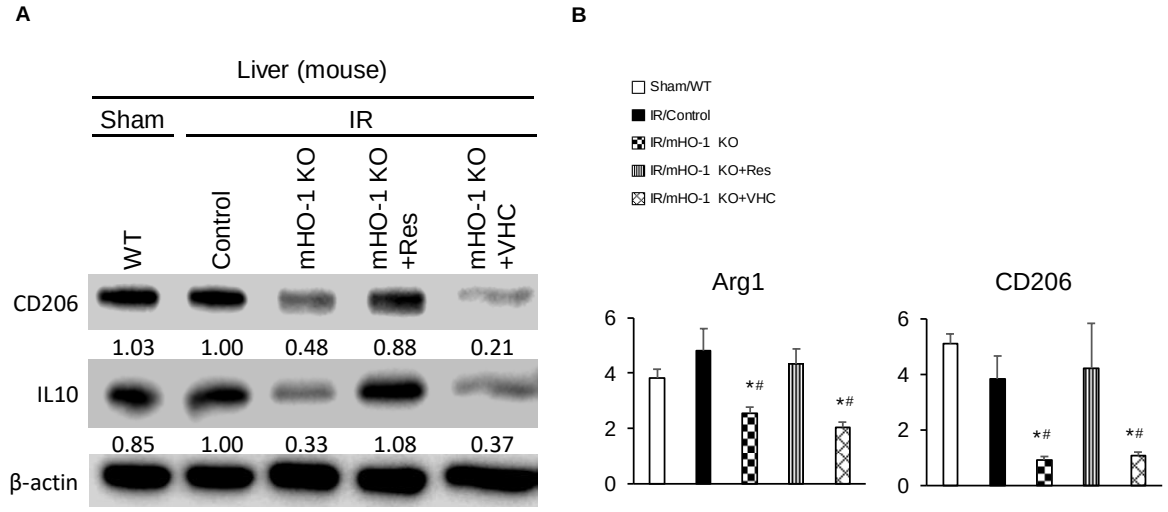
Supplementary Fig. 5



Supplementary Fig. 5: Myeloid-specific HO-1 deficiency enhanced hepatocellular apoptosis, leukocyte infiltration and oxidative stress while adjunctive SIRT1 activation conferred resistance to exacerbated local inflammation/liver damage.

Groups of control and myeloid-specific HO-1 knockout (mHO-1 KO) mice were subjected to 90min of liver partial warm ischemia followed by 6h reperfusion. Some mice were pretreated with Res (25mg/kg i.p. at -1h) or VHC. (**A**) Representative immunohistochemical staining of TUNEL+ cells (original magnification, ×400). (**B**) Representative immunohistochemical staining of CD11b+ and Ly6G+ liver infiltrating cells (original magnification, ×400). (**C**) Quantification of TUNEL, CD11b and Ly6G positive cells/HPF (n=4/group). Data are presented as the means ± SD. * p<0.05 vs. IR/Control, # p<0.05 vs. IR/mHO-1 KO+Res (1-way ANOVA). (**D**) Immunohistochemical staining of 4-Hydroxynonenal (4HN). Representative of three experiments is shown.

Supplementary Fig. 6



Supplementary Fig. 6: Myeloid-specific HO-1 deficiency depressed anti-inflammatory program in liver IRI while adjunctive Resveratrol (Res) restored anti-inflammatory phenotype.

Groups of control and myeloid-specific HO-1 knockout (mHO-1 KO) mice were subjected to 90min of liver partial warm ischemia followed by 6h reperfusion. Some mice were treated with Res (25mg/kg i.p. at -1h) or VHC. (**A**) Western blot-assisted detection of CD206 and IL10. β-actin expression serves as an internal control for normalization. The values under the bands represent the relative ratio of normalized intensity compared to IR/Control. Representative of three experiments is shown. (**B**) Quantitative RT-PCR-assisted detection of mRNA coding for Arg1 and CD206. Data were normalized to HPRT gene expression (n=4/group). Data are presented as the means±SD. * $p<0.05$ vs. IR/Control, # $p<0.05$ vs. IR/mHO-1 KO+Res (1-way ANOVA).

Supplementary table 1: Donor and recipient demographics.

Donor	high HO-1 (n=11)	low HO-1 (n=10)	p value
Age (years)	34.0±17.3	41.7±15.9	p=0.18
Gender (M/F)	6 / 5	6 / 4	p=1.00
Weight (kg)	77.9±16.7	82.8±10.3	p=0.36
BMI (kg/m^2)	26.2±5.5	29.3±4.7	p=0.18
Cold Ischemic Time (min)	441±160	487±100	p=0.32
Donation status	All of the donors were donation after brain death		

Recipient	high HO-1 (n=11)	low HO-1 (n=10)	p value
Age (years)	50.5±12.1	55.5±6.2	p=0.55
Gender (M/F)	8 / 3	6 / 4	p=0.66
Weight (kg)	82.4±30.5	92.2±27.2	p=0.26
BMI (kg/m^2)	27.9±6.9	31.0±7.8	p=0.43
Race			p=0.43
Asian	1	1	
Black/African American	0	0	
Caucasian/White	6	3	
Hispanic/Latino(a)	3	6	
other	0	1	
Disease ethiology			p=0.31
Alchol	4	1	
HCV	2	5	
NASH	1	2	
other	4	2	
HCC (with/without)	2 / 9	3 / 7	p=0.64
ABO			p=1.00
identical	10	9	
compatible	1	1	
MELD score	34.7±62	34.9±6.4	p=1.00
Pre-transplant ALT (IU/L)	55.3±33.9	61.7±45.4	p=1.00
Pre-transplant AST (IU/L)	115.5±88.7	87.1±57.6	p=0.62
Pre-transplant T-Bil (mg/dl)	14.6±14.9	18.7±14.2	p=0.53

Twenty-one post-transplant human liver biopsy samples were divided based on the relative HO-1 expression levels (high - n=11 or low - n=10). Correlations between donor and recipient demographic parameters and post-transplant HO-1 levels were analyzed using Fisher's exact test for categorical variables and Mann–Whitney U test for continuous values. Values are expressed as means ± SD.

Supplementary table 2: Primer sequences used for Real-Time Quantitative PCR

Gene	Forward	Reverse
HO-1	5’-TCCCAGACACCGCTCCTCCAG-3’	5’-GGATTTGGGGCTGCTGGTTTC-3’
SIRT1	5’-ATCGGCTACCGAGACAAC-3’	5’-GTCACTAGAGCTGGCGTGT-3’
Noxa	5’-CCCACTCCTGGGAAAGTACA-3’	5’-AATCCCTTCAGCCCTTGATT-3’
p21	5’-TCTCAGGGCCGAAAACGGAG-3’	5’-ACACAGAGTGAGGGCTAAGG-3’
TNFα	5’-GCCTCTTCTCATTCCTGCTTGT-3’	5’-GATGATCTGAGTGTGAGGGTCTG-3’
IL1β	5’-TGTAATGAAAGACGGCACACC-3’	5’-TCTTCTTTGGGTATTGCTTGG-3’
MCP1	5’-CATCCACGTGTTGGCTCA-3’	5’-GATCATCTTGCTGGTGAATGAGT-3’
iNOS	5’-GTTCTCAGCCCAACAATACAAGA-3’	5’-GTGGACGGGTCGATGTCAC-3’
CCL5	5’-CAGCAGCAAGTGCTCCAATCTT-3’	5’-TTCTTGAACCCACTTCTTCTCTGG-3’
CXCL10	5’-GCTGCCGTCATTTTCTGC-3’	5’-TCTCACTGGCCCGTCATC-3’
GzmB	5’-AGAGAGCAAGGACAACACTC-3’	5’-ATCGAAAGTAAGGCCATGTAG-3’
IL12p40	5’-TCATCAGGGACATCATCAAAC-3’	5’-TGAGGGAGAAGTAGGAATGGG-3’
Arg1	5'-GGAAAGCCAATGAAGAGCTG-3'	5'-GATGCTTCCAACTGCCAGAC-3'
CD206	5'-TGCAAGGATCATACTTCCCT-3'	5'-TGATGTTCTCCAGTAGCCAT-3'
HPRT	5’-TCAACGGGGGACATAAAAGT-3’	5’-TGCATTGTTTTACCAGTGTCAA-3’
B2M	5’-TGACCGGCTTGTATGCTATC-3’	5’-CACATGTCTCGATCCCAGTAG-3’

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