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ORIGINAL ARTICLE

AJT

Recipient HO-1 inducibility is essential for posttransplant hepatic HO-1 expression and graft protection: From bench-to-bedside

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By documenting potent antioxidative and anti-inflammatory functions, preclinical studies encourage heme oxygenase-1 (HO-1)-inducing regimens in clinical orthotopic liver transplantation (OLT). We aimed to determine the importance of recipient-derived HO-1 in murine and human OLTs. Hepatic biopsies from 51 OLT patients were screened for HO-1 expression (Western blots) prior to put-in (basal) and post reperfusion (stressed) and correlated with the hepatocellular function. In parallel, livers from HO-1 proficient mice (WT; C57/BL6), subjected to ex vivo cold storage (18 hour), were transplanted to syngeneic myeloid HO-1 deficient (mHO-1 KO) or FLOX (control) hosts, and sampled postreperfusion (6 hour). In human OLT, post-transplant but not pretransplant HO-1 expression correlated negatively with ALT levels ($P = .0178$). High posttransplant but not pretransplant HO-1 expression trended with improved OLT survival. Compared with controls, livers transplanted into mHO-1 KO recipient mice had decreased HO-1 levels, exacerbated hepatic damage/frequency of TUNEL+ cells, increased mRNA levels coding for TNF α /CXCL1/CXCL2/CXCL10, higher frequency of Ly6G+/4HN+ neutrophils; and enhanced MPO activity. Peritoneal neutrophils from mHO-1 KO mice exhibited higher CellRox+ ratio and increased TNF α /CXCL1/CXCL2/CXCL10 expression. By demonstrating the importance of posttransplant recipient HO-1 phenotype in hepatic macrophage/neutrophil regulation and function, this translational study identifies recipient HO-1 inducibility as a novel biomarker of ischemic stress resistance in OLT.

KEYWORDS

basic (laboratory) research/science, immunobiology, ischemia reperfusion injury (IRI), liver disease: immune/inflammatory, liver transplantation/hepatology, organ perfusion and preservation, protocol biopsy, tissue injury and repair, translational research/science

Abbreviations: 4HN, 4-hydroxynonenal; ALT, alanine aminotransferase; AST, aspartate transaminase; BMDM, bone marrow-derived macrophages; Bx, biopsy; CIT, cold ischemia time; GFP, green fluorescent protein; HE, hematoxylin and eosin; HO-1, heme oxygenase-1; IRI, ischemia-reperfusion injury; mHO-1, myeloid-specific HO-1; MPO, myeloperoxidase; OLT, orthotopic liver transplantation; POD, postoperative day; ROS, reactive oxygen species; Tg, transgenic; TUNEL, TdT-mediated dUTP nick end labeling; UW solution, University of Wisconsin solution; WT, wild type.

Shoichi Kageyama, Hirofumi Hirao, Kojiro Nakamura share coauthorship.

1 | INTRODUCTION

Orthotopic liver transplantation (OLT) has become the standard care for patients with end-stage liver disease and those with hepatic malignancies.¹ Ischemia-reperfusion injury (IRI), a leading cause of early graft dysfunction, represents a major risk factor in the development of acute/chronic rejection, and contributes to the shortage of donor organs.² Thus, novel IR-targeted strategies are needed to improve clinical outcomes and expand donor organ pool. Despite its clinical importance, however, the mechanisms that account for liver IRI are not fully appreciated.³

Liver IR damage, an innate immune-driven inflammation, is followed by the hepatocellular death. The cellular IR stress primes secretion of damage-associated molecular patterns, which trigger inflammatory cytokines to further hepatocyte death. In addition to macrophages, neutrophils also serve as early effectors in hepatic IRI by generating/diffusing reactive oxygen species (ROS) and disturbing homeostasis to trigger mitochondrial dysfunction and cell death.^{4,5} Indeed, systemic neutrophil depletion attenuated liver IRI by suppressing hepatic neutrophil accumulation.⁶

Heme oxygenase-1 (HO-1; *HMOX1*; *hsp32*), the inducible isoform of heme oxygenase catalyzing the degradation of heme into biliverdin, iron, and carbon monoxide, exerts antioxidative and anti-inflammatory functions.⁷ We reported on benefits of HO-1 induction in hepatic IRI rodent models, using gene transfer,^{8,9} and macrophage-based therapies.^{10,11} With macrophage recognized as a key mediator of innate inflammation, HO-1 cytoprotection is attributed to its regulation in IR-stressed liver.^{12,13} Others have shown that cobalt protoporphyrin (chemical HO-1 inducer) decreased neutrophil superoxide production and suppressed neutrophil migration,^{14,15} implying neutrophil regulation as an integral part of the HO-1 cytoprotective phenotype. However, by decreasing BACH1 and increasing NRF2 protein levels, cobalt protoporphyrin acts indirectly on HO-1,¹⁶ whereas gene specific HO-1 function in neutrophil regulation remains to be defined.

We have reported that posttransplant HO-1 expression negatively correlated with the severity of IRI in liver transplant patients.¹¹ Because macrophages are the main source of HO-1 in IR-stressed livers,¹² posttransplant HO-1 phenotype in OLT may include liver-resident (Kupffer cell, donor origin) and liver-infiltrating (recipient origin) macrophages. Indeed, Devey et al reported that Kupffer cells may dictate the hepatic HO-1 levels in a mouse warm liver IRI model.¹⁷ By contrast, in a mouse cold IRI-OLT model, adjunctive infusion of HO-1-overexpressing bone marrow-derived macrophages (BMDM) increased graft HO-1 levels; whereas HO-1-silenced (siRNA) BMDM decreased graft HO-1 expression, as compared with unmodified BMDM infusion.^{10,18} Although in murine models, liver-infiltrating host BMDM may affect HO-1 expression/function at the graft site, it remains unknown whether recipient-derived HO-1 may influence hepatic HO-1 levels and IRI severity in OLT. In humans, genetic HO-1 induction seems to govern its expression profile, with

studies focusing on donor basal HO-1 levels, and no insights into recipients' HO-1 inducibility.^{19,20}

In this study, we analyzed whether recipient-derived HO-1 may affect liver graft HO-1 levels and function. In the experimental arm, we used myeloid-specific HO-1 deficient mice as recipients of HO-1 proficient (WT) livers to highlight how HO-1 macrophage/neutrophil regulation may affect IRI in OLT. Our clinical arm reinforces the importance of recipient HO-1 phenotype by documenting the need for pretransplant HO-1 enhancement in OLT protection. In the context of preclinical studies paving the way for HO-1 cytoprotective regimens and the need of future inclusion criteria for clinical responders, the current study provides important insights into the role of recipient HO-1 inducibility as a novel biomarker of hepatocellular resistance against IR stress in liver transplantation.

2 | METHODS

2.1 | Clinical liver transplant study

Fifty-one adult primary OLT recipients were recruited under an institutional review board protocol (13-000143; May 2013–August 2015).^{11,21} 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 Management System. Livers were perfused with and stored in University of Wisconsin (UW) solution (ViaSpan; Bristol-Meyers Squibb Pharmaceuticals, New York City, NY). Cold ischemia time was defined as the time from the perfusion of the donor liver with UW solution to its removal from the cold storage for implantation. Recipient venous blood was collected within the hour prior to the transplant and on postoperative day 1 (POD1). The hepatocellular injury was evaluated by serum alanine aminotransferase (sALT). Protocol Tru-Cut needle biopsies (Bx) from the left hepatic lobe were obtained during back-table preparation (prior to the put-in); and 2 hours after portal reperfusion (prior to the abdominal closure).

2.2 | Animals

Myeloid-specific HO-1 deficient (mHO-1 KO; C57BL/6) mice were generated.²² In brief, floxed HO-1 KO mice were crossed with *lysM* (lysozyme M) Cre transgenic mice.²³ Homozygous mice for floxed and Cre transgenic alleles (HO-1^{fl/fl}, *lysM* Cre^{+/+}) were used as mHO-1 KO, and HO-1^{fl/fl}, *lysM* Cre^{-/-} served as controls.¹² We confirmed depressed HO-1 expression in liver-resident and bone marrow-derived macrophages from mHO-1 KO mice. Wild-type (WT; C57BL/6) and green fluorescent protein (GFP) transgenic (Tg) mice were obtained from Jackson Laboratory (Bar Harbor, ME). Animals were housed at UCLA under pathogen-free conditions and received humane care according to criteria outlined in the "Guide for the Care and Use of Laboratory Animals" (National Institutes of Health [NIH] publication 86-23 revised 1985).

2.3 | Mouse orthotopic liver transplantation

We used a mouse model of ex vivo hepatic cold storage and transplantation.^{21,24} To mimic “marginal” human OLT, donor livers were stored in UW solution at 4°C for 18 hours prior to transplant into syngeneic recipients. Liver graft and serum were collected at 6 hours of reperfusion, the peak of hepatocellular damage in this model. The sham group underwent the same procedures except for OLT.

2.4 | Hepatocellular function assay

Mouse serum alanine transaminase (sALT) and aspartate transaminase (sAST), an indicator of hepatocellular injury, were measured by IDEXX Laboratories (Westbrook, ME).

2.5 | OLT histology and IRI grading

Formalin-fixed paraffin-embedded liver sections (5 μ m) were stained with hematoxylin and eosin (H&E). The severity of IRI was graded using Suzuki’s criteria.²⁵

2.6 | TdT-mediated dUTP nick end labeling (TUNEL) assay

Cell death in formalin-fixed paraffin-embedded liver sections (5 μ m) was detected by Apop Tag 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.

2.7 | Immunofluorescence

Mouse livers were stained with rabbit anti-CD11b Ab (Abcam, Cambridge, MA), rat anti-Ly6G Ab (BD Biosciences, San Jose, CA), rabbit anti-4-hydroxynonenal Ab (Abcam), rabbit anti-HO-1 Ab (Enzo Life Sciences, Farmingdale, NY), and rat anti-CD68 Ab (Bio Rad, Hercules, CA). Human livers were stained with rabbit anti-HO-1 Ab (Enzo Life Sciences) and mouse anti-CD68 Ab (BD Biosciences). Signals were visualized with secondary Alexa Fluor Abs. Liver-infiltrating CD11b+ and Ly6G+ cells were scored semiquantitatively by blindly counting positive cells in 10 HPF/section (x400).

2.8 | Bone marrow-derived macrophage culture

Bone marrow-derived macrophages were generated, as described.¹⁰ In brief, bone marrow cells were obtained from the femurs and tibias, cultured in 15% L929-conditioned medium for 7 days, and used for in vitro study.

2.9 | Neutrophil isolation and flow cytometry

Casein-elicited peritoneal neutrophils were purified by Percoll density gradient centrifugation (Neutrophil Isolation Kit, Cayman Chemical, Ann Arbor, MI).²⁶ Neutrophils were incubated in a 5 μ M

concentration of the CellROX Green Reagent (Invitrogen, Carlsbad, CA) and stained with the surface marker of PerCP/Cy5.5 conjugated Ly6G (BioLegend, San Diego, CA). Multiparameter FACS was performed using a SORP BD LSRII analytic flow cytometer (BD Bioscience) and results were analyzed using BD FACSDiva software (BD Bioscience).

2.10 | Western blot assay

Proteins were extracted from tissue/cell samples and their concentration measured using BCA Protein Assay Kit (Thermo Scientific, Waltham, MA). An equal amount of protein was electrophoresed, blotted, incubated with primary Ab, secondary HRP-conjugated Ab, and developed. Primary Ab detecting HO-1 (Enzo Life Sciences, Farmingdale, NY), cleaved caspase 3, and β -actin (Cell Signaling Technology, Danvers, MA) were used. To compare protein expression in multiple human OLT samples, densitometry quantification was conducted as reported.^{12,27} Briefly, in a preliminary study, one of the Bx samples expressing all target proteins was chosen and assigned as a “control” sample. Equal amount of protein lysate from each sample was applied to each well/gel, and the target band intensity was expressed as relative band intensity to that of the positive control in the same gel. The target relative protein value was normalized according to β -actin intensity.

2.11 | Quantitative RT-PCR analysis

RNA extracted with RNase Mini Kit (Qiagen, Germantown, MD) was reverse-transcribed into cDNA. PCR was performed using QuantStudio 3 (Applied Biosystems, Foster City, CA). The primer sequences are listed (Table S1). The target gene expression was normalized to housekeeping HPRT or β -actin.

2.12 | ELISA

Serum MCP1/TNF α concentration was measured by ELISA kits (Thermo Scientific), according to the manufacturer’s protocol.

2.13 | Myeloperoxidase (MPO) activity assay

The presence of MPO was used as an index of neutrophil accumulation in the liver. The change in absorbance was measured spectrophotometrically at 655 nm. One unit of MPO activity was defined as the quantity of enzyme degrading 1 μ mol peroxide per minute at 25°C per gram of tissue.²⁸

2.14 | Statistical analysis

Group comparisons were performed using a Student *t* test for mouse experiments, and Mann-Whitney U test was used for human data. Spearman’s correlation coefficient (*r*) was used to evaluate the strength of linear relationship between variables. The cumulative survival rate was analyzed by Kaplan-Meier method, and differences

were compared using a log-rank test. JMP for Windows 8.0 (SAS Institute, Cary, NC) was used for statistical analyses. A $P < .05$ was considered statistically significant.

3 | RESULTS

3.1 | Posttransplant but not pretransplant HO-1 expression negatively correlates with hepatocellular function in human OLT

Although Buis et al reported that pretransplant hepatic high HO-1 expression associated with improved human OLT survival,²⁰ others failed to identify increased pretransplant HO-1 level as a biomarker of preserved OLT function.¹⁹ Having documenting negative correlation between posttransplant HO-1 levels and IRI severity in clinical OLT,^{11,12} we now aimed to compare the relationship between pretransplant/posttransplant HO-1 expression and IR-triggered hepatocellular damage in 51 OLT patients. Pretransplant liver Bx was

collected after cold storage (prior to put-in) and posttransplant Bx was obtained 2 hours after portal reperfusion (prior to the abdominal closure) (Figure 1A). Representative pretransplant (basal) and posttransplant (IR-induced) Western blot-assisted HO-1 expression profiles are shown in Figure 1B. There was no significant correlation between basal hepatic HO-1 expression and donor demographics, preprocurement blood tests, duration of brain ischemia, or cold ischemia times (Table S2). We also found no significant correlation between posttransplant HO-1 levels and donor/recipient demographics, preoperative blood tests, duration of brain or cold ischemia time, race, disease etiology, presence of hepatocellular carcinoma, ABO-compatibility, model for end-stage liver disease (MELD) score, pretransplant dialysis, or intra-operative blood loss (Table S3). Unlike basal HO-1 levels, which failed to significantly correlate with sALT at POD1 ($r = -0.2138$, $P = .1320$, Figure 1C), posttransplant HO-1 correlated negatively with sALT at POD1 ($r = -0.3306$, $P = .0178$, Figure 1D). This indicates that posttransplant rather than basal HO-1 levels were essential for improved hepatocellular function in OLT recipients.

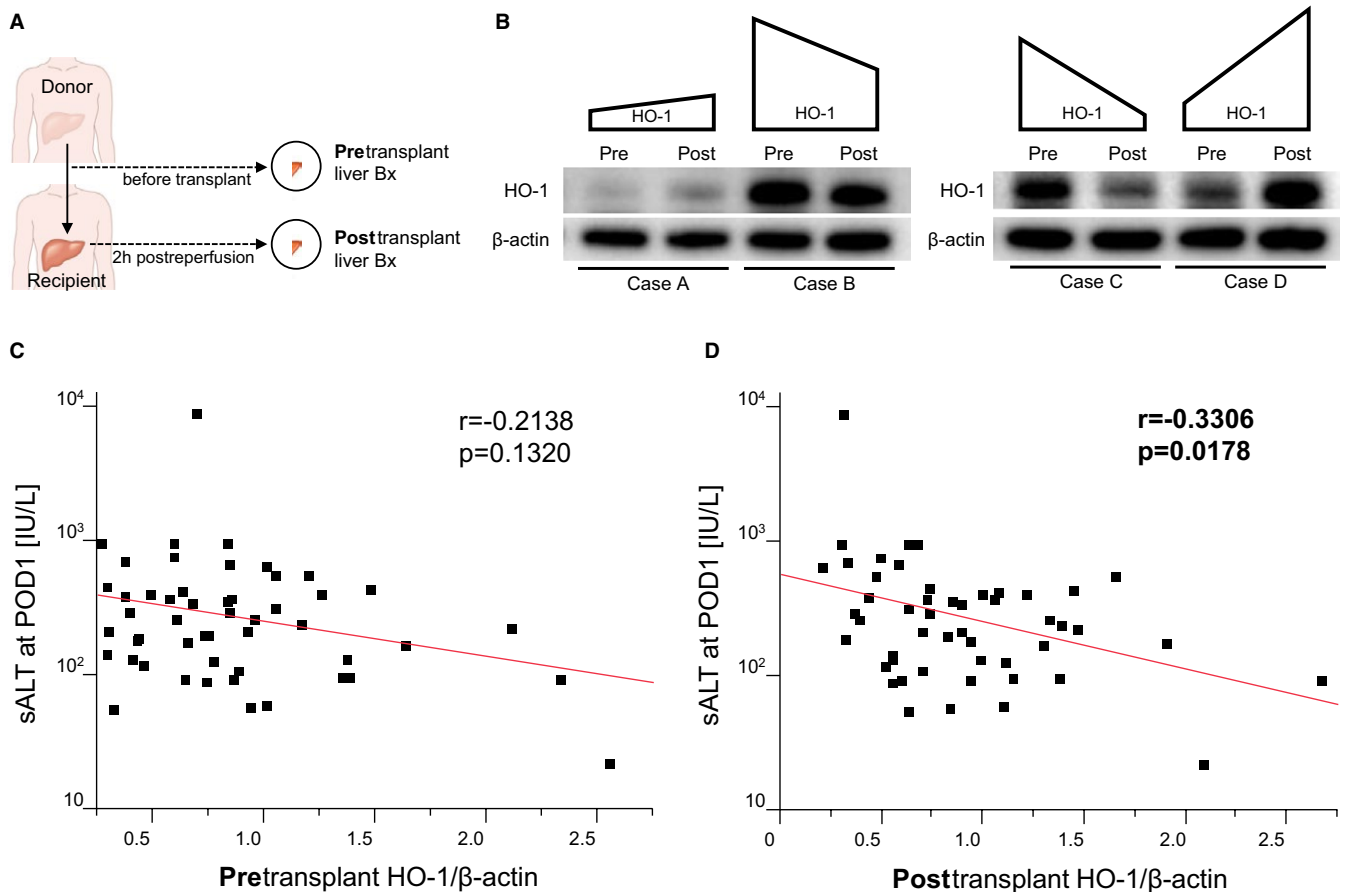


FIGURE 1 Posttransplant but not pretransplant graft HO-1 expression correlates negatively with the hepatocellular damage in liver transplant patients. (A) Pretransplant (prior to put-in) and posttransplant (2 hours after reperfusion) protocol liver biopsies (Bx) were collected from 51 liver transplant patients. HO-1 expressions in Bx samples were analyzed by Western blots with β -actin normalization, as described in Methods. (B) Representative perioperative HO-1 profiles (Pre: pretransplant, Post: posttransplant, case A vs case B: pretransplant HO-1 level rather than perioperative HO-1 increase determined posttransplant HO-1 expression; case C vs case D: perioperative HO-1 enhancement rather than pretransplant HO-1 was crucial for posttransplant HO-1 level). (C) Relationship between pretransplant HO-1 and sALT level at postoperative day 1 (POD1). (D) Relationship between posttransplant HO-1 level and sALT level at POD1. r , Spearman's correlation coefficient [Color figure can be viewed at wileyonlinelibrary.com]

We next evaluated the influence of pretransplant vs posttransplant hepatic HO-1 levels on recipient OLT survival, with a median follow-up of 740 days (range, 4-1432 days). None of the patients underwent secondary liver transplantation. Based on Western blot-assisted basal HO-1 quantification, patients were classified into pre-low ($n = 26$) and pre-high ($n = 25$) HO-1 expression groups (Figure 2A). There was a tendency for pre-high HO-1 having inferior survival as compared with pre-low HO-1 group (2-year: pre-high = 81.1% vs pre-low = 92.4%; $P = .2356$; Figure 2B). However, when OLT patients were divided into post-low ($n = 26$) and post-high ($n = 25$) HO-1 expression groups (Figure 2C), the latter showed a trend toward improved survival, compared with the post-low HO-1 cohort (2-year: post-high = 92.6% vs pre-low = 80.7%; $P = .1963$; Figure 2D). Despite lacking statistical significance (Figure 2B/D), these findings are consistent with the notion that posttransplant HO-1 expression profile is likely important for OLT protection against IR-stress, whereas pretransplant basal HO-1 levels seem to be a less reliable predictor of the clinical outcome (Figure 1C/D).

3.2 | Both pretransplant HO-1 levels and peritransplant HO-1 enhancement are essential for posttransplant HO-1 levels

Having demonstrated the importance of posttransplant HO-1 levels in graft cytoprotection, we envisioned two putative scenarios.

Comparing clinical case A and B (Figure 1B left panel), despite IR-stress enhanced graft HO-1 in case A but not in case B, the HO-1 level was lower in case A after reperfusion, indicating basal HO-1 but not perioperative HO-1 increase determined posttransplant HO-1 phenotype. In contrast, comparing case C and D (Figure 1B right panel), although basal HO-1 expression was higher in case C, marked HO-1 enhancement after reperfusion was noted in case D but not in case C, suggesting perioperative HO-1 increase but not basal HO-1 was crucial for posttransplant HO-1 levels. To verify the importance of these two factors, we tested the relationship between pretransplant and posttransplant HO-1 expression (Figure 3A) as well as the correlation between posttransplant/pretransplant HO-1 ratio (an indicator of peritransplant HO-1 enhancement) and posttransplant HO-1 expression (Figure 3B). Posttransplant HO-1 expression significantly correlated with basal HO-1 levels ($r = 0.5564$, $P < .0001$), implying pretransplant HO-1 expression influenced posttransplant HO-1 levels. On the other hand, despite posttransplant/pretransplant HO-1 ratio was calculated with division by basal HO-1 (which showed strong correlation with posttransplant HO-1 [Figure 3A]), the posttransplant HO-1 levels nonetheless showed significant correlation with posttransplant/pretransplant HO-1 ratio ($r = 0.3696$, $P = .0076$, Figure 3B). Thus, posttransplant HO-1 expression was dictated not only by basal HO-1 steady state but also by IR-stress triggered HO-1 increase.

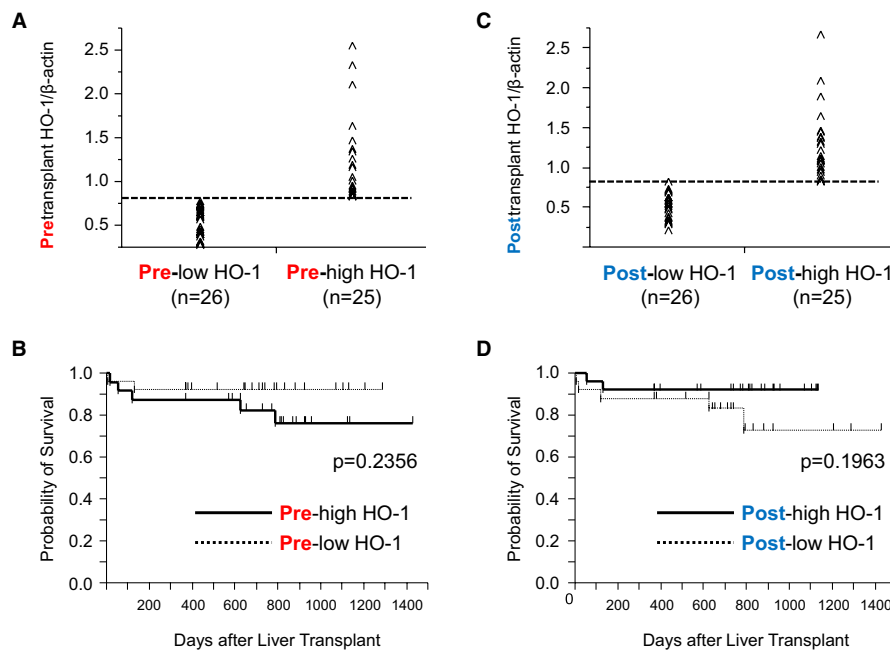


FIGURE 2 Relationship between basal or posttransplant HO-1 expression and liver transplant patient survival. Pretransplant (prior to put-in) and posttransplant (2 hours after reperfusion) liver biopsies (Bx) were collected from 51 liver transplant patients. HO-1 expression in Bx samples was analyzed by Western blots with β -actin normalization. (A) Based on Western blot-assisted HO-1 expression in pretransplant liver Bx, human OLT recipients were classified into “pre-low HO-1” ($n = 26$) and “pre-high HO-1” ($n = 25$) groups. (B) The cumulative probability of posttransplant survival (Kaplan-Meier method). Solid line indicates pre-high HO-1, and the dotted line pre-low HO-1 groups (log-rank test). (C) Based on Western blot-assisted HO-1 expression in posttransplant liver Bx, recipients were classified into “post-low HO-1 ($n = 26$)” and “post-high HO-1 ($n = 25$)” groups. (D) The cumulative probability of posttransplant survival (Kaplan-Meier method). Solid line indicates post-high HO-1, and the dotted line post-low HO-1 groups (log-rank test) [Color figure can be viewed at wileyonlinelibrary.com]

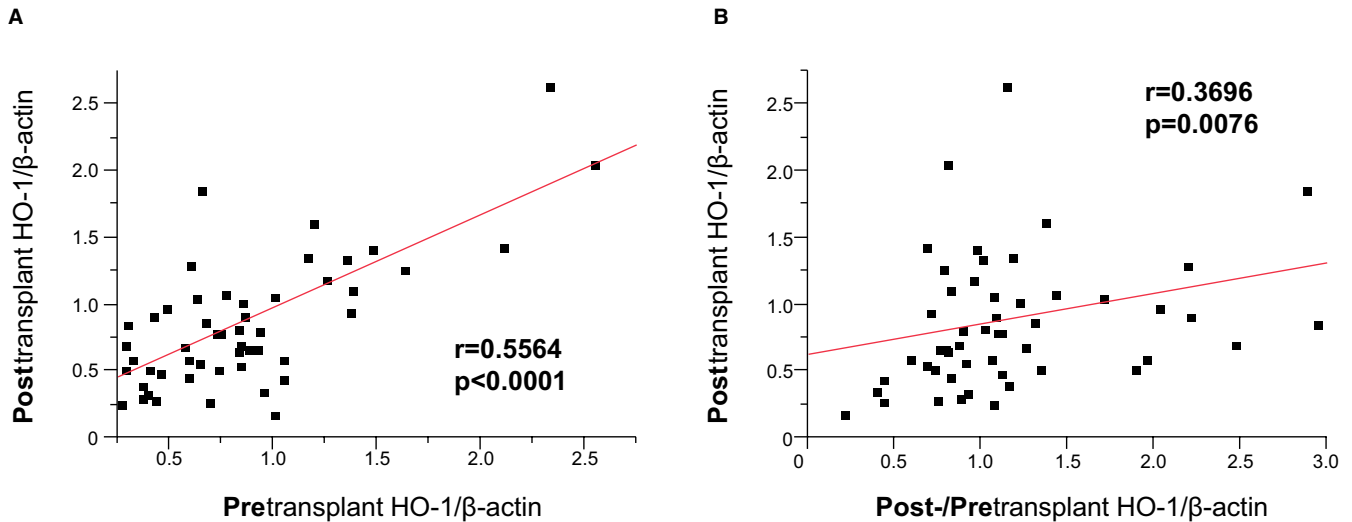


FIGURE 3 Both basal HO-1 level and peritransplant HO-1 enhancement are important for postreperfusion hepatic HO-1 expression in liver transplant patients. Pretransplant (prior to put-in) and posttransplant (2 hours after reperfusion) liver biopsies (Bx) were collected from 51 liver transplant patients. HO-1 expression in Bx samples was analyzed by Western blots with β -actin normalization. (A) Relationship between pretransplant HO-1 and posttransplant HO-1 levels. (B) Relationship between posttransplant/pretransplant HO-1 ratio and posttransplant HO-1 level. r , Spearman's correlation coefficient [Color figure can be viewed at wileyonlinelibrary.com]

3.3 | Recipient myeloid HO-1 deficiency decreases HO-1 expression in mouse OLT

Consistent with published data,^{12,19} we found CD68 macrophages are the primary source of HO-1 in IR-stressed human and mouse OLT (Figure S1). Because postreperfusion liver grafts contain both donor-origin resident (Kupffer cells) and recipient-origin infiltrating macrophages, theoretically both cell types can produce HO-1 and contribute to IR-triggered peritransplant HO-1 enhancement, which is one of posttransplant HO-1 determinant factors (Figure 3B). However, with previous studies focused on HO-1 expression in donor-derived resident macrophages,^{17,19,20} the importance of recipient-derived infiltrating macrophages on graft HO-1 expression remains to be elucidated. To determine HO-1 origin (donor vs recipient) in OLT, we first transplanted WT livers, subjected to 18 hours cold storage, into GFP-Tg recipient mice. By 6 hours of reperfusion, we observed HO-1 expression in donor-derived GFP-negative cells (Figure S2A/S2b) as well as in recipient-derived GFP-positive cells (Figure S2C/S2d), indicating both donor- and recipient-origin macrophages were the source of HO-1 in IR-stressed OLT. Next, to determine the impact of recipient HO-1 on graft HO-1 expression, we transplanted HO-1 proficient WT livers (cold-stored for 18 hours) into groups of myeloid-specific HO-1 knockout (mHO-1 KO) vs HO-1 proficient (control) mice. We confirmed that compared with controls, HO-1 protein was almost undetectable in BMDM cultures from mHO-1 KO mice (Figure S3). As shown in Figure 4A, myeloid-specific HO-1 recipient deficiency decreased graft HO-1 levels by almost half at 6 hours of reperfusion as compared with controls, indicating IR-stressed recipient macrophage HO-1 was a critical determinant for posttransplant graft HO-1 levels. On the other hand, HO-1 levels in WT livers

transplanted into mHO-1 KO mice were enhanced by fivefold as compared with sham livers, indicating liver-resident macrophages achieved fivefold HO-1 enhancement in IR-stressed OLT without recipient-derived macrophage HO-1. Hence, liver-resident macrophage HO-1 as well as recipient macrophage HO-1 are both essential for posttransplant hepatic HO-1 phenotype.

3.4 | Recipient myeloid HO-1 deficiency accelerates hepatocellular damage in mouse OLT

We next asked whether suppression of HO-1 at the graft site, resulting from recipient myeloid HO-1 deficiency, may influence IR-damage in OLT. At 6 hours postreperfusion, WT livers transplanted to mHO-1 KO mice displayed enhanced sinusoidal congestion, edema/vacuolization and hepatocellular necrosis, as compared with OLT in HO-1 proficient controls (Figure 4B). This correlated with increased Suzuki's histological grading of liver IRI (control = 3.5 ± 0.6 vs mHO-1 KO = 6.0 ± 1.4 , $n = 4-5$, $P = .0066$, Figure 4C); elevated sALT/sAST levels (sALT: control = 8895 ± 5411 vs mHO-1 KO recipient = $23\,880 \pm 13\,891$ IU/L, $P = .0370$; sAST: control = 5984 ± 2433 vs mHO-1 KO = $12\,405 \pm 4323$ IU/L, $P = .0142$; $n = 4-5$, Figure 4C); enhanced cleaved caspase-3 ($P = .0077$, Figure S4A); and increased frequency of TUNEL+ cells ($P = .0011$, Figure S4B). Thus, suppression of graft HO-1 levels due to recipient myeloid HO-1 deficiency exacerbated IR damage in OLT.

3.5 | OLTs in mHO-1 KO recipients exhibit increased proinflammatory IR-signature

As the release of proinflammatory cytokines is critical for the continuum of immune cascade culminating in the hepatocellular death,

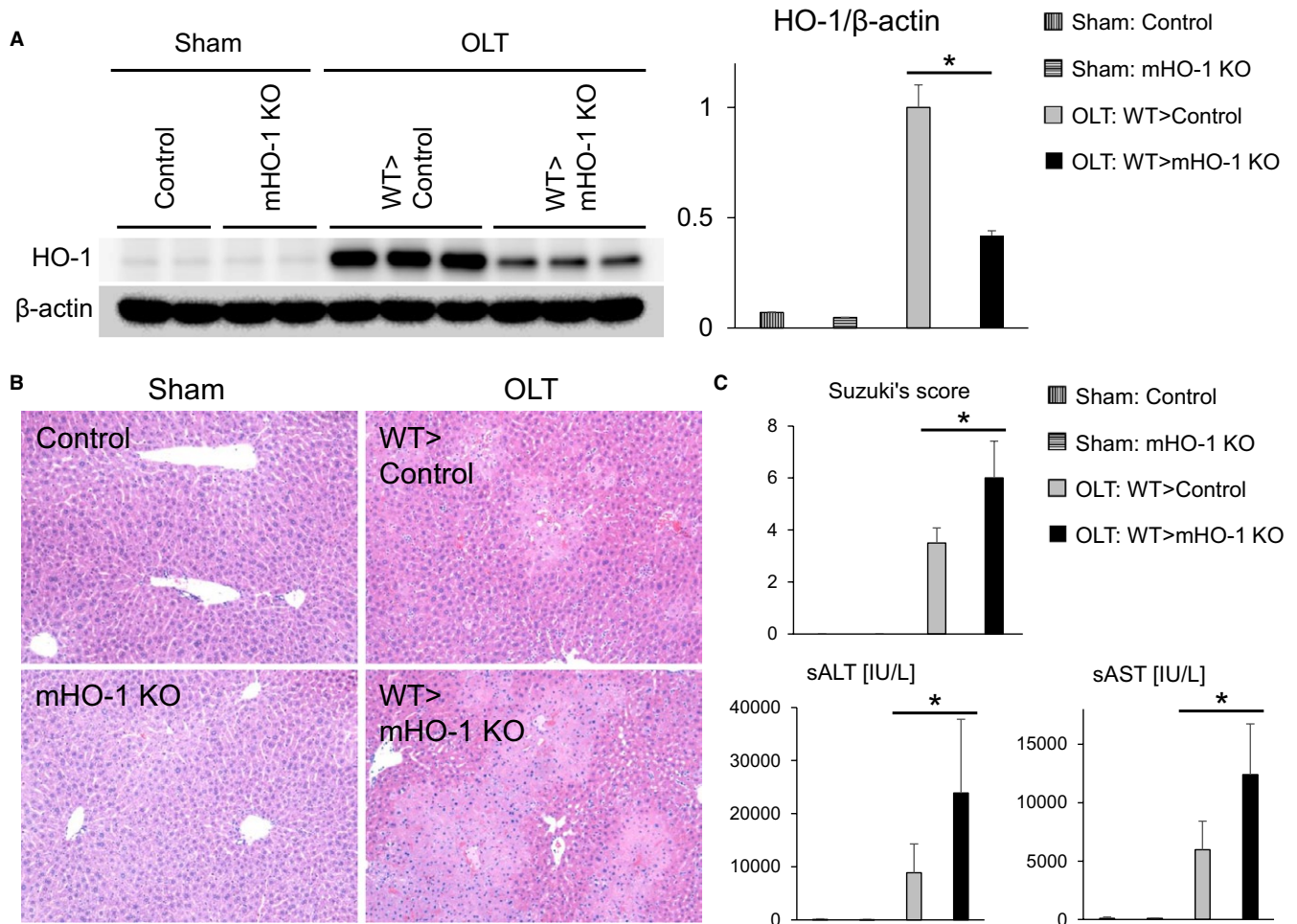


FIGURE 4 Recipient myeloid HO-1 deficiency decreases graft HO-1 expression and aggravates hepatocellular damage in mouse OLT. WT mouse (C57/Bl6) livers subjected to 18 hours of cold storage were transplanted orthotopically to HO-1 proficient control and myeloid-specific HO-1 knockout (mHO-1 KO) recipient mice. Liver grafts and serum samples were analyzed at 6 hours post-OLT. The sham group underwent the same procedures except for OLT. (A) Western blot-assisted detection and relative intensity ratio of HO-1. β -actin expression served as an internal control and used for normalization. (B) Representative H&E staining (original magnification, $\times 100$). (C) Serum ALT/AST levels (IU/L) and Suzuki's histological grading of liver IRI ($n = 3\text{-}5/\text{group}$). Data shown as mean \pm SD ($*P < .05$, Student *t* test) [Color figure can be viewed at wileyonlinelibrary.com]

we next focused on cytokine profile in our IRI-OLT model. At 6 hours postreperfusion, WT livers transplanted into mHO-1 KO recipients showed higher levels of TNF α , MCP1, CXCL1, CXCL2 and CXCL10 ($P < .05$, Figure 5A/B), and increased frequency of infiltrating macrophages (CD11b; $P = .0002$, Figure S5A/B). These *in vivo* findings were corroborated by BMDM cultures (data not shown), consistent with the notion that HO-1 inhibits macrophage proinflammatory phenotype,^{12,13} a dominant HO-1 producer (Figure S1) and the key regulator of innate immune response in IR-stressed livers. Thus, recipient myeloid HO-1 deficiency enhanced inflammation in IR-stressed OLT.

3.6 | Recipient myeloid HO-1 deficiency enhances neutrophil activation in mouse OLT

As macrophages play a critical role in IRI, exacerbated inflammation in WT livers transplanted to mHO-1 KO recipients (Figure 5A/B) could be most likely attributed to disrupted macrophage regulation

by HO-1.^{12,13} However, by producing cytotoxic ROS, neutrophils may also be essential in IRI-OLT pathogenesis. With their role largely understudied, we then focused on the impact of HO-1 deficiency upon the function of OLT-infiltrating neutrophils. By 6 hours of reperfusion, OLTs in mHO-1 KO mice exhibited increased neutrophil (Ly6G) sequestration (Figure S5A/B, $P < .0001$) and higher MPO activity (Figure S5C, $P = .0013$), as compared with controls. Enhanced neutrophil OLT sequestration in mHO-1 KO recipients was accompanied by increased 4-hydroxynonenal (4HN) expression, one of the key oxidative metabolites (Figure 6).

3.7 | HO-1 deficient neutrophils exhibit increased ROS and proinflammatory phenotype

We next focused on the neutrophil function in our model by analyzing the influence of HO-1 disruption in casein-elicited peritoneal neutrophil population. Indeed, Ly6G⁺ sorted neutrophils from mHO-1 KO mice were characterized by increased levels of ROS as

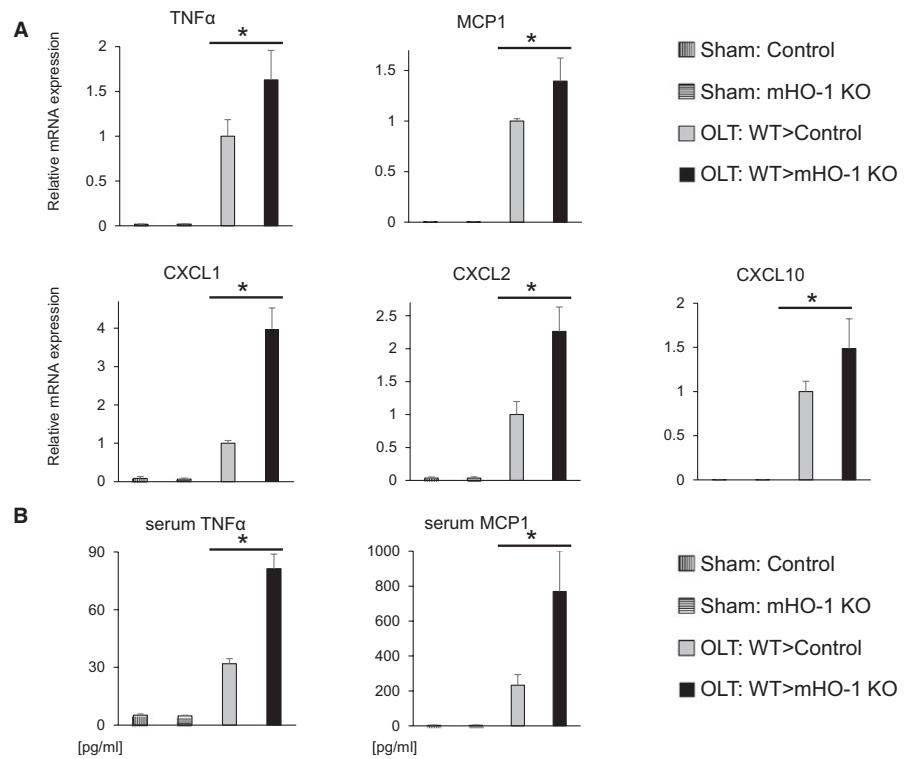


FIGURE 5 Recipient myeloid HO-1 deficiency enhances inflammatory response in IR-stressed mouse OLT. WT mouse livers subjected to 18 hours of cold storage were transplanted into HO-1 proficient control and myeloid-specific HO-1 knockout (mHO-1 KO) recipient mice, followed by serum/graft sampling at 6 hours after reperfusion. (A) qRT-PCR-assisted detection of mRNA coding for TNF α , MCP1, CXCL1, CXCL2, and CXCL10 in OLTs. Data were normalized to HPRT gene expression ($n = 3-4$ /group). (B) ELISA-assisted examination of serum TNF α and MCP1 levels (pg/mL, $n = 3-5$ /group). Data shown as mean \pm SD ($*P < .05$, Student t test)

compared with those from HO-1 proficient (control) mice, evidenced by CellRox staining by FACS (Figure 7A, $P = .0021$). In addition, neutrophils from mHO-1 KO mice displayed enhanced mRNA levels coding for TNF α , CXCL1, CXCL2, CXCL10, and concomitantly depressed HO-1 (Figure 7B, $P < .05$). These findings indicate neutrophil HO-1 is essential to regulate ROS and proinflammatory gene programs.

4 | DISCUSSION

This is the first study, to the best of our knowledge, which demonstrates the hepatoprotective function of recipient myeloid cell-specific HO-1. In the clinical arm, we found that posttransplant low HO-1 level was a reliable predictor of exacerbated hepatic IR-damage (Figure 1D). Posttransplant HO-1 reflected pretransplant HO-1 expression (Figure 3A), whereas the relationship between sALT (POD1) and basal HO-1 was weaker as compared with posttransplant HO-1 levels (Figure 1C/D), implying that steady state HO-1 level was not a singular decisive factor for posttransplant HO-1 phenotype. To bridge the gap, we have identified perioperative HO-1 increase as one of the critical factors for postreperfusion HO-1 expression in human OLT (Figure 3B). In the experimental arm, recipient myeloid HO-1 deficiency increased hepatic tissue injury histology scores, release of liver enzymes (Figure 4C), cell death (Figure S4), proinflammatory cytokine phenotype (Figure 5), and leukocyte infiltration in IR-stressed mouse OLT (Figure S5). To establish macrophage HO-1 regulatory axis,^{12,13} we now show the regulatory function of HO-1 in neutrophil ROS and proinflammatory gene expression programs (Figure 7). Taken together, our study documents

the importance of recipient HO-1 inducibility (ie, the ability to trigger HO-1 under IR-stress) for perioperative HO-1 enhancement and OLT cytoprotection.

Prolonged cold ischemia time (CIT) represents an independent risk factor for OLT loss.²⁹ In our current 51 human OLT cohort, livers with CIT < 8 hours showed significantly better post-OLT survival as compared with those subjected to CIT ≥ 8 hours (Figure S6A, $P < .05$). Although HO-1 is a stress-inducible gene, we found no correlations between pretransplant/posttransplant HO-1 levels and CIT (Figure S6B-S6E), suggesting CIT is an unlikely determinant factor for graft HO-1 expression. Moreover, we found no significant correlations between graft HO-1 levels and other clinical parameters, including liver function (AST/ALT/T-Bil/INR), duration of brain ischemia, MELD score, intraoperative blood loss, all of which possibly reflect stress severity (Table S2/S3). In contrast, others have reported that cerebral cortex/hippocampus HO-1 levels in normal human brains showed a significant positive correlation with age,³⁰ whereas unstressed livers from aged mice had significantly more HO-1 compared with young counterparts.³¹ Likewise, despite lacking statistical correlation, pretransplant HO-1 levels in our clinical study trended toward a positive correlation with donor age ($r = 0.2771$, $P = .0514$, Table S2), whereas the relationship between donor age and HO-1 weakened in postreperfusion biopsies ($r = 0.1422$, $P = .3245$, Table S3). Noteworthy, human liver grafts with perioperative HO-1 increase (at 2 hour postreperfusion) had significantly lower preoperative HO-1 levels (Figure S7). Similarly, Geuken et al reported that human liver grafts with abundant pretransplant HO-1 showed decreased HO-1 expression after reperfusion (by 23%), whereas those with low pretransplant HO-1 levels were able to induce HO-1 at reperfusion.¹⁹

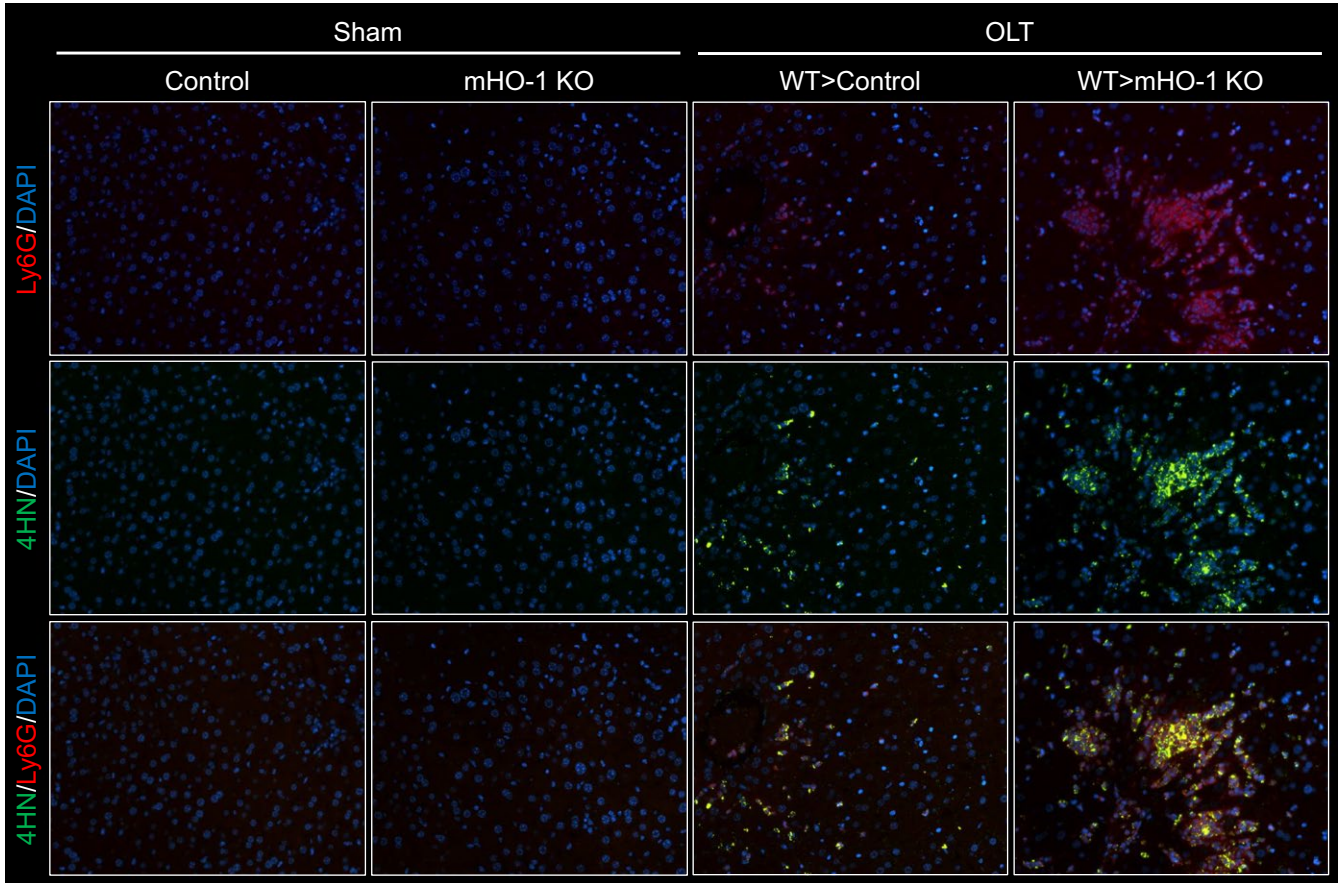


FIGURE 6 Recipient myeloid HO-1 deficiency increases neutrophil 4-Hydroxynonenal (4HN) expression in mouse OLT. WT mouse livers subjected to 18 hours of cold storage were transplanted into HO-1 proficient control and myeloid-specific HO-1 knockout (mHO-1 KO) recipient mice, followed by hepatic sampling at 6 hours after reperfusion. Immunohistochemical detection of Ly6G (red), 4HN (green), and DAPI (blue) in OLTs. The sham group underwent the same procedures except for OLT. Representative of three experiments is shown [Color figure can be viewed at wileyonlinelibrary.com]

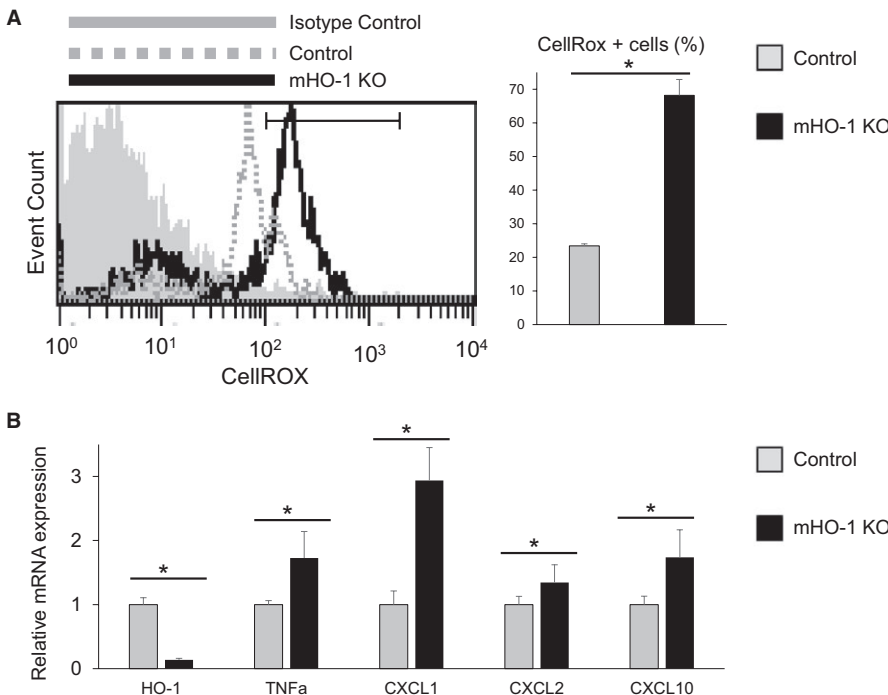


FIGURE 7 Neutrophil HO-1 deficiency enhances reactive oxygen species (ROS) production and inflammatory gene phenotype. Casein-elicited peritoneal neutrophils were obtained from HO-1 proficient control and myeloid-specific HO-1 knockout (mHO-1 KO) mice. (A) ROS level in neutrophil was analyzed by flow cytometry using fluorescent CellROX probe (n = 4/group). (B) qRT-PCR-assisted detection of mRNA coding for HO-1, TNF α , CXCL1, CXCL2, and CXCL10. Data were normalized to β -actin gene (n = 4/group). Data shown as mean \pm SD (*P < .05, Student t test)

As unlike in “young” mice, oxidative stress failed to increase hepatic HO-1 levels in “old” mice,³¹ donor age might be one of the factors contributing to discrepant HO-1 dynamics and the aforementioned gap between pretransplant and posttransplant HO-1 levels.

Human HO-1 gene expression is modulated by two functional polymorphisms in the gene promoter. First, a short (GT)_n repeat polymorphism has been associated with enhanced transcriptional HO-1 activity.³² Indeed, a short (GT)_n repeat in the kidney graft was accompanied by a favorable posttransplant renal function and survival.^{33,34} Second, A(-413)T single nucleotide polymorphism (SNP) has also been identified as a functionally relevant variation of the HO-1 gene, whereas A-allele rather than T-allele of this SNP correlated with a higher promoter activity.³⁵ Interestingly, two studies^{19,20} failed to find a correlation between donor (GT)_n repeat polymorphism and pretransplant HO-1 mRNA level in OLT, whereas donor livers with at least one A-allele of A(-413)T SNP could be characterized by higher pretransplant mRNA levels coding for HO-1.²⁰ This suggests that A(-413)T SNP may be dominant over (GT)_n repeat polymorphism in the hepatic HO-1 promoter activity. Our study suggests the importance of recipient HO-1 inducibility in the mechanism of liver graft protection. A single clinical study reported to date, examining the impact of recipient (GT)_n repeat polymorphism but without looking into HO-1 levels, failed to show significant differences on OLT outcomes.³⁶ Further studies on the impact of two functional polymorphisms in organ donor and recipient on posttransplant HO-1 expression levels in OLT patients, are warranted. With previous studies focusing on donor HO-1 polymorphisms, a possibility that recipient polymorphisms may influence HO-1 function in the graft itself is a novel and attractive idea. Indeed, determining the recipient HO-1 polymorphism could be useful to identify prospective transplant patients with poor perioperative HO-1 inducibility and then utilize, if needed, HO-1 induction regimens beforehand to minimize the risk of a subsequent allograft failure.

We acknowledge the limitations of our study. First, despite recipient HO-1 inducibility affecting postreperfusion graft HO-1 levels and IRI severity in mouse OLT, we were unable to examine donor/recipient gene polymorphisms in the current patient cohort, or analyze whether putative varieties of HO-1 inducibility in human OLT are indeed decisive to graft HO-1 levels/clinical outcomes. In addition, we found no significant clinical factors related to graft HO-1 levels (Table S2/S4). Second, although posttransplant HO-1 levels negatively correlated with sALT at POD1 (Figure 1D, $r = -0.3306$, $P = .0178$), the survival differences between post-high and post-low HO-1 groups failed to reach statistical significance (Figure 2D, $P = .1963$). Moreover, although OLTs experiencing biopsy-proven rejection ($n = 5$) had lower HO-1 expression as compared with rejection-free counterparts ($n = 46$), the differences failed to reach statistical significance (data not shown). As many factors influence post-OLT patient/graft survival, encompassing analyses of possible confounders in large patient cohort are required to conclusively determine the importance of HO-1 signaling for OLT clinical outcomes.

We have reported on macrophage HO-1 regulatory functions in the mechanism of liver IRI,^{12,13,37} Consistently, IR-stressed mHO-1

KO livers as well as BMDM cultures generated from mHO-1-deficient mice exhibit increased M1 and decreased M2 gene expression programs as compared with controls (Zhang and Nakamura, unpublished data), confirming that HO-1 signaling can drive the phenotypic shift to anti-inflammatory M2 phenotype.^{37,38} Of note, HO-1 was highly expressed in hemorrhage-specific macrophages (Mhem), a newly identified antioxidative and anti-inflammatory subset in human atherosclerotic plaques.^{39,40} Because activating transcription factor 1 drives macrophage adaptation to intraplaque hemorrhage while inducing HO-1, HO-1 may well be crucial for atheroprotective Mhem function.⁴⁰ Thus, mHO-1 KO mouse used in the current study may be useful to further investigate distinct macrophage states while searching for new immunomodulatory approaches in widely diverse human diseases.

Although Kupffer cells are principal ROS producers early post-reperfusion, neutrophil oxidative burst becomes the main source of ROS in the later IRI phase.^{41,42} Indeed, by 6 hours of reperfusion (the peak of hepatocellular damage in our murine model), OLT-infiltrating Ly6G-positive neutrophils elaborated large amounts of ROS metabolite, 4HN (Figure 6), the levels of which along the frequency of CellROX in casein-elicited peritoneal neutrophils, increased further in myeloid-deficient HO-1 hosts (Figure 7A). These findings, consistent with our data on neutrophil regulation,^{43,44} reinforce the role of neutrophil-targeted therapy against liver IRI. Although neutrophil depletion (mAb clone: 1F12) prior to the ischemia insult did not affect hepatocellular damage, despite reducing their infiltration by 60%, repeated mAb treatment alleviated IRI, implying early neutrophil regulation was insufficient for hepatoprotection.⁴⁵ Paradoxically, disruption of neutrophil signaling (mAb clone: 1F12) was accompanied by activation of Kupffer cells to release toxic oxygen-derived metabolites *in vivo* and *in vitro*,⁴⁶ whereas neutrophil depletion (mAb clone: 1A8)⁴⁷ decreased sALT levels at 4 hours postreperfusion.⁴⁸ Moreover, adoptive transfer of WT but not TLR4/TRL9 deficient neutrophils, recreated neutrophil extracellular traps (NETs) and cell damage indicating the importance of TLR-dependent netosis in the pathogenesis of liver IRI.⁴⁹ By shedding light on unappreciated role of neutrophils in the early IRI phase and novel neutrophil-NETs axis in hepatic inflammatory enhancement, these findings further advance the therapeutic potential of neutrophil management in OLT.

In conclusion, this translational study documents the importance of recipient myeloid HO-1 in postreperfusion HO-1 function, neutrophil regulation, and graft protection against IR stress. In the context of encouraging preclinical data on HO-1 inducing regimens in organ transplantation, and the need of inclusion criteria for prospective clinical responders, our study highlights the recipient HO-1 inducibility as one of potentially important biomarkers of hepatic resistance against IR-stress in OLT.

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CONFLICTS OF INTEREST

The authors of this manuscript have no conflicts of interest to disclose as described by the *American Journal of Transplantation*.

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SUPPORTING INFORMATION

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