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NATIVE MACROPHAGES GENETICALLY MODIFIED TO EXPRESS HEME OXYGENASE-1 PROTECT RAT LIVER TRANSPLANTS FROM ISCHEMIA/REPERFUSION INJURY

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Abstract

We investigated whether native macrophages overexpressing heme oxygenase-1 (HO-1) may protect rat orthotopic liver transplants (OLT) from cold ischemia/reperfusion injury (IRI). Livers from SD rats were stored at 4 C in University of Wisconsin (UW) solution for 24 h, and then transplanted to syngeneic recipients. Bone marrow-derived macrophages (BMMs) transfected ex-vivo with Ad-HO-1, Ad-s-gal or HO-1siRNA were infused directly to OLTs before reperfusion. Controls consisted of OLT conditioned with unmodified or scrambled siRNA-transfected cells. Transfer of Ad-HO-1/BMMs increased OLT survival to 100% (vs. 40–50% in controls), decreased sALT levels and histological features of the hepatocellular damage. In contrast, infusion of macrophages transfected with HO-1 siRNA/Ad-s-gal failed to affect IRI. Gene therapy-induced HO-1 suppressed TLR4 expression, decreased pro-inflammatory TNF- α , IL-1 β , MCP-1, CXCL-10, and attenuated endothelial ICAM-1 expression with resultant diminished OLT leukocyte sequestration. Although Ad-HO-1/BMMs decreased the frequency of TUNEL+ apoptotic cells and ameliorated caspase-3 activity, the expression of IL-10 and anti-apoptotic Bcl-2/Bcl-xL increased in well-functioning OLTs. Thus, transfer of native macrophages transfected ex vivo with HO-1 can rescue rat iso-OLTs from IRI. Our study validates a novel and clinically attractive concept in which native macrophages transfected ex vivo with anti-oxidant HO-1 can be applied at the time of transplantation, to mitigate otherwise damaging Ag-independent liver inflammation and damage resulting from the peri-transplant “harvesting” insult. If proven effective in allo-OLT recipients, this refined new strategy should be considered in the clinical settings to increase the supply of usable donor organs and ultimately improve the overall success of liver transplantation.

Keywords

Liver ischemia/reperfusion injury; liver transplantation; heme-oxygenase-1; toll-like receptor-4; gene therapy

Introduction

Liver transplantation has been established as the definitive therapy for patients with end-stage liver disease (1). However, the full potential for liver replacement is far from being realized because increasing number of donor livers become discarded due to a poor quality.

Indeed, “marginal” livers are particularly susceptible to ischemia and reperfusion injury (IRI) (2). The IRI syndrome represents major component of the “harvesting” insult in organ transplantation. Ag-independent hepatocellular damage surrounding liver removal, storage and engraftment is critical to primary nonfunction or early dysfunction of orthotopic liver transplants (OLT). Moreover, IRI may influence allo-immune responses and the development of late OLT dysfunction leading to chronic rejection. Hence, minimizing the adverse effects of IRI should improve short and long term OLT outcomes, as well as increase the donor pool and thus number of patients that successfully undergo transplantation.

The mechanisms of liver IRI in OLT recipients involve complex interactions that include Kupffer cell activation, cytokine release, neutrophil activation, increased endothelial expression of adhesion molecules, sinusoidal endothelial cell death, followed by hepatocyte death (3,4). Although the etiology of post-OLT failure is multifactorial, the oxidative stress contributes directly to the initial hepatocellular damage through protein oxidation/degradation and subsequent innate inflammation responses. Heme oxygenase-1 (HO-1), a rate-limiting stress-responsive protein in heme catabolism, provides potent cytoprotective defense response against oxidative injury (5). Back in 1999 our group was the first to demonstrate the efficacy of HO-1 induction to ameliorate rat liver damage due to cold ischemia and subsequent reperfusion in OLT recipients (6). Although local HO-1 induction exerts potent anti-inflammatory and anti-apoptotic cytoprotective functions in several experimental transplant models (7), new clinically applicable, safe and effective therapeutic regimens need to be explored. Recently, we have shown the efficacy of *ex vivo* modified HO-1 expressing primary bone marrow-derived macrophages (BMMs) in a mouse model of liver damage due to warm IR (8). However, as both “warm” and “cold” components of the “procurement” insult contribute to the pathophysiology of hepatic IRI during liver transplantation, in this study we employed an established rat liver model of prolonged (24 h) hepatic cold ischemia followed by orthotopic syngeneic transplant (6,9). We investigated the therapeutic potential of and putative mechanisms by which *ex-vivo* HO-1 transfected native BMMs affect IR-mediated OLT outcomes, the hepatocellular function, and associated local innate inflammation responses.

Materials and Methods

BMMs

Bone marrow cells, isolated from donor rat femurs and tibias, were cultured (5×10^7 /well) in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum (Gemini Bio-Products), 100 U/ml penicillin, 100 µg/ml streptomycin, and 2 mmol/l l-glutamine (Invitrogen), with the addition of 15% L929-conditioned medium for 7 days with 5% CO₂ and 95% air at 37°C.

Ex-vivo transfection

Five $\times 10^7$ cells/well were incubated with Ad-HO-1 or Ad-β-gal (10) for 1 h in Dulbecco's modified Eagle's medium + 2% fetal calf serum. Cells were transfected for 24 h with HO-1 siRNA or scrambled siRNA by using lipofectamine 2000 reagent (Invitrogen). After washing, transfected cells were harvested for *in-vivo* transfer. For *in-vitro* gene expression assay, cells were incubated with Cobalt protoporphyrin (CoPP; 10 µg/ml; Porphyrin Products, Logan, UT), a known HO-1 inducer (11).

Animals

Male Sprague–Dawley (SD) rats (220–280g, Harlan Sprague–Dawley, Inc., San Diego, CA) were maintained under conditions approved by the UCLA Chancellor's Animal Research

Committee. Animals were housed in micro-isolator cages in virus-free facilities and fed laboratory chow *ad libitum*.

Liver transplantation and *in-vivo* BMMs transfer

Livers from SD rat donors were stored at 4° C in UW solution for 24 h, and then transplanted orthotopically into syngeneic recipients, as described (6,9). Five $\times 10^7$ of BMMs transfected *ex-vivo* with Ad-HO-1 or Ad- β -gal (2.5×10^9 pfu) were infused to OLT via portal vein immediately (<1 min) prior to reperfusion. Separate OLT groups were treated with HO-1 siRNA/BMMs, scrambled HO-1 siRNA/BMMs or unmodified BMMs. The vast majority of deaths occur within 48 h post-transplant due to liver failure in this syngeneic rat OLT model.

In-vitro and *in-vivo* X-Gal staining

Five $\times 10^7$ BMMs/well were transfected with Ad- β -gal for 48 h, and stained with X-Gal (5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside). Cells expressing β -gal cleave X-Gal to yield a blue chromophore in the cytoplasm. For *in vivo* X-Gal staining, OLT were harvested at day 1, embedded with O.C.T. freezing compound, and snap-frozen in liquid nitrogen. Cryosections (5- μ m thick) were fixed with 1.25% glutaraldehyde at 4 °C, and stained with X-Gal at 37 °C (19). Representative areas were scored with light microscope for percentage of infected (blue) cells.

Hepatocellular function assay

Serum alanine aminotransferase (sALT) levels, indicator of hepatocellular injury, were measured using an auto analyzer (ANTECH Diagnostics, Los Angeles, CA).

Histology

OLT specimens were fixed in 10% buffered cut into 5- μ m section, and stained with hematoxylin and eosin (H&E). Histological severity of liver injury was graded using Suzuki's criteria where sinusoidal congestion, hepatocyte necrosis/ballooning degeneration are graded from 0 to 4 (12). No necrosis, congestion/centrilobular ballooning is given a score of 0, whereas severe congestion/ballooning degeneration with >60% lobular necrosis is given a value of 4.

MPO activity assay

The presence of MPO was used as an index of neutrophil OLT accumulation (13). The change in absorbance was measured spectrophotometrically at 655 nm (Bio-tek Instruments, Winooski, VT). One unit of MPO activity was defined as the quantity of enzyme degrading 1 μ mol peroxide/min/gram of tissue at 25°C.

Quantitative RT-PCR

Quantitative PCR was performed using the DNA Engine with Chromo 4Detector (MJ Research, Waltham, MA). In a final reaction volume of 20 μ L, the following were added: 1 $\times$ SuperMix (Platinum SYBR Green qPCR Kit, Invitrogen, Carlsbad, CA), complementary DNA, and 10 μ M of each primer. Amplification conditions were: 50°C (2 min), 95°C (5 min), followed by 50 cycles of 95°C (15 sec), 60°C (30 sec). Primers used to amplify specific gene fragments have been listed (14,15). Target gene expressions were calculated by their ratios to the housekeeping gene HPRT.

Apoptosis assay

Apoptosis in 5- μ m OLT paraffin sections was detected by the TUNEL method using the FragEL™ DNA Fragmentation Detection kit (Calbiochem, Gibbstown, NJ) according to the manufacturer's protocol. Negative control was prepared by omission of terminal deoxynucleotidyl transferase. Positive controls were generated by treatment with DNase. TUNEL-positive cells were counted in 10 HPF/section under light microscopy ($\times 200$).

Caspase-3 activity assay

Caspase-3 activity was determined by an assay kit (Calbiochem, La Jolla, CA), and by measuring the absorbance at a wavelength of 405 nm with a plate reader (BioTek Instruments, Winooski, VT).

Western blots

Western blots were performed with liver proteins (30 μ g/sample), and polyclonal Ab against mouse HO-1 (StressGen, Victoria, Canada), TLR4 (Imgenex, San Diego, CA), cleaved caspase-3, Bcl-2, Bcl-xL, and β -actin (Cell Signaling, Danvers, MA), as described (16). The protein quantities, determined by densitometer, were expressed in absorbance unit (AU).

Statistical analysis

All values are expressed as mean $\pm$ SD. Animal survival was evaluated by Kaplan–Meier method and the log-rank test. Data were analyzed using one-way analysis of variance or Student's *t* test for unpaired data. All differences were considered statistically significant at the *P* value of <0.05 .

Results

Characterization of BMMs transfected *ex-vivo* with Ad-HO-1

To determine the effects of *ex-vivo* Ad-HO-1 gene transfer on primary rat macrophages, we analyzed β -gal expression after transfection of BMMs with Ad- β -gal. The X-Gal staining at 48 h of BMM transfection was $>90\%$ (blue spots; Fig. 1B), compared with control non-transfected BMMs (Fig. 1A). The liver X-Gal staining 24 h post infusion of BMMs transfected with Ad- β -gal was 30–40% (Fig. 1D). Then, we performed Western blots to analyze cytoprotective proteins in BMMs (AU). As shown in Fig. 1E, the levels of HO-1 were markedly enhanced in Ad-HO-1/BMMs (2.0–2.2; lane 4a), as compared with Ad- β -gal/BMMs (0.2–0.4; lane 5a). We performed additional *in-vitro* experiments in which we found diminished HO-1 expression in BMMs pretreated with HO-1 siRNA plus CoPP, an exogenous HO-1 inducer (0.2–0.3; lane 2a), but not in BMMs conditioned with scrambled HO-1 siRNA plus CoPP (1.5–1.7; lane 3a). As shown in Fig. 1E, Ad-HO-1/BMMs expressed increased Bcl-2 and Bcl-xL (1.9–2.1 and 1.6–1.8, respectively; lane 4b&c), compared with Ad- β -gal (0.4 and 0.6, respectively; lane 5b&c). However, in the presence of CoPP, HO-1 siRNA diminished both Bcl-2/Bcl-xL (0.2 and 0.1, respectively; lane 2b&c) levels, compared with BMMs cultured with scrambled HO-1 siRNA plus CoPP (1.8 and 1.5, respectively; lane 3b&c).

Ad-HO-1/BMMs prolong OLT survival and ameliorate liver IRI

After characterizing the cytoprotective phenotype of Ad-HO-1 transfected BMMs *in-vitro*, we then asked whether *ex-vivo* genetically modified macrophages might exert cytoprotection *in-vivo*. We have chosen a well-established rat liver transplant model of prolonged 24 h cold ischemia followed by syngeneic OLT (6,9). As shown in Fig. 2A, only 50% (5/10) of OLT survived 14 days after intra-portal infusion of BMMs (5×10^7) transfected with Ad- β -gal, comparable with untreated controls. In marked contrast, rat OLT recipients receiving the

same number of BMM transfected with Ad-HO-1 showed 100% (10/10) survival at 14 days ($p<0.05$). Of note, OLT were infused directly via portal vein immediately prior to reperfusion. Consistent with HO-1 mediated cytoprotection, siRNA-facilitated knock-down of HO-1 in BMMs prior to the adoptive transfer decreased OLT survival to 40% (4/10; $p<0.05$).

Prolonged survival of OLT subjected to 24 h cold ischemia and intra-portal BMMs transfer was accompanied by significantly improved hepatocellular function (Fig. 2B). The sALT levels (IU/L) in Ad-HO-1/BMMs group were significantly decreased throughout, as compared to those conditioned with Ad- β -gal or HO-1 siRNA (6 h: 814 ± 21 vs. 2327 ± 680 and 4946 ± 692 [$p<0.01$]; day 1: 441 ± 146 vs. 2343 ± 241 and 3200 ± 382 [$p<0.01$]; day 3: 56 ± 10 vs. 137 ± 12 and 322 ± 36 $p<0.05$). We have set up two additional control groups of OLT that were infused with BMMs transfected with scrambled HO-1 siRNA, and with unmodified, i.e., non-transfected BMMs. Markedly increased sALT levels in the latter groups (Fig. 2B) confirm the efficacy of Ad-HO-1/BMMs to ameliorate IR-induced OLT damage.

Finally, we assessed the severity of IR/OLT injury by using the Suzuki classification, an established histological measure of the hepatocellular damage in OLT (Fig. 2C). Livers infused with Ad- β -gal, HO-1 siRNA, scrambled siRNA, or control non-transfected BMMs revealed massive sinusoidal/vascular congestion as well as cytoplasmic vacuolization, with 12.5–33% hepatocyte necrosis by day 1 post-OLT (panel b, c, d, e: score: 2.9 ± 0.5 , 3.2 ± 0.7 , 2.8 ± 0.6 and 2.7 ± 0.4 , respectively). In marked contrast, OLT transfected with Ad-HO-1 showed good preservation of lobular architecture with minimal sinusoidal congestion or necrosis (panel a; score = 1.0 ± 0.2 ; $p<0.01$).

Ad-HO-1/BMMs depress pro-inflammatory cytokines but up-regulate IL-10 in OLT

We used qRT-PCR to analyze cytokine/chemokine mRNA expression pattern in OLT. As shown in Fig. 3A&B, transfer of Ad-HO-1/BMMs significantly decreased intrahepatic expression of mRNA coding for TNF- α , IL-1 β , MCP-1 and CXCL-10 at both 6 h and 1 day after reperfusion, as compared with transfer of Ad- β -gal, HO-1 siRNA, scrambled HO-1 siRNA or control BMMs ($p<0.05$ – 0.01). Interestingly, OLT expression of IL-10 after Ad-HO-1/BMMs transfer was consistently upregulated at 6 h ($p<0.01$), day 1 ($p<0.01$) and day 3 ($p<0.05$), as compared with all the controls.

Ad-HO-1/BMMs inhibit adhesion molecule expression and leukocyte traffic in OLT

We assessed mRNA levels coding for ICAM-1 that mediates early adhesive leukocyte - endothelium cross talk at the inflammation sites. Ad-HO-1/BMMs attenuated OLT expression of ICAM-1 after reperfusion, as compared with Ad- β -gal, HO-1 siRNA, scrambled HO-1 siRNA and non-transfected BMMs (Fig. 3B, $p<0.05$). As depicted in Fig. 3C, the myeloperoxidase (MPO) activity, reflecting neutrophil infiltration, was decreased in Ad-HO-1/BMMs livers at 6 h (1.7 ± 0.25 vs. 4.30 ± 0.39 , 6.55 ± 0.46 , 4.66 ± 0.30 , 4.52 ± 0.34 ; $p<0.01$), day 1 (0.8 ± 0.21 vs. 3.00 ± 0.23 , 3.77 ± 0.14 , 3.19 ± 0.14 , 3.24 ± 0.10 ; $p<0.01$) and day 3 (0.27 ± 0.03 vs. 0.37 ± 0.02 , 0.39 ± 0.03 , 0.36 ± 0.03 , 0.37 ± 0.03 ; $p<0.05$), as compared with Ad- β -gal, HO-1 siRNA, scrambled HO-1 siRNA and control BMMs groups.

Ad-HO-1/BMM attenuate apoptosis and caspase-3 activity in OLT

We used TUNEL assay to examine IR-triggered apoptotic events in OLT. As shown in Fig. 4A&B, Ad-HO-1 transfected BMMs (panel a) reduced OLT frequency of TUNEL+ cells at 1 day after reperfusion (1.18 ± 0.4 vs. 18.13 ± 5.56 , 40.5 ± 13.27 , 15.3 ± 6.11 and 12.45 ± 1.55 ($p<0.01$), as compared with Ad- β -gal (panel b), HO-1 siRNA (panel c), scrambled HO-1 siRNA (panel d) or control BMMs (panel e), respectively. Furthermore, as shown in Fig. 4C,

administration of Ad-HO-1/BMMs depressed caspase-3 activity (U) at day 1 post-OLT (0.66 ± 0.22), as compared with Ad- β -gal (2.88 ± 0.26 , $p < 0.001$), HO-1 siRNA (3.57 ± 0.23 , $p < 0.001$), scrambled HO-1 siRNA (1.48 ± 0.37 , $p < 0.05$), or control BMMs (2.68 ± 0.18 , $p < 0.001$) groups. Indeed, HO-1 siRNA/BMMs enhanced caspase-3 activity compared with scrambled HO-1 siRNA/BMMs ($p < 0.001$).

Ad-HO-1/BMMs induce cytoprotective molecules and inhibit TLR4 expression in OLT

We used Western blots to evaluate the expression of anti-oxidant (HO-1), anti-apoptotic (Bcl-2/Bcl-xl), pro-apoptotic (cleaved caspase-3), and TLR4 gene products in OLT; the relative expression levels in absorbance units (AU) were analyzed by densitometry. As shown in Fig. 5, Ad-HO-1/BMMs up-regulated the expression of HO-1, Bcl-2 and Bcl-xl (6 h: 2.2–2.4 AU, 1.5–1.7 AU, and 1.4–1.6; 24 h: 1.4–1.5, 1.3–1.4 and 1.0–1.1, respectively) simultaneously inhibiting cleaved caspase-3 and TLR4 (6 h: 0.1–0.2 AU; 24 h: 0.2–0.3 AU, respectively) levels in OLT. In contrast, the corresponding liver samples in Ad- β -gal, HO-1 siRNA, scrambled HO-1 siRNA, and control BMMs groups revealed higher levels of cleaved caspase-3 (1.2–2.0) and TLR4 (1.2–2.6), but markedly decreased expression of HO-1 (0.05–0.3), Bcl-2 (0.05–0.2), and Bcl-xl (0.05–0.3).

Discussion

This is the first study, which demonstrates the striking efficacy of HO-1 expressing primary macrophages to prevent the hepatocellular damage in a stringent rat model of prolonged donor liver cold ischemia followed by orthotopic liver iso-transplantation. Intra-portal infusion of native bone marrow-derived macrophages transfected *ex-vivo* with Ad-HO-1 successfully rescued rat OLT from IRI, as evidenced by: (i) one hundred percent recipient survival; (ii) improved OLT hepatocellular function and preserved hepatic architecture; (iii) diminished leukocyte sequestration, depressed pro-inflammatory cytokine/chemokine/adhesion molecule programs but increased IL-10 expression in OLT; (iv) suppressed apoptosis by inhibiting caspase-3 activity and up-regulating anti-apoptotic (Bcl-2/Bcl-xl) and anti-oxidant (HO-1) molecules; (v) inhibited TLR4 expression. These results are in agreement with our recent report (8) documenting the efficacy of *ex-vivo* HO-1 modified BMMs to prevent IRI in a non-transplant mouse liver model of partial “warm” ischemia and reperfusion. However, unlike the former, the current study involves OLT subjected to prolonged (24 h) period of cold (4°C) ischemia, and hence reflecting better the real life liver transplant scenario. This study extends our previous findings, and provides the rationale for a clinically attractive strategy in which recipient’s macrophages, collected ahead of time, can be transfected *ex-vivo* with “cytoprotective” molecule, and then infused if needed directly to the donor liver at the time of transplantation. Based on our results, we expect this novel approach should mitigate IR-triggered local inflammation and hepatocellular damage that affect early and late OLT outcomes.

Recently, the Edinburgh group has used differential deletion approach to show that mouse tissue-resident rather than circulating macrophages protect livers against IR damage via HO-1 dependent mechanism (17). This suggests that in the warm ischemia model, HO-1 expression by Kupffer cells *in-situ* may be more important than that by peripheral macrophages. In our study, BMMs transfected *ex-vivo* with HO-1 migrated efficiently to rat OLT and exerted potent local cytoprotective functions. Hence, in liver cold ischemia/OLT setting, peripheral macrophages can be manipulated genetically *ex-vivo* to mimic liver-resident Kupffer cells’ HO-1 cytoprotective potential, a novel finding of potential practical importance.

Back in 2002, we proposed that HO-1 system should be considered as a novel therapeutic concept in organ transplantation (7). Indeed, accumulating evidence point to the ability of

HO-1 overexpression, by pharmacological or genetic means, to diminish organ IRI in a plethora of animal models (18), data supported by findings in the transplant patients (19,20). However, there are major drawbacks with currently used HO-1-based therapies. First, exogenous HO-1 inducers, such as protoporphyrins may exert non-specific effects (21), whereas targeted HO-1 gene therapies are far from being organ selective. Second, the current means of HO-1 overexpression *in-vivo* do require at least 24 h pretreatment schedule, raising practical concerns from a clinical standpoint. In this respect, our current findings are of particular interest. First, the striking efficacy of Ad-HO-1 (one hundred percent OLT/recipient survival vs. 40–50% in controls) was achieved after local infusion of Ad-HO-1/BMMs directly to the donor liver via portal vein at the time of liver transplantation. Recreation of the fulminant hepatocellular damage after siRNA-facilitated HO-1 gene silencing, confirm the specificity of Ad-HO-1/BMMs therapeutic effect. Second, our X-gal assay showing high (>90%) Ad- β -gal transfection by BMMs *in vitro*, combined with the preferential (30–40% X-gal staining) migration of Ad- β -gal/BMMs to ischemic tissue *in-vivo*, confirms the rationale for using Ad-HO-1/BMMs approach to target liver IR-mediated OLT damage. Others have shown protection against IRI in rat livers infused with Kupffer cells transduced by Ad-superoxide dismutase (22), with ca. 20% injected cells localizing in the liver. Collectively, these data supports the prominent role of the liver as an ultimate target organ for cell-based gene therapy.

Ischemia-activated macrophages are the key players in liver innate-dominated immune response, leading to the production of reactive oxygen species (ROS) and pro-inflammatory cytokine programs that facilitate the hepatocellular damage following reperfusion (2–4). Interestingly, as the major source of anti-oxidant HO-1 (23), circulating macrophages may also represent one of the most suitable delivery systems for cytoprotective gene transfer. Our results show that hepatic cytoprotection resulting from HO-1 overexpression in rat BMMs transfected with Ad-HO-1 could be abolished after HO-1 silencing. Although siRNA-treated BMMs produced no deterioration of OLT function compared with untreated controls, HO-1 deletion or inhibition in a non-transplant liver warm IRI model did accelerate local injury, as compared with controls (17). Obvious differences between animal species and liver IRI models may explain these disparate findings.

Macrophages were used before for local IL-4/IL-10 delivery and to successfully reduce inflamed injury in a rat model of experimental glomerulonephritis (24,25). Our study shows that BMM-facilitated HO-1 overexpression affected leukocyte trafficking, evidenced by diminished ICAM endothelial expression, along with interrupted neutrophil infiltration/activity in OLT. IR-mediated macrophage/Kupffer cell activation results in the release of TNF- α , IL-1 β , CXCL10, and CCL2 (MCP-1), the “signature” markers of liver IRI (4). Transfer of Ad-HO-1/BMMs has blunted macrophage pro-inflammatory/chemotactic gene expression programs, with the exception of IL-10, which was simultaneously and selectively heightened. Such an altered local inflammation response was shown by us to be responsible for cytoprotection in mouse liver model of warm ischemia, as i/IL-10 neutralization restored inflammation/hepatocellular damage in otherwise IR resistant mice deficient of CXCL10 (14), and ii/vIL-10 gene transfer prevented hepatic IR insult in WT recipients in association with depressed Th1 cytokine/chemokine programs (26). Perhaps, by virtue of selective IL-10 overexpression, Ad-HO-1/BMMs might raise the defensive threshold to inflammatory response in IR-exposed OLT.

Our findings suggest the dynamic cross talk between HO-1 and IL-10 signaling, which might contribute to the protective effects of HO-1 in response to IR insult. Interestingly, HO-1 has been shown to mediate anti-inflammatory effects of IL-10, implying that HO-1 may represent one of IL-10 downstream effector molecules (27). Although mice homozygous for a targeted disruption of the HO-1 gene (HO-1^{-/-}) have been shown by

others to express a more definitive proinflammatory phenotype compared with heterozygous controls (HO-1^{-/+}) (17), we have successfully used the latter to demonstrate that decreased basal HO-1 levels, rather than its total absence, affects liver IRI (28). Unlike in WT, exogenous HO-1 induction with CoPP largely failed to reduce liver IRI in HO-1^{-/+} KO mice. We also found increased IL-10 levels in HO-1^{-/+} as compared with WT counterparts, which could only be partially mitigated by CoPP. The decreased baseline HO-1 frequency may augment IL-10 expression, perhaps to counter the increased susceptibility to oxidative stress and pro-inflammatory stimuli. The inability of CoPP to prevent liver IRI after IL-10 neutralization strengthens the role of and the requirement for IL-10 in the hepatocytprotection, consistent with accelerated liver IRI seen in WT mice pretreated with neutralizing anti-IL-10 mAb (26).

TLR4 is expressed in the liver primarily on Kupffer cells and some hepatocytes (29,30). In our studies, TLR4 signaling was required in initiating liver inflammation and hepatocellular damage, whereas disruption of TLR4 pathway reduced IRI and improved hepatic function (10,31). Moreover, as defective TLR4 pathway increases HO-1 expression, it is plausible that TLR4 might function as putative HO-1 repressor (26). Our data shows that infusion of BMMs with defective HO-1 (after HO-1 siRNA pretreatment) restored hepatic injury, evidenced by diminished survival, increased sALT levels, histological damage, neutrophil infiltration, and pro-inflammatory cytokine/chemokine program. Moreover, defective HO-1 increased TLR4 expression, leading to liver inflammatory injury. We have shown that vIL-10 gene transfer not only reversed the detrimental effect of HO-1 deficit, but also diminished TLR4, and ameliorated hepatic damage (26). Our present findings further support the innate – adaptive feedback mechanism between TLR4 and HO-1 in the context of IL-10 in the pathophysiology of liver IRI.

Cell apoptosis constitutes a critical mechanism in IR-triggered liver injury, with the key morphological changes being mediated by caspases, the intracellular cysteine proteases (32). Ad-HO-1-modified BMMs decreased caspase-3 activity and the frequency of TUNEL+ cells in ischemic rat OLT. These are consistent with enhanced IR-induced apoptosis following HO-1 siRNA treatment, and inhibition of apoptosis via caspase 3-dependent pathway (15). Thus, HO-1 expressing BMMs represent major anti-oxidant mediators with both anti-inflammatory and anti-apoptotic defense mechanisms in IR-induced liver transplant damage. In addition, HO-1-modified macrophages selectively up-regulated the expression of anti-apoptotic Bcl-2 and Bcl-xL, consistent with their ability to inhibit IR-induced apoptosis mediated by ROS, cytosolic cytochrome c release, or caspase-3 activation (33,34). The induction of Bcl-2/Bcl-xL via HO-1 represents an important cytoprotective function that prevents the hepatocellular and sinusoidal endothelial cells apoptosis, ameliorates IRI and prolongs OLT/recipients survival.

Thus, our study validates a novel and clinically attractive concept in which native macrophages transfected *ex-vivo* with anti-oxidant HO-1 can be applied *in-vivo*, at the time of liver transplantation, to mitigate otherwise damaging Ag-independent local inflammation resulting from the peri-transplant “harvesting” insult. We are currently applying this therapeutic principle in IRI/allo-OLT rat model. If proven effective, this refined new strategy should be considered in the clinical settings to increase the supply of usable donor organs and ultimately improve the overall success of transplantation.

Acknowledgments

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Abbreviations

BMM	bone marrow-derived macrophages
HO-1	heme oxygenase-1
IRI	ischemia and reperfusion injury
OLT	orthotopic liver transplantation
TLR	toll-like receptor
UW solution	University of Wisconsin solution

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Figure 1.

(A, B) The expression of β -gal in bone marrow-derived macrophages (BMMs). Cells were transfected with Ad- β -gal, incubated for 48 h, and stained with X-Gal. *Note:* >90% expression of β -gal (blue cells) in BMMs (B) vs. control untreated BMMs (A).

(C, D) The X-Gal staining in rat liver at 24 h after *in vivo* adoptive transfer of BMMs with Ad- β -gal. *Note:* The expression of β -gal was 30–40% (D) vs. normal untreated liver (C). Representative of three experiments; magnification, $\times 200$.

(E) Western blot analysis of HO-1, Bcl-2, and Bcl-xL expression in BMM. Lane 1, BMMs alone; lane 2, BMMs transfected with HO-1 siRNA plus CoPP (10 μ g/ml); lane 3, BMMs transfected with scrambled HO-1 siRNA plus CoPP (10 μ g/ml); lane 4, BMMs transfected with Ad-HO-1; lane 5, BMMs transfected with Ad- β -gal; lane 6, BMMs plus CoPP (10 μ g/ml). *Note:* Selectively increased expression of HO-1, Bcl-2/Bcl-xL in Ad-HO-1-treated BMMs (lane 4), as compared with Ad- β -gal (lane 5). Diminished expression of HO-1, Bcl-2/Bcl-xL in BMMs treated with HO-1 siRNA plus CoPP (lane 2), compared with scrambled HO-1 siRNA plus CoPP (lane 3). Anti- β -actin Ab was used to assure equal protein amounts between samples. Data shown are representative of three experiments.

Figure 2.

(A) OLT survival. SD rat livers were stored at 4°C in UW solution for 24 h prior to transplantation to syngeneic SD recipients. Livers were infused via portal vein immediately prior to reperfusion with BMMs transfected with: (■) Ad-HO-1; (◆) Ad-β-gal; (▲) HO-1 siRNA. *Note:* OLT survival at 14 days: 100% in Ad-HO-1/BMMs group vs. 40–50% in HO-1 siRNA/Ad β-gal transfected BMMs groups (N=10/group; $p<0.05$).

(B). sALT levels in OLT recipients from (A) experimental series, at 6 h, 1 day and 3 days after reperfusion. *Note:* sALT levels were lower in Ad-HO-1/BMMs group (grid bars), compared with Ad-β-gal (hatched bars), HO-1 siRNA (white bars), scrambled HO-1 siRNA (black bars) or control non-transfected BMMs (dotted bars) groups. N=4–5/group. Mean $\pm$ SD are shown. $P< *0.05$; $**0.01$.

(C). OLT histology (day 1 post-transplant). Panel: B, C, D, E. The donor livers infused with BMMs transfected with: (b) Ad-β-gal; (c) HO-1 siRNA; (c) scrambled HO-1 siRNA; or control non-transfected BMMs: sinusoidal and vascular congestion/cytoplasmic vacuolization with 12.5–33% of hepatocyte necrosis (Suzuki score: 2.9 ± 0.5 , 3.2 ± 0.7 , 2.8 ± 0.6 and 2.7 ± 0.4 , respectively); (a): Ad-HO-1/BMMs: preservation of lobular architecture with minimal necrosis/sinusoidal congestion. (Suzuki score = 1.0 ± 0.2 ; $p<0.01$). (f) sham control. (Magnification $\times 100$, H&E stain; representative of $n=4-5$ /group).

Figure 3.

(A, B) Quantitative RT-PCR assisted detection of mRNA coding for TNF-α, IL-1β, MCP-1, CXCL-10, ICAM-1 and IL-10 in OLT at 6h, 1 day and 3 days after reperfusion. *Note:* Adoptive transfer of Ad HO-1/BMMs (grid bars) diminished the expression of TNF-α, IL-1β, MCP-1, CXCL-10 and ICAM-1 but increased the levels of IL-10, as compared with Ad-β-gal (hatched bars), HO-1 siRNA (white bars), scrambled HO-1 siRNA (black bars) or non-transfected BMMs (dotted bars). N=4–5/group; p<*0.05; **< 0.01.

(C). MPO-assisted detection of neutrophil enzymatic activity in OLT. *Note:* Treatment with Ad HO-1- transfected BMMs (grid bars) decreased MPO activity (6 h: p<0.01, 1 day: p<0.01, and 3 days: p<0.05) after reperfusion, as compared with Ad-β-gal (hatched bars), HO-1 siRNA (white bars), scrambled HO-1 siRNA (black bars) or control non-transfected BMMs (dotted bars). Each column represents the mean±SD; N=4–5 samples/group.

Figure 4.

(A, B) TUNEL-assisted detection of apoptosis in OLTs at day 1 after reperfusion. *Note:* Apoptotic cells in Ad-HO-1/BMMs group (panel a) were diminished after reperfusion, as compared with Ad-β-gal (panel b), HO-1 siRNA (panel c), scrambled HO-1 siRNA (panel d), and control BMMs (panel e) groups. Panel f: sham. ** $p < 0.01$). Original magnification $\times 400$.

(C). Caspase-3 activity in OLT. *Note:* Administration of Ad-HO-1/BMMs markedly decreased caspase-3 activity (** $p < 0.001$), as compared with Ad-β-gal, HO-1 siRNA and control BMMs. In contrast, infusion of HO-1 siRNA/BMMs enhanced caspase-3 activity as compared with scrambled HO-1 siRNA/BMMs ($p < 0.001$). Mean $\pm$ SD are shown ($n = 4-5$ /group).

Figure 5.

Western blot analysis of anti-oxidant (HO-1), anti-apoptotic (Bcl-2/Bcl-xl), pro-apoptotic (cleaved caspase-3), and TLR4 proteins in OLT (6 h and day 1 post-reperfusion). *Note:* Transfer of Ad-HO-1/BMMs up-regulated the expression of HO-1 and Bcl-2/Bcl-xl but inhibited cleaved caspase-3 and TLR4 levels at 6 h and day 1. In contrast, the expression of cleaved caspase-3 and TLR4 increased, whereas that of HO-1 and Bcl-2/Bcl-xl simultaneously decreased in Ad-β-gal, HO-1 siRNA, scrambled HO-1 siRNA, and control BMMs groups. The data are representative of three separate experiments.