

# Adoptive Transfer of Heme Oxygenase-1 (HO-1)-Modified Macrophages Rescues the Nuclear Factor Erythroid 2-Related Factor (Nrf2) Antiinflammatory Phenotype in Liver Ischemia/Reperfusion Injury

Jing Huang,\* Xiu-Da Shen, Shi Yue, Jianjun Zhu, Feng Gao, Yuan Zhai, Ronald W Busuttill, Bibo Ke, and Jerzy W Kupiec-Weglinski

Dumont-UCLA Transplant Center, Department of Surgery, David Geffen School of Medicine at UCLA, Los Angeles, California, United States of America. \*Current address: Department of Hepatobiliary-Pancreatic Surgery, Lihuili Hospital, Ningbo University School of Medicine, Ningbo, China

Macrophages are instrumental in the pathophysiology of liver ischemia/reperfusion injury (IRI). Although *Nrf2* regulates macrophage-specific heme oxygenase-1 (HO-1) antioxidant defense, it remains unknown whether HO-1 induction might rescue macrophage *Nrf2*-dependent antiinflammatory functions. This study explores the mechanisms by which the *Nrf2*-HO-1 axis regulates sterile hepatic inflammation responses after adoptive transfer of *ex vivo* modified HO-1 overexpressing bone marrow-derived macrophages (BMMs). Livers in *Nrf2*-deficient mice preconditioned with Ad-HO-1 BMMs, but not Ad- $\beta$ -Gal-BMMs, ameliorated liver IRI (at 6 h of reperfusion after 90 min of warm ischemia), evidenced by improved hepatocellular function (serum alanine aminotransferase (sALT) levels) and preserved hepatic architecture (Suzuki histological score). Treatment with Ad-HO-1 BMMs decreased neutrophil accumulation, proinflammatory mediators and hepatocellular necrosis/apoptosis in ischemic livers. Moreover, Ad-HO-1 transfection of *Nrf2*-deficient BMMs suppressed M1 (*Nos2*<sup>+</sup>) while promoting the M2 (*Mrc-1/Arg-1*<sup>+</sup>) phenotype. Unlike in controls, Ad-HO-1 BMMs increased the expression of *Notch1*, *Hes1*, phosphorylation of *Stat3* and *Akt* in IR-stressed *Nrf2*-deficient livers as well as in lipopolysaccharide (LPS)-stimulated BMMs. Thus, adoptive transfer of *ex vivo* generated Ad-HO-1 BMMs rescued *Nrf2*-dependent antiinflammatory phenotype by promoting *Notch1*/*Hes1*/*Stat3* signaling and reprogramming macrophages toward the M2 phenotype. These findings provide the rationale for a novel clinically attractive strategy to manage IR liver inflammation/damage.

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## INTRODUCTION

Ischemia/reperfusion injury (IRI) has been recognized as a major cause of organ dysfunction and failure after liver transplantation. Hepatic IR activates

Kupffer cells and triggers reactive oxygen species (ROS) production, leading to the release of proinflammatory cytokines/chemokines and cell apoptosis (1). Macrophages are key mediators, which

initiate toll-like receptor-4 (*TLR4*) response during the inflammatory phase of hepatic IRI (2). We have shown that disruption of *TLR4* signaling regulated macrophage inflammation (2) and ameliorated IR-induced liver damage *in vivo* (3). Activation of HO-1 exerts potent antiinflammatory and antiapoptotic functions in transplant models of liver allograft rejection (4) and IRI (5–7). We and others have shown that HO-1 is expressed primarily by liver macrophages and Kupffer cells (8,9), whereas depletion of Kupffer cells diminished HO-1 expression and increased IR hepatic damage (10). HO-1 is one of the antioxidant response element (ARE)-dependent phase II detoxifying enzymes and antioxidants (11), which are regulated by

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**Address correspondence to** Bibo Ke, Dumont-UCLA Transplant Center, Department of Surgery, David Geffen School of Medicine at UCLA, Box 957054, Los Angeles, CA. Phone: 310-794-7557; Fax: 310-236-2367; E-mail: [bke@mednet.ucla.edu](mailto:bke@mednet.ucla.edu); or Jerzy W Kupiec-Weglinski, Dumont-UCLA Transplant Center, Department of Surgery, David Geffen School of Medicine at UCLA, Box 957054, Los Angeles, CA. Phone: 310-825-4196; Fax: 310-267-2358; E-mail: [jkupiec@mednet.ucla.edu](mailto:jkupiec@mednet.ucla.edu).

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the redox-sensitive transcription factor nuclear factor erythroid 2-related factor (*Nrf2*) (12). Activation of *Nrf2* induces HO-1 expression (13), suggesting *Nrf2* is essential for HO-1-mediated cytoprotection against IR liver injury.

*Nrf2*, a “master regulator” of cellular resistance to oxidative stress (14), controls both basal and induced expression of ARE-dependent genes to regulate cell defense systems (15). Under normal conditions, *Nrf2* is kept in the cytoplasm by *Keap1* (Kelch-like ECH-associated protein 1) and Cullin 3, which degrade *Nrf2* by ubiquitination (16). Activation of *Nrf2* leads to *Nrf2* translocation into the nucleus and binding to the ARE, which initiates transcription of *Nrf2* target genes (17). Increased *Nrf2* activity promotes cell survival and mitigates oxidative stress-induced damage (18,19), whereas disruption of *Nrf2* enhances hypoxia-induced acute lung and oxidative stress-mediated inflammation (20). Thus, *Nrf2* might be functioning as a key regulator of inflammatory responses against oxidative stress-induced organ injury.

The present study was designed to explore the mechanisms by which the *Nrf2*-HO-1 axis regulates inflammation response in a mouse model of liver IRI. Our results show that adoptive transfer of *ex vivo* generated Ad-HO-1-modified bone marrow-derived macrophages (BMMs) ameliorated liver IRI in *Nrf2*-deficient mice by enhancing the Notch1/Hes1/Stat3 signaling and promoting the M2 phenotype. Thus, HO-1-modified macrophages can be applied to rescue *Nrf2*-dependent antiinflammatory functions.

## MATERIALS AND METHODS

### Animals

The *Nrf2*-deficient (*Nrf2*<sup>-/-</sup>; KO) male mice (C57BL/6) at 6–8 wks of age were used (provided by T Kensler, Johns Hopkins University, Baltimore, MD, USA). Animals were housed in the UCLA animal facility under specific pathogen-free conditions and received humane care according to the criteria outlined in the

*Guide for the Care and Use of Laboratory Animals* (NIH publication 86-23, revised 1985) (21).

### Mouse Liver IRI Model/Treatment

We used a mouse model of warm hepatic ischemia followed by reperfusion (22). The *Nrf2* KO mice were injected with heparin (100 U/kg), and an atraumatic clip was used to interrupt the arterial/portal venous blood supply to the cephalad liver lobes. After 90 min, the clip was removed, and mice were sacrificed at 6 h of reperfusion. In the treatment groups, each mouse was injected via a portal vein with  $5 \times 10^6$  of transfected BMMs with Ad-HO-1/Ad- $\beta$ -gal ( $2.5 \times 10^9$  plaque-forming units [pfu]) or untreated BMMs 24 h before the onset of warm ischemia. After adoptive transfer of Ad- $\beta$ -gal-transfected BMMs, the X-Gal staining in the ischemic liver lobes at 6 h of reperfusion was 40–50%, as compared with control livers (23).

### Hepatocellular Function Assay

Serum alanine aminotransferase (sALT) levels, an indicator of hepatocellular injury, were measured by IDEXX Laboratories (Westbrook, ME, USA).

### Histology/Immunohistochemistry

Liver sections (5  $\mu$ m) were stained with hematoxylin and eosin (H&E). The severity of IRI was graded by using Suzuki criteria on a scale from 0 to 4 (24). Liver neutrophils were detected by using primary rat anti-mouse Ly6G monoclonal antibody (BD Biosciences, San Jose, CA, USA). The secondary, biotinylated goat anti-rat IgG (Vector, Burlingame, CA, USA) was incubated with immunoperoxidase (ABC Kit; Vector), according to the manufacturer's instructions. Positive cells were counted blindly in 10 fields per section.

### Myeloperoxidase Activity Assay

The presence of myeloperoxidase (MPO) was used as an index of hepatic neutrophil accumulation (25). The change in absorbance was measured spectrophotometrically at 655 nm. One unit of MPO

activity was defined as the quantity of enzyme degrading 1  $\mu$ mol peroxide per minute at 25°C per gram of tissue.

### Terminal Deoxynucleotidyl Transferase dUTP Nick End Labeling (TUNEL) Assay

The Klenow-FragEL DNA Detection Kit (EMD Chemicals, Gibbstown, NJ, USA) was used to detect the DNA fragmentation characteristic of apoptosis in formalin-fixed paraffin-embedded liver sections (2). The results were scored semiquantitatively by averaging the number of apoptotic cells/field. Ten fields were evaluated per tissue sample.

### Quantitative Real-Time Polymerase Chain Reaction Analysis

Quantitative real-time polymerase chain reaction (RT-PCR) was performed by using the DNA Engine with Chromo 4 Detector (MJ Research, Waltham, MA, USA). In a final reaction volume of 25  $\mu$ L, the following were added: 1 $\times$  SuperMix (Platinum SYBR Green qPCR Kit; Invitrogen/Life Technologies [Thermo Fisher Scientific Inc., Waltham, MA, USA]), cDNA and 10  $\mu$ mol/L of each primer. Amplification conditions were as follows: 50°C (2 min) and 95°C (5 min), followed by 40 cycles of 95°C (15 s) and 60°C (30 s). Primers used to amplify specific gene fragments are shown in Supplementary Table S1.

### Western Blot Assay

Proteins (30  $\mu$ g/sample) from cell cultures or liver samples were subjected to 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to nitrocellulose membrane (Bio-Rad, Hercules, CA, USA). Polyclonal rabbit anti-mouse HO-1 (StressGen Biotech, Victoria, BC, Canada) and monoclonal rabbit anti-mouse *Notch1*, *Hes1*, *phos-Akt*, *Akt*, *phos-Stat3*, *Stat3*, *NF- $\kappa$ B*, *Bcl-2*, *Bcl-xL*, *cleaved caspase-3* and  *$\beta$ -actin* antibodies were used (Cell Signaling Technology, Danvers, MA, USA).

### Cell Isolation/*In Vitro* Cultures

Murine BMMs were generated, as described (23). Bone marrow cells were re-

moved from the femurs and tibias of *Nrf2* KO mice and cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal calf serum (FCS) and 15% L929-conditioned medium. Cells ( $5 \times 10^6$ /well) cultured for 7 d were added with Ad-HO-1 or Ad- $\beta$ -gal (at a multiplicity of infection [MOI] of 10) and incubated for 1 h. The medium was removed and changed to DMEM + 2% FCS. After 36–48 h, cells were harvested for *in vivo* adoptive transfer. The X-Gal staining by 48 h of transfection was >90% in BMMs, compared with control BMMs (23). For the *in vitro* assay, these transfected cells were incubated with lipopolysaccharide (LPS; 100 ng/mL) for an additional 6 h.

### Statistical Analysis

Data are expressed as mean  $\pm$  SD. Statistical comparisons were analyzed by the Student *t* test. Differences were statistically significant at  $p < 0.05$ .

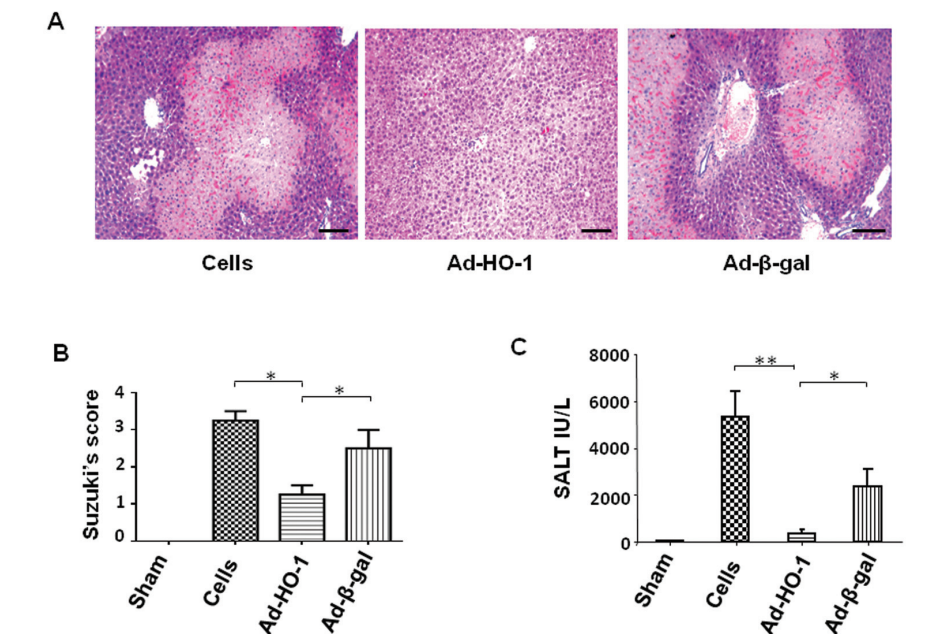
All supplementary materials are available online at [www.molmed.org](http://www.molmed.org).

## RESULTS

### Ad-HO-1 BMMs Ameliorate Liver IRI in *Nrf2*-Deficient Mice

We used a mouse model of hepatic warm ischemia (90 min) followed by reperfusion (22). By 6 h of reperfusion, livers in the control *Nrf2*-deficient mice preconditioned (at d -1) with Ad- $\beta$ -gal BMMs, or untreated BMMs, revealed significant edema, severe sinusoidal congestion, cytoplasmic vacuolization and extensive hepatocellular necrosis (30–50%; Figures 1A, B; Suzuki score  $2.5 \pm 0.45$  and  $3.15 \pm 0.25$ , respectively). In contrast, livers in *Nrf2*-deficient hosts that received Ad-HO-1 BMMs had well-preserved hepatic architecture, with minimal sinusoidal congestion, without edema, vacuolization or necrosis (Figures 1A, B; Suzuki score  $1.33 \pm 0.29$ ;  $p < 0.05$ ).

The hepatocellular function in *Nrf2* KO mice, assessed by sALT levels (IU/L), improved after adoptive transfer of Ad-HO-1 BMMs, as compared with Ad- $\beta$ -gal BMMs or untreated BMM groups (Figure 1C;



**Figure 1.** Adoptive transfer of Ad-HO-1 BMMs prevents IR-induced hepatocellular damage in *Nrf2*-deficient mice. BMMs transfected ( $5 \times 10^6$ ) with Ad-HO-1 or Ad- $\beta$ -gal ( $2.5 \times 10^9$  pfu) or untreated BMMs were infused into *Nrf2* KO mice (via portal vein) at 24 h before 90 min of ischemia. Animals were killed at 6 h of reperfusion. (A) Representative histological staining (H&E) of ischemic liver tissue. Scale bars indicate 50  $\mu$ m. (B) Liver damage, evaluated by Suzuki histological score. \* $p < 0.05$ . (C) Hepatocellular function, assessed by sALT levels (IU/L). \* $p < 0.005$ ; \*\* $p < 0.0005$ . Mean  $\pm$  SD are shown ( $n = 4$ –6/group).

$378.8 \pm 84.8$  versus  $2,358 \pm 366.9$  and  $5,321 \pm 558.3$ , respectively;  $p < 0.005$ ).

### Ad-HO-1 BMMs Inhibit Neutrophil Activation in *Nrf2*-Deficient IR-Stressed Livers

By 6 h of reperfusion after 90 min of ischemia, livers in *Nrf2* KO mice pretreated with Ad-HO-1 BMMs showed reduced infiltration by Ly6G<sup>+</sup> neutrophils (Figures 2A, B;  $5 \pm 1.2$ ) compared with Ad- $\beta$ -gal ( $24.33 \pm 1.39$ ,  $p < 0.05$ ) or BMMs ( $26.33 \pm 1.53$ ,  $p < 0.05$ ). Furthermore, the MPO assay (Figure 2C), reflecting hepatic neutrophil activity (U/g), was decreased from  $4.26 \pm 0.37$  in Ad- $\beta$ -gal BMMs and  $3.78 \pm 0.3$  in BMMs, to  $1.45 \pm 0.06$  in the Ad-HO-1 BMMs group ( $p < 0.05$ ).

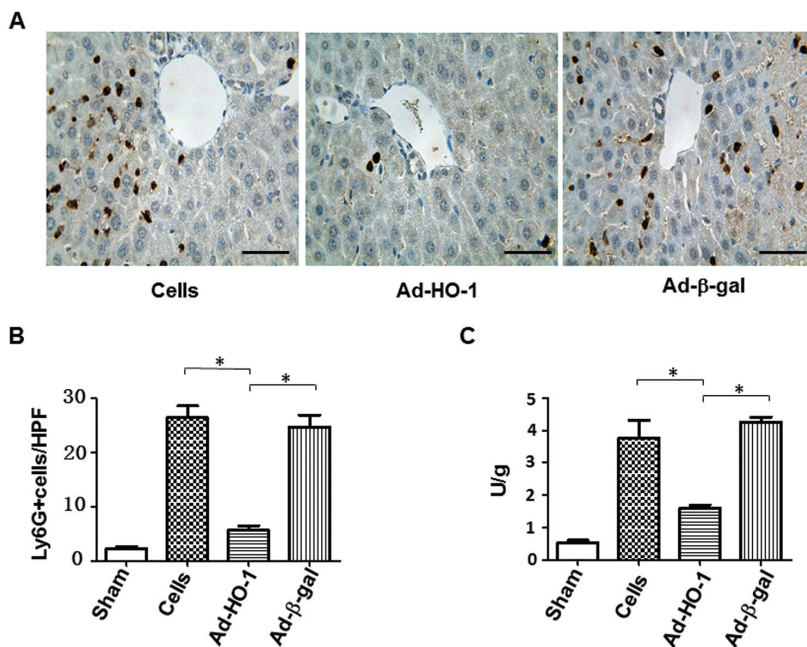
### Ad-HO-1 BMMs Upregulate Notch Signaling in *Nrf2*-Deficient IR-Stressed Livers

Notch signaling has been shown to regulate macrophage inflammatory

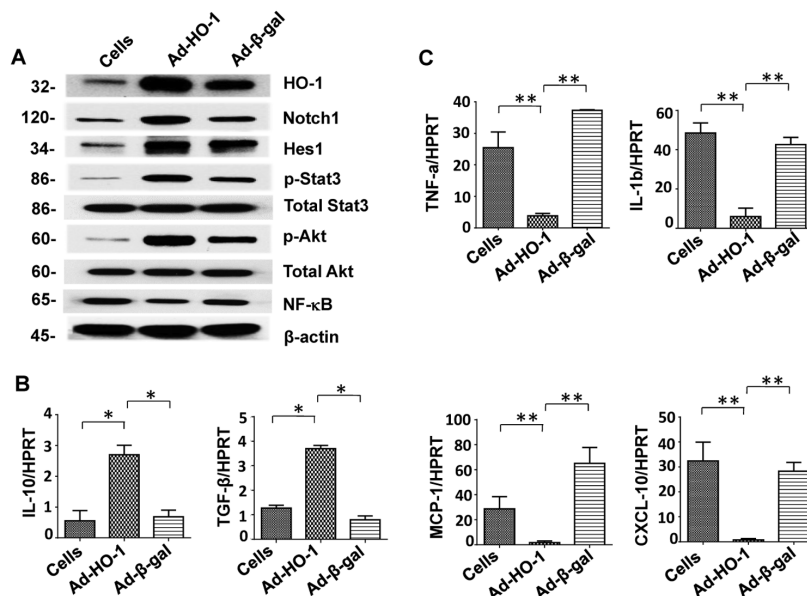
responses (26). In our liver IRI model in *Nrf2*-deficient mice, infusion of Ad-HO-1 BMMs upregulated Western-assisted hepatic expression of *Notch1*, *Hes1*, *p-Stat3* and *p-Akt*, yet downregulated *NF- $\kappa$ B*, as compared with Ad- $\beta$ -gal or BMMs groups (Figure 3A). Furthermore, unlike in controls, Ad-HO-1 BMMs increased mRNA levels coding for interleukin (IL)-10 and transforming growth factor (TGF)- $\beta$  (Figure 3B,  $p < 0.05$ ), while decreasing tumor necrosis factor (TNF)- $\alpha$ , IL-1 $\beta$ , monocyte chemoattractant protein (MCP)-1 and C-X-C motif chemokine 10 (CXCL10) expression (Figure 3C,  $p < 0.05$ ).

### Ad-HO-1 BMMs Prevent Apoptosis in *Nrf2*-Deficient IR-Stressed Livers

Western-assisted analysis of ischemic tissue has shown that unlike in controls, Ad-HO-1 BMMs decreased the expression of cleaved caspase-3 while increasing *Bcl-2* and *Bcl-xL* levels (Figure 4A). Furthermore, while *Nrf2*-deficient livers



**Figure 2.** Adoptive transfer of Ad-HO-1 BMMs inhibits hepatic neutrophil activation in *Nrf2*-deficient mice (6 h of reperfusion after 90 min of ischemia). (A, B) Liver neutrophil accumulation by immunohistochemistry. Scale bars indicate 50  $\mu$ m. (C) Hepatic neutrophil activity analyzed by MPO assay (U/g). \* $p < 0.05$ . Mean  $\pm$  SD are shown ( $n = 4$ –6/group).



**Figure 3.** Adoptive transfer of Ad-HO-1 BMMs upregulates Notch signaling and regulates IR inflammation in *Nrf2*-deficient livers (6 h of reperfusion after 90 min of ischemia). (A) Western blot analysis of hepatic *HO-1*, *Notch1*, *Hes1*, *p-Stat3*, total *Stat3*, *p-Akt*, total *Akt* and *NF-κB* expression in *Nrf2* KO mice pretreated with Ad-HO-1/Ad-β-gal-transfected BMMs or untreated BMMs. Anti-β-actin antibody was used to assure equal protein amounts between samples. Data are representative of three experiments. (B, C) Quantitative real-time PCR-assisted detection of cytokine/chemokine programs in ischemic livers. \* $p < 0.05$ ; \*\* $p < 0.005$ . Mean  $\pm$  SD;  $n = 3$ –4/group.

pretreated with Ad-β-gal BMMs or untreated BMMs showed increased frequency of TUNEL<sup>+</sup> cells (Figures 4B, C;  $28.33 \pm 2.1$  and  $24.67 \pm 2.1$ ), the number of hepatic TUNEL<sup>+</sup> cells markedly dropped after infusion of Ad-HO-1 BMMs ( $2 \pm 0.4$ ,  $p < 0.001$ ).

### Nrf2–HO-1 Axis Promotes Notch1/Hes1/Stat3 Signaling to Regulate Inflammatory Responses *In Vitro*

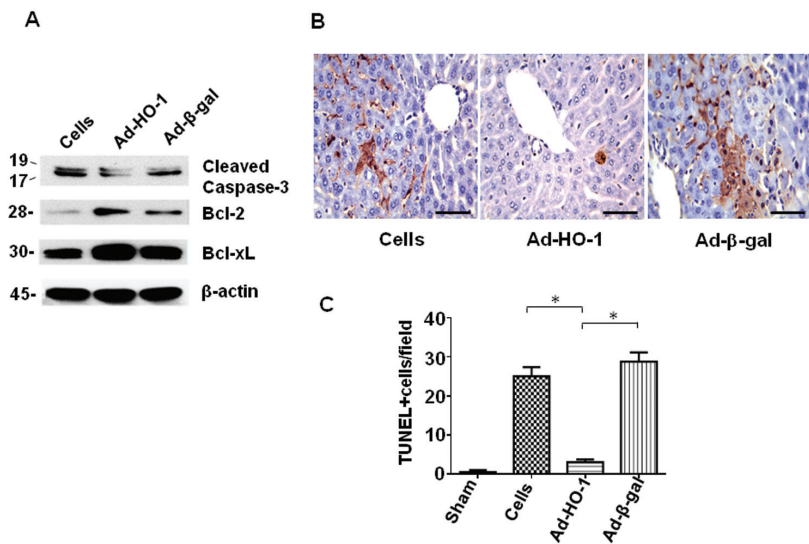
Our *in vivo* data suggest that HO-1 overexpression may rescue *Nrf2*-dependent antiinflammatory functions, which in turn diminishes liver IR inflammation. We then used an LPS-stimulated BMM culture system to test a hypothesis that the Nrf2–HO-1 axis regulates inflammatory response through the Notch1/Hes1/Stat3 signaling pathway. Indeed, Ad-HO-1 transfection upregulated *HO-1*, *Notch1*, *Hes1*, *p-Stat3* and *p-Akt* but depressed *NF-κB* in *Nrf2*-deficient BMMs (Figure 5A), compared with controls. Consistent with these data, Ad-HO-1 transfection consistently reduced mRNA levels coding for *TNF-α*/*IL-1β* and *MCP-1/CXCL10* in *Nrf2*-deficient BMMs, compared with Ad-β-gal BMMs or untreated BMMs (Figure 5B).

### Nrf2–HO-1 Axis-Mediated Notch1/Hes1/Stat3 Signaling Regulates Macrophage Differentiation and Function *In Vitro*

To test whether HO-1 might regulate alternative macrophage activation/function in *Nrf2*-deficient BMMs, we focused on the M1 (*Nos2*<sup>+</sup>) and M2 (*Mrc-1*<sup>+</sup> and *Arg-1*<sup>+</sup>) macrophage phenotype. Indeed, Ad-HO-1 transfection in *Nrf2*-deficient BMMs inhibited *Nos2*<sup>+</sup> while enhancing *Mrc-1* and *Arg-1*<sup>+</sup> expression (Figure 6A). Consistent with these data, the expression of TGF-β and IL-10 was consistently increased in Ad-HO-1-transfected BMMs compared with controls (Figure 6B).

## DISCUSSION

*Nrf2* is a key transcription factor that regulates oxidative stress and inflammation through activation of its targeting

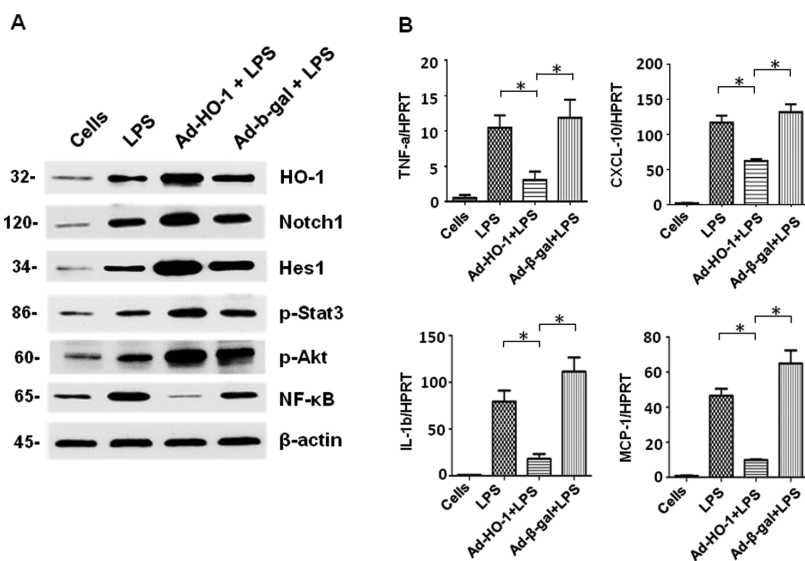


**Figure 4.** Adoptive transfer of Ad-HO-1 BMMs ameliorates hepatocellular apoptosis in *Nrf2*-deficient mice (6 h of reperfusion after 90 min of ischemia). (A) Representative (n = 3) Western blot analysis of hepatic *cleaved caspase-3*, *Bcl-2* and *Bcl-xL* after infusion of Ad-HO-1- or Ad-β-gal-transfected BMMs or untreated BMMs. (B) Representative (n = 3) TUNEL-assisted detection of apoptosis in ischemic liver tissue. Scale bars indicate 50 μm. (C) TUNEL+ cells were scored semiquantitatively by averaging the number of apoptotic cells (mean ± SD) per field. A minimum of 10 fields was evaluated/sample. \**p* < 0.001. There were 4–6 mice/group.

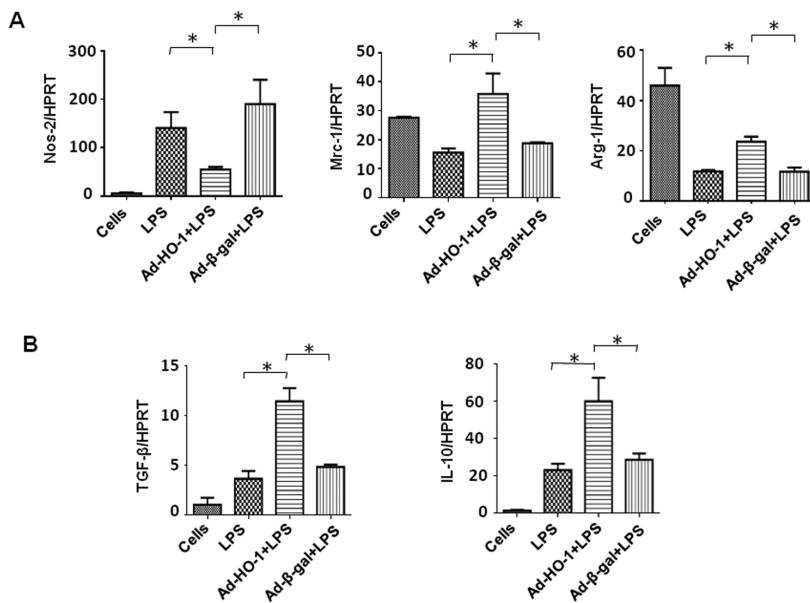
genes encoding antioxidant and detoxifying molecules (27). In this study, adoptive transfer of *ex vivo* genetically modified HO-1-overexpressing BMMs ameliorated liver IRI in *Nrf2* KO mice, suggesting HO-1 can successfully impose an antiinflammatory phenotype in *Nrf2*-deficient IR-stressed livers.

The major mechanism of *Nrf2*-dependent cellular defense against oxidative stress is activation of *Nrf2*-ARE signaling (28). Many antioxidative genes involved in cellular protection are under the transcriptional control of *Nrf2*-ARE (29). *Nrf2* promotes cell survival by binding ARE in the promoter of downstream target genes that induce antioxidant molecules and scavenging of ROS. Indeed, activation of *Nrf2* induces HO-1 expression to regulate oxidative stress while inactivating the transcriptional repressor *Bach1* (13,30). Increasing cellular levels of HO-1 enhances its antiinflammatory effects in macrophages (31). The ability of Ad-HO-1 BMMs to impose cytoprotection in IRI-susceptible *Nrf2*-deficient livers, as shown in the current study, underlines the regulatory function of the *Nrf2*-HO-1 axis in sterile-type inflammation response, consistent with our previous findings that *Nrf2* mediates its cytoprotection by inducing HO-1 (32).

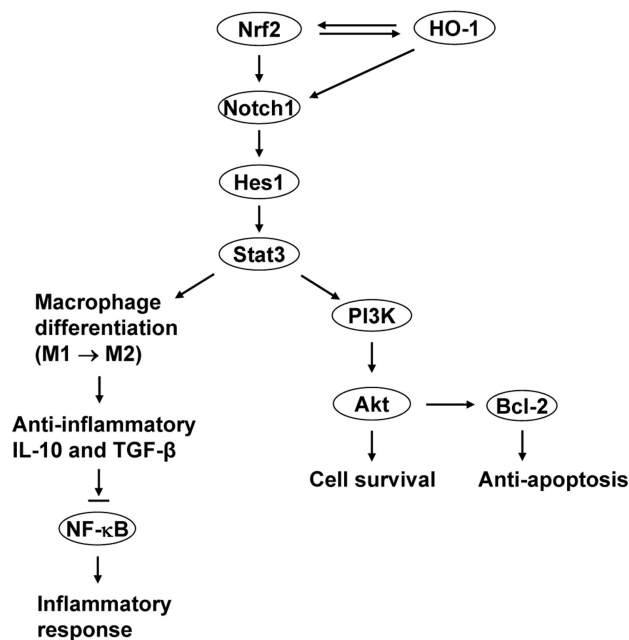
Because *Nrf2* activity is required to counteract the oxidative stress via induction of HO-1 in the cell defense system (33), the regulatory mechanism of *Nrf2*-HO-1 axis may involve multiple intercellular signaling pathways. We found that adoptive transfer of Ad-HO-1 BMMs into *Nrf2* KO mice activated *Notch* signaling, a highly conserved pathway that controls cell fate, including cell growth, differentiation and survival (34). Although IR-triggered injury upregulated *Notch1* expression in our study, HO-1 induction further enhanced *Notch* signaling after hepatic IR (Supplementary Figure S1). Indeed, activation of *Notch* signaling has been shown to increase hepatocyte DNA synthesis and cell survival, whereas disruption of *Notch* suppressed hepatocyte proliferation during liver regeneration after partial hepatectomy (35). Moreover,



**Figure 5.** *Nrf2*-HO-1 axis promotes *Notch1*/*Hes1*/*Stat3* signaling to regulate inflammatory response *in vitro*. *Nrf2*-deficient BMMs were transfected with Ad-HO-1 or Ad-β-gal (at an MOI of 10). After 36–48 h, cells were incubated with LPS (100 ng/mL) for 6 h. (A) Proteins isolated from Ad-HO-1/Ad-β-gal transfected or untreated BMMs were stained for *HO-1*, *Notch1*, *Hes1*, *phos-Akt*, *phos-Stat3* and *NF-κB*. Anti-β-actin antibody was used to assure equal protein amounts between samples. Data are representative of three experiments. (B) Cytokine/chemokine programs analyzed by quantitative real-time PCR. \**p* < 0.05. Mean ± SD; n = 3–4/group.



**Figure 6.** Nrf2–HO-1 axis regulates macrophage differentiation and function *in vitro*. Nrf2-deficient BMMs were transfected with Ad-HO-1 or Ad-β-gal (at an MOI of 10). After 36–48 h, cells were incubated with LPS (100 ng/mL) for an additional 6 h. Quantitative RT-PCR assisted analysis of macrophage: (A) phenotype (*Nos2*<sup>+</sup>, *Mirc-1*/*Arg-1*<sup>+</sup>); (B) TGF-β/IL-10 levels. \**p* < 0.05. Mean ± SD; n = 3–4/group.



**Figure 7.** Scheme of signaling pathways by which the Nrf2–HO-1 axis may regulate inflammatory responses in liver IRI. HO-1 rescues Nrf2-dependent antiinflammatory functions by activating Notch1/Hes1/Stat3 and reprogramming macrophages toward the M2 phenotype. This step, in turn, upregulates antiinflammatory IL-10/TGF-β, leading to inhibition of NF-κB activation and inflammatory responses in IR-stressed livers. Furthermore, HO-1-mediated Stat3 activation promotes PI3K/Akt signaling to increase cell survival and enhance antiapoptotic activity, which may also exert hepatoprotection in IR-stressed livers.

blocking *Notch* increased ROS production and aggravated IR-liver damage. In contrast, enhancing *Notch* activation reduced intracellular ROS and protected hepatocytes from IR-triggered apoptosis through the activation of *STAT3* (36). Consistent with *Notch*-mediated cytoprotection, our study has revealed that *Nrf2* deficiency increased IR-triggered liver damage (Supplementary Figure S2). Overexpression of HO-1 after adoptive transfer of genetically modified BMMs into *Nrf2*-deficient livers upregulated the expression of *Hes1*, a *Notch* downstream effector molecule crucial for the regulation of cell cycle and survival (37,38). Indeed, as a transcriptional regulator, *Hes1* has been shown to control differentiation/proliferation of neuronal, endocrine and T-lymphocyte progenitors during cell development (39–41). Induction of *Hes1* increases cell survival by directly controlling the activity of transcriptional repression of a cyclin-dependent kinase inhibitor *p27<sup>Kip1</sup>* (42). Thus, Nrf2–HO-1 axis promotes Notch1/Hes1 signaling, which contributes to IR-hepatocellular protection.

Adoptive transfer of Ad-HO-1 BMMs induced Akt activation and enhanced antiapoptotic gene expression in *Nrf2*-deficient livers, accompanied by decreased frequency of apoptotic cells. In marked contrast, *Nrf2* deficiency itself without infusion of Ad-HO-1 BMMs increased hepatocyte apoptosis in parallel with depressed *Akt* phosphorylation. Indeed, Akt promotes cell survival by phosphorylating Bcl-2/Bcl-xL-associated death promoter (*BAD*), a pro-apoptotic protein of the *Bcl-2* family, and inhibiting caspase-mediated cell death (43). Moreover, *Akt* promotes cell survival in a variety of apoptotic paradigms (44,45). Activation of *Akt* inhibits cell apoptosis by reducing apoptosis signal-regulating kinase 1 (*ASK1*) activity (46). Consistent with our previous findings that *Nrf2* activation induces HO-1 to promote phosphatidylinositol 3-kinase (PI3K)/Akt in hepatocyte protection (32), our current results support the cytoprotective role of the Nrf2–HO-1 axis in IR-induced liver apoptosis.

We have shown that macrophages may serve as a cellular delivery system for HO-1 gene transfer in the ischemic livers (23). Indeed, adoptive transfer of HO-1-modified BMMs markedly increased HO-1 mRNA and protein expression levels in ischemic livers (23), suggesting the macrophage–HO-1 delivery system functions as a therapeutic targeting gene in IR-stressed liver. However, because macrophages are pivotal for IR-liver inflammatory injury, the question arises as to whether the Nrf2–HO-1 axis can regulate macrophage activation and induce an alternative macrophage polarization in our model? We analyzed the molecular mechanisms by which Nrf2–HO-1 axis may regulate Notch1/Hes1/Stat3 signaling upon macrophage polarization during inflammatory response *in vitro*. First, we found that Nrf2-deficient BMMs transfected with Ad-HO-1 showed increased expression of HO-1, Notch1 and Hes1, as well as enhanced phosphorylation of Stat3, critical for macrophage differentiation and function (47). Indeed, activation of Notch signaling, which protects hepatocytes against hepatic IR, is required for Hes-dependent Stat3 activation (36), whereas Hes can bind Stat3 to regulate cell differentiation (48). Here, we show that induction of Hes1 enhanced Stat3 phosphorylation, leading to selective inhibition of the M1 phenotype (Nos2<sup>+</sup>) while simultaneously increasing the M2 phenotype (Mrc-1/Arg-1<sup>+</sup>), along with the antiinflammatory (TGF-β/IL-10) cytokine program in Ad-HO-1-transfected BMMs. In contrast, Nrf2 deficiency in LPS-stimulated control BMMs increased Nos2<sup>+</sup> while decreasing Mrc-1<sup>+</sup>/Arg-1<sup>+</sup> and the antiinflammatory cytokine program. Consistent with our *in vivo* findings that infusion of Ad-HO-1 BMMs increased antiinflammatory phenotype to limit IR-induced inflammation in Nrf2-deficient livers, our results are consistent with the key regulatory role of Nrf2–HO-1 axis in Notch1/Hes1/Stat3 signaling to promote alternative macrophage activation toward an antiinflammatory M2 phenotype during hepatic IRI.

## CONCLUSION

Adoptive transfer of Ad-HO-1 BMMs rescued Nrf2-dependent antiinflammatory phenotype *in vivo* and *in vitro*. The Nrf2–HO-1 axis promoted Notch1/Hes1/Stat3 signaling to regulate macrophage activation, enhance antiinflammatory cytokine program and inhibit liver IRI. The present data, in agreement with our recent report (23), confirm the validity of a novel investigative tool in which native macrophages can be transfected *ex vivo* with cytoprotective HO-1 and then infused, if needed, to prospective recipients to mitigate IR-mediated inflammation, such as during liver transplantation, resection or trauma. Moreover, as decreasing donor organ quality represents one of the most challenging problems, our findings provide the rationale for a new and clinically attractive gene targeted strategy to “rejuvenate” extended criteria suboptimal donor livers and thus improve their function in transplant recipients.

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## DISCLOSURE

The authors declare that they have no competing interests as defined by *Molecular Medicine*, or other interests that might be perceived to influence the results and discussion reported in this paper.

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