Heme Oxygenase-1 Regulates Myeloid Cell Trafficking in AKI

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ABSTRACT
Renal ischemia-reperfusion injury is mediated by a complex cascade of events, including the immune response, that occur secondary to injury to renal epithelial cells. We tested the hypothesis that heme oxygenase-1 (HO-1) expression, which is protective in ischemia-reperfusion injury, regulates trafficking of myeloid-derived immune cells in the kidney. Age-matched male wild-type (HO-1+/+), HO-1–knockout (HO-1−/−), and humanized HO-1–overexpressing (HBAC) mice underwent bilateral renal ischemia for 10 minutes. Ischemia-reperfusion injury resulted in significantly worse renal structure and function and increased mortality in HO-1−/− mice. In addition, there were more macrophages (CD45+ CD11bhi F4/80lo) and neutrophils (CD45+ CD11bhi MHCII2 Gr-1hi) in HO-1−/− kidneys than in sham and HO-1+/+ control kidneys subjected to ischemia-reperfusion. However, ischemic injury resulted in a significant decrease in the intrarenal resident dendritic cell (DC; CD45+ MHCII+ CD11blo F4/80hi) population in HO-1−/− kidneys compared with controls. Syngeneic transplant experiments utilizing green fluorescent protein–positive (GFPL+) HO-1+/+ or HO-1−/− donor kidneys and green fluorescent protein–negative HO-1+/+ recipients confirmed increased migration of the resident DC population from HO-1−/− donor kidneys, compared to HO-1+/+ donor kidneys, to the peripheral lymphoid organs. This effect on renal DC migration was corroborated in myeloid-specific HO-1−/− mice subjected to bilateral ischemia. These mice also displayed impaired renal recovery and increased fibrosis at day 7 after injury. These results highlight an important role for HO-1 in orchestrating the trafficking of myeloid cells in AKI, which may represent a key pathway for therapeutic intervention.

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AKI is an intractable clinical condition associated with significant morbidity and mortality.1,2 Epithelial injury and death, circulatory dysfunction, and immunologic factors from the circulation or within the kidney influence the integrity of the renal parenchyma and determine the outcome of AKI.3,4 The innate immune system, which includes cells of the myeloid lineage such as neutrophils, circulating monocytes, tissue macrophages, dendritic cells (DCs), and their precursors, is a key contributor to the pathogenesis and resolution of AKI.5–7 Ischemia-reperfusion injury (IRI) is a common cause of AKI in clinical settings such as major cardiac surgery, hypovolemic shock, and renal transplantation.8–11 The
pathogenesis of IRI involves crosstalk between the kidney parenchyma and the immune system, with effects on renal hemodynamics and tubular injury.12,13

Heme oxygenase-1 (HO-1) is an important cytoprotective enzyme that regulates the inflammatory response to tissue injury.14,15 Its induction is a protective response in renal IRI and other forms of AKI.16,17 HO-1 expression decreases neutrophil infiltration after acute tissue injury.18 In addition, HO-1 expression in DCs regulates their ability to undergo maturation and antigen presentation in lymphoid tissues.14,19 HO-1 expression decreases neutrophil infiltration after IRI (Figure 1C). The proportion of bone marrow–derived (CD45−) HO-1+/+ and (GFP+) HO-1+/− donor kidneys into green fluorescent protein–negative (GFP−) HO-1+/− recipients to evaluate the role of HO-1 in trafficking of myeloid cells after IRI.

RESULTS

HO-1 Deficiency Increases Susceptibility to Renal IRI

HO-1–deficient mice are extremely susceptible to renal IRI.23 Therefore, we used a short ischemia time (10 minutes), which was sublethal in HO-1+/+ mice, but resulted in 50% mortality after 1 day of reperfusion, and 60% mortality by day 2 in HO-1−/− mice (P<0.05) (Figure 1A). Serum creatinine was significantly elevated by day 1 in HO-1−/− mice compared with HO-1+/+ mice (P=0.01) and sham-operated control mice (Figure 1B). In HO-1−/− mice that survived to day 7, serum creatinine was not significantly different from baseline levels or HO-1+/+ controls. Overexpression of human HO-1 in HBAC mice rescued the mortality and decline in kidney function observed in HO-1−/− mice (Figure 1).

HO-1 Expression Prevents Tubulointerstitial Damage and Renal Fibrosis after IRI

There appeared to be no differences in renal histology in HO-1+/+, HO-1−/−, and HBAC mice subjected to sham surgery. However, significant injury was evident in HO-1−/− kidneys at day 1 after IRI, with extensive brush border loss in the proximal tubules, cast formation, and necrotic tubules. These features were not present in HO-1+/+ and HBAC kidneys subjected to IRI (Figure 2A). At day 7 after IRI, cast formation was increased in HO-1−/− mice compared with HO-1+/+ controls. Analysis of renal fibrosis by picrosirius red staining 7 days after IRI revealed increased collagen deposition in HO-1−/− kidneys compared with HO-1+/+ and HBAC kidneys (Figure 2B). Western blotting demonstrated increased expression of both α smooth muscle actin (Figure 2C) and fibronectin (Figure 2D) in HO-1−/− kidneys subjected to IRI.

HO-1 Deficiency Alters the Trafficking of Myeloid-Derived Immune Cells after IRI

Single cell suspensions from perfused kidneys harvested 1 day after 10 minutes of ischemia were analyzed by flow cytometry (Figure 3A). The proportion of bone marrow–derived (CD45+) cells was significantly elevated in HO-1−/− kidneys subjected to IRI compared with kidneys from HO-1+/+ and HBAC mice (P<0.01) (Figure 3, B and D). Further characterization of CD45+ cells revealed significant neutrophil (CD45+ CD11bhi Gr-1hi) and macrophage (CD45+ CD11bhi MHCIIhi) recruitment (Figure 3A) in HO-1−/− kidneys compared with HO-1+/+ and HBAC kidneys subjected to IRI (P<0.001) and sham kidneys (Figure 3, C and E). Seven days after ischemia, there were no significant differences in neutrophil infiltration (data not shown).

IRI also resulted in a striking decrease in the proportion of CD45+ CD11bhi MHCII+ cells (hereafter called RMNCs; Figure 3, A, C, and F) in HO-1−/− kidneys compared with HO-1+/+ and HBAC mice (P<0.001). There was no significant difference in sham-operated mice, suggesting that the decrease of RMNCs in HO-1−/− mice is a specific response to kidney injury. The observed decrease in RMNCs between HO-1−/− kidneys subjected to sham and IRI (P<0.001) may be explained by trafficking of RMNCs from the injured kidney.

Figure 1. HO-1−/− mice exhibit increased susceptibility to renal IRI. (A) Kaplan–Meier survival plot demonstrating mortality over 7 days in HO-1−/− (n=10), HO-1+/− (n=12), and HBAC (n=6) mice subjected to 10 minutes of IRI. **P<0.01 versus HO-1+/− and HBAC. (B) Serum creatinine levels in sham-operated mice and after 1 or 7 days of IRI. Results are presented as the mean±SEM. **P<0.01 in HO-1−/− versus HO-1+/− and HBAC mice at day 1. n=6–12 for IRI groups and n=3–4 for sham groups.
The predominant population of immune cells in the quiescent kidney is F4/80 \textsuperscript{hi} CD11b \textsuperscript{lo}, although a second smaller population of F4/80 \textsuperscript{lo} CD11b \textsuperscript{hi} cells is also present and significantly increases after IRI.\textsuperscript{24} These cells have been previously defined as DCs and macrophages, respectively, and will be referred to as such hereafter.\textsuperscript{24,25} We confirmed this previously reported finding by analyzing RMNC expression of F4/80 in the quiescent kidney and after IRI (Figure 4, Supplemental Figure 1). As expected, the F4/80 \textsuperscript{hi} CD11b \textsuperscript{lo} population (DCs) predominates in the kidneys of mice subjected to sham surgery, with no significant difference between macrophages or DCs in HO-1\textsuperscript{+/+} and HO-1\textsuperscript{2/2} mice (Supplemental Figure 1). However, relative to HO-1\textsuperscript{+/+} or HBAC mice, HO-1 deficiency results in a striking decline in the proportion of DCs (P<0.001) and a concomitant increase in the proportion of macrophages (P<0.001) after IRI (Figure 4, A–C). The increase in macrophages and decrease in DCs in HO-1\textsuperscript{2/2} kidneys was also confirmed by analyzing changes in absolute cell number in mice subjected to renal IRI (Figure 4, D and E).

**HO-1–Deficient RMNCs Exhibit Dysregulated Cytokine Gene Expression**

RMNCs (CD45\textsuperscript{+} CD11b\textsuperscript{+} MHCII\textsuperscript{+} Gr-1 \textsuperscript{−}) isolated from kidneys 1 day after IRI demonstrated robust induction of HO-1 expression compared with RMNCs isolated from sham kidneys (P<0.001) (Figure 5A). Cytokine expression was also assessed, which revealed marked induction of proinflammatory IL-6 expression (P<0.05) in HO-1\textsuperscript{−/−} compared with HO-1\textsuperscript{+/+} and HBAC RMNCs (Figure 5B). The expression of the proinflammatory cytokine TNF\alpha was suppressed in HO-1\textsuperscript{−/−} RMNCs (Figure 5C), likely as a result of decreased DCs within the RMNC population after IRI (Figures 3 and 4).\textsuperscript{16} Interestingly, RMNCs from HBAC mice overexpress IL-10 relative to HO-1\textsuperscript{+/+} and HO-1\textsuperscript{2/2} RMNCs (P<0.01) (Figure 5D).

**HO-1 Deficiency Increases Trafficking of Resident RMNCs from the Kidney after IRI**

To mitigate the confounding variable of a systemic proinflammatory milieu and deranged immune system in HO-1\textsuperscript{2/2} mice,\textsuperscript{26,27} we employed syngeneic kidney transplant experiments, utilizing GFP\textsuperscript{2} HO-1\textsuperscript{+/+} recipients and donor kidneys from their GFP\textsuperscript{+} HO-1\textsuperscript{2/2} or GFP\textsuperscript{+} HO-1\textsuperscript{+/+} littermates (Figure 6A, Table 1). This model provides a clear means to delineate kidney-resident immune cells (GFP\textsuperscript{+} CD45\textsuperscript{+}) from recipient-derived immune cells (GFP\textsuperscript{−} CD45\textsuperscript{−}) that infiltrate the graft after injury, which was induced by 25 minutes of warm ischemia (Figure 6B). Flow cytometry analysis of GFP\textsuperscript{+} HO-1\textsuperscript{−/−} grafts demonstrated a significant decrease in the proportion of resident GFP\textsuperscript{+} CD45\textsuperscript{+} cells compared with GFP\textsuperscript{+} HO-1\textsuperscript{+/+} grafts (P=0.001) (Figure 6C) 3 days after transplantation, supporting our previous finding (Figure 3) and suggesting increased trafficking of RMNCs from HO-1\textsuperscript{−/−} kidneys after ischemic injury. However, there was no significant difference in the proportion of graft-infiltrating cells (GFP\textsuperscript{−} CD45\textsuperscript{+}) in the HO-1\textsuperscript{−/−} graft compared with the HO-1\textsuperscript{+/+} control (Figure 6D). The vast majority of GFP\textsuperscript{+} CD45\textsuperscript{−} cells in...
HO-1+/+ grafts were RMNCs (Figure 6E). The proportion of RMNCs decreased significantly in HO-1−/− grafts (P<0.05), likely owing to a decrease in the resident DC population (GFP+ F4/80hi CD11blo) (P<0.05) compared with HO-1+/+ grafts (Figure 6F). The proportion of resident macrophages (GFP+ F4/80lo CD11bhi) was significantly higher in HO-1−/− grafts (Figure 6G).

HO-1 Deﬁciency Increases RMNC Trafﬁcking to the Peripheral Lymphoid Organs

We next examined whether the decreased proportion of intrarenal resident DCs in HO-1−/− kidneys after IRI (Figure 4) or syngeneic transplantation (Figure 6) resulted from increased trafﬁcking of these cells to the peripheral lymphoid organs. Kidneys from GFP+ HO-1+/+ or GFP+ HO-1−/− mice were transplanted into GFP− HO-1−/− or GFP− HO-1+/+ mice to delineate the effects of HO-1 deﬁciency within the renal parenchyma versus the RMNC (Table 1), we utilized mice with myeloid-restricted expression of Cre-recombinase (LysM-Cre) that were bred to mice containing biallelic ﬂocked HO-1

Figure 3. HO-1 deﬁciency alters the trafﬁcking of myeloid-derived immune cells after IRI. One day after sham surgery or 10 minutes of bilateral IRI, kidneys are explanted and homogenized for analysis by ﬂow cytometry. (A) Scheme depicting the sequential gating used to identify neutrophils (CD45+ CD11bhi MHCIi− Gr-1hi) and RMNCs (CD45+ CD11b+ MHCIi+) in the kidney of HO-1+/+, HO-1−/−, and HBAC mice. (B and C) Representative ﬂow cytometry histograms depicting the proportion of bone marrow–derived cells (CD45+) (B) or neutrophils and RMNCs (C) in the kidney after IRI. (D) Quantiﬁcation of CD45+ cells in the kidney, presented as a proportion of total cells. (E and F) Quantiﬁcation of neutrophils (E) and RMNCs (F) in the kidney, presented as a proportion of CD45+ cells. *P<0.05; **P<0.01; ***P<0.001 versus the indicated group. Results are expressed as the mean ±SEM. n=4 per group.
constructs to generate mice with myeloid-specific HO-1 deficiency, hereafter called HO-1^{lysM−/−}. These mice lack HO-1 expression in cells of the myeloid lineage, which includes macrophages, monocytes, neutrophils, and tissue-resident DCs. HO-1 flox mice lacking LysM-Cre (HO-1^{lysM+/+}) were used as controls. Twenty-five minutes of bilateral renal ischemia was sublethal (0% mortality; data not shown) but caused a significant increase in serum creatinine at days 1 and 3 in both HO-1^{lysM−/−} and HO-1^{lysM+/+} mice compared with baseline (Figure 8A). Compared with baseline values, serum creatinine at days 5 and 7 remained significantly elevated in HO-1^{lysM−/−} mice but not HO-1^{lysM+/+} control mice. Three days after IRI, cast formation, proximal tubular brush border loss, and tubule necrosis were evident but not significantly different between kidneys from HO-1^{lysM−/−} and HO-1^{lysM+/+} mice (Figure 8, B and C). Seven days after IRI, compared with HO-1^{lysM+/+} kidneys, there were significantly more casts in HO-1^{lysM−/−} kidneys (21.3±2.6 versus 5.98±1.14 in HO-1^{lysM+/+}, P<0.01; Figure 8D) and delayed regeneration of the proximal tubule brush border (Figure 8D, inset). The apparent delay in recovery from IRI in HO-1^{lysM−/−} kidneys was accompanied by a striking increase in renal fibrosis, as evidenced by increased picrosirius red staining for collagen (Figure 8E) and fibronectin expression (Figure 8F).

**DISCUSSION**

HO-1 modulates trafficking of three types of myeloid immune cells: neutrophils, macrophages, and tissue-resident DCs. First, HO-1 deficiency results in early (day 1) infiltration of neutrophils after IRI consistent with previous studies showing a role for HO-1 expression in trafficking of neutrophils.31,32 We have shown that HO-1 overexpression in the heart prevents injury-induced neutrophil infiltration occurring secondary to myocyte dysfunction and death.18 In this study, we demonstrate significantly increased neutrophil infiltration after AKI in HO-1^{−/−} kidneys, but not in HO-1^{lysM−/−} mice, which supports a protective role for

**Figure 4**. The intrarenal resident F4/80^{lo} CD11b^{lo} subpopulation of RMNCs specifically decreases after IRI. (A) RMNCs (intrarenal CD11b^{+} MHCII^{+}) from HO-1^{+/+}, HO-1^{−/−}, and HBAC mice subjected to IRI are further characterized based on their expression of F4/80. (B–D) F4/80^{lo} CD11b^{hi} macrophages (MΦ) (B) and F4/80^{hi} CD11b^{lo} DCs (C) are presented as a proportion of total RMNCs and as absolute cell number per gram of kidney weight for macrophages (D) and DCs (E). *P<0.05; **P<0.01; ***P<0.001 versus the indicated group. Results are expressed as the mean±SEM. n=4 per group.
HO-1 against neutrophil infiltration at the level of the tissue parenchyma.

Although many studies have described the protection conferred upon parenchymal cells by HO-1 expression, relatively few have used targeted approaches such as cell tracking and cell-sterred upon parenchymal cells by HO-1 expression, relatively few

myeloid HO-1 expression is necessary for proinflammatory macropage skewing in the adipose tissue of mice on a high-fat diet. In this study, HO-1 expression appears to be important within intrarenal resident DCs and in macrophages. At day 3, transplant experiments with HO-1+/− renal grafts (HO-1+/− renal parenchyma and RMNCs, but HO-1+/+ systemic immune system; Table 1) demonstrate a significant decrease in resident (GFP+) DCs, yet no significant increase in the infiltration of monocyte-derived macrophages from the circulation (GFP−). However, HO-1LysM−/− mice (HO-1+/− myeloid cells, including RMNCs, but HO-1+/+ renal parenchyma; Table 1) subjected to IRI exhibit a significant decrease of DCs and a significant increase in Ly6C+ proinflammatory macrophages 3 days after IRI. These results argue that myeloid HO-1 regulates DC emigration and directly modulates the macrophage response to AKI, independent of HO-1 expression in the renal parenchyma. In addition, we observed no proinflammatory milieu in HO-1+/− mice could effectively prime DCs in the quiescent kidney to be more likely to emigrate after injury. Finally, HO-1 deficiency in DCs could have an effect on migratory capacity, resulting in increased trafficking secondary to IRI. To address each of these possibilities, we used myeloid-specific HO-1+/− mice and syngeneic transplant experiments with HO-1+/+ recipient mice. Regarding the first possibility, we confirmed previous findings demonstrating that the mild ischemia time (10 minutes) causes a significant injury in HO-1+/− mice without causing significant changes in HO-1+/+ mice. This raises the possibility that our finding of DC emigration after 10 minutes of IRI, which only occurred in HO-1+/− kidneys, may simply result from significantly less AKI in HO-1+/+ mice. In subsequent studies utilizing HO-1LysM−/− mice, 25 minutes of bilateral ischemia induced significant changes in serum creatinine at day 1 and 3 compared to baseline levels. In these studies, although the extent of injury was no different between HO-1LysM−/− mice and HO-1LysM+/+ controls, significant DC emigration and macrophage infiltration was still evident in HO-1LysM−/− kidneys. This observation suggests that our earlier findings are specific to HO-1 expression in RMNCs and circulating myeloid cells, as opposed to the extent of AKI.

We have also provided evidence suggesting that the second possibility is unlikely. After syngeneic kidney transplantation, increased DC emigration from the graft is observed in HO-1+/− kidneys compared to HO-1+/+ grafts. Because the recipients in significant differences in serum creatinine or tubulointerstitial damage between HO-1LysM−/− and HO-1LysM+/+ mice 3 days after IRI, which suggests that these effects on myeloid trafficking are independent of the extent of tissue injury and subsequent functional changes. However, at later time points (e.g., day 7), myeloid HO-1 deficiency impairs the resolution of renal injury, resulting in increased renal fibrosis, persistence of tubular casts, and delayed regeneration of the proximal tubule brush border. Collectively, these data suggest that myeloid HO-1 expression is important in regulating the early (e.g., day 3) trafficking of DCs from and macrophages to the kidney after AKI, and that these events have an important effect at later time points in the resolution of kidney injury.

Figure 5. HO-1−deficient RMNCs exhibit dysregulated cytokine gene expression. Gene expression in electronically sorted RMNCs is analyzed by real-time PCR. (A) HO-1 expression is assessed in HO-1+/+ and HO-1−/− mice subjected to sham or 10 minutes of ischemia and 1 day of reperfusion. (B–D) Expression of the cytokines IL-6 (B), TNFα (C), and IL-10 (D) is quantified in HO-1+/+, HO-1−/−, and HBAC RMNC 1 day after 10 minutes of renal ischemia. Analysis is performed in triplicate and results are normalized to GAPDH and expressed as the mean±SEM. *P<0.05; **P<0.01; ***P<0.001. n=3 per group.
the transplant experiments are HO-1+/+, the effect of the systemic proinflammatory milieu in HO-1−/− mice is nullified, thus allowing for the isolation of events that are caused by IRI in the HO-1−/− deficient renal parenchyma or resident RMNC network. Our findings support previous studies that utilized pharmacologic modulation of HO-1 expression to demonstrate a role for HO-1 in immune cell trafficking.34,35 Collectively, these findings support a role for myeloid HO-1 in regulating the trafficking of intrarenal resident DCs (the third possibility). Although the decrease in intrarenal DCs could also be the result of a phenotypic switch, whereby DCs acquire the phenotypic characteristics of renal macrophages by reciprocally regulating their expression of CD11b and F4/80, this possibility is less likely, because syngeneic transplant experiments confirm increased DC emigration from the kidney to the peripheral lymphoid organs.

Figure 6. The resident RMNC population declines in HO-1−/− kidneys after IRI induced by syngeneic transplantation. (A) HO-1−/− or HO-1+/+ kidneys are harvested from GFP+ donors and transplanted into GFP+ HO-1+/+ littermates. IRI is modeled by 25 minutes of intraoperative ischemia. (B) Grafts are harvested 3 days after transplantation and analyzed by flow cytometry for the emigration of GFP+ CD45+ donor-derived resident cells or infiltration of GFP+ CD45+ recipient-derived inflammatory cells. (C and D) Emigration of GFP+ CD45+ donor-derived cells (C) and infiltration of GFP+ CD45+ recipient-derived cells (D), expressed as a proportion of the total renal cell population. (E–G) Donor-derived intrarenal resident cells are further characterized as CD11b+ MHCII+ RMNCs (E), F4/80hi CD11blo DCs (F), or F4/80lo CD11bhi macrophages (G) and expressed as a proportion of GFP+ CD45+ cells. *P<0.05; ***P≤0.001. n=3 per group. Results are expressed as the mean±SEM.
It is important to note technical differences between our experiments utilizing 25 minutes of bilateral IRI and those using syngeneic transplantation. In both instances, the warm ischemia time was carefully controlled and limited to precisely 25 minutes. In addition, the ensuing inflammatory response in both models was sterile in nature. Using inbred mouse strains and littermates as donor/recipient pairs in the transplant experiments eliminated the potential of a confounding alloresponse. However, several inherent differences exist, and deserve mention. First, transplant surgery is technically more complex than IRI surgery. Blood flow through the aorta and inferior vena cava just proximal to the bifurcation is stopped briefly during the vascular anastomosis. In addition, although the right kidney is removed during the transplant procedure, modeling bilateral IRI is not possible in the transplant model. Although these considerations may cause slight variations in the systemic immune response, their effect on our experimental observations is expected to be minimal.

Even mild 10-minute ischemic insult had a clear effect on HO-1+/+ mice at the level of the RMNC, as evidenced by robust induction of HO-1 expression (Figure 6). Further gene expression studies in RMNCs after IRI demonstrated several interesting findings. As expected, and consistent with previous studies, expression of the proinflammatory cytokine IL-6 was significantly elevated in HO-1−/− mice. The finding of decreased TNFα expression in the RMNCs of HO1−/− mice after IRI is consistent with our finding of DC emigration from the kidney, because resident DCs are the primary source of this proinflammatory cytokine in AKI. Overexpression of IL-10 in RMNCs sorted from HBAC mice is likely associated with HO-1 overexpression in these mice, because the production of anti-inflammatory IL-10 appears to be tightly coupled and proportional to HO-1 expression levels. DCs are the most abundant RMNCs in the quiescent kidney and are distributed throughout the parenchyma as an interconnected network of cells that likely contribute to tissue homeostasis and serve as sentinels to tissue insult. Macrophage deple-

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*For control strains, see the text.

**Table 1. Mouse models of HO-1 expression**
**Figure 7.** HO-1−/− RMNCs exhibit increased trafficking to the peripheral lymphoid organs. (A) RMNC trafficking is tracked using flow cytometry after syngeneic transplantation of GFP+ HO-1+/+ or GFP+ HO-1−/− donor kidneys. (B and C) One day (B) or 3 days (C) after transplantation, the spleen, renal LNs, and mesenteric (Mes.) LNs are harvested from kidney transplant recipients and donor-derived RMNCs that had emigrated from the graft are identified as GFP+ CD45+. (D) Increased trafficking from HO-1−/− grafts is confirmed in frozen tissue sections from the spleen, renal LNs, and mesenteric LNs from transplant recipients, utilizing the GFP+ signal from donor-derived RMNCs. *P≤0.05; **P≤0.01. n=3 per group. Results are expressed as the mean±SEM.
Cross-clamped for 10 or 25 minutes using an atraumatic vascular clamp (catalog number 18055-05; Fine Science Tools, Foster City, CA). Immediate blanching of the kidney confirmed ischemic induction. Body temperature was maintained at 37°C during surgery and ischemia time. Kidneys were inspected for color change within 1 minute of clamp removal to ensure uniform reperfusion. Control mice were subjected to sham operation, wherein both kidney pedicles were exposed but not clamped. Renal function was assessed by serum creatinine levels that were measured as previously described using liquid chromatography coupled to tandem mass spectrometry.29

**Syngeneic Kidney Transplantation Model**

All mice used in transplant experiments were littermates from the same inbred colony to ensure syngeneic transplantation. Donors were GFP$^{+}$ HO-1$^{-/-}$ or GFP$^{-}$ HO-1$^{-/-}$ and, in all experiments, recipients were GFP$^{-}$ HO-1$^{-/-}$. Orthotopic kidney transplantation was performed as previously described using the University of Alabama at Birmingham-University of California San Diego (UCSD) O’Brien Center Resource for preclinical studies (details in the Supplemental Methods).41 The donor mouse was perfused with saline to removed intravascular immune cells. Intraoperative warm ischemia time was held constant at 25 minutes for each transplant.

**Western Blot Analyses**

Western blotting was performed as previously described.18 Briefly, kidneys were homogenized in RIPA buffer (50 mmol/L Tris/HCl, 1% NP-40, 0.25% deoxycholic acid, 150 mmol/L NaCl, 1 mmol/L EGTA, 1 mmol/L sodium orthovanadate, and 1 mmol/L sodium phosphate) and proteins were separated on 4–12% Bis-Tris gels and transferred to a polyvinylidene difluoride membrane. After blocking, membranes were incubated with primary antibodies overnight at 4°C. After washing, membranes were incubated with horseradish peroxidase-conjugated secondary antibodies for 1 hour at room temperature. Signals were developed using the chemiluminescence method and quantified using ImageJ software. Experimental conditions were normalized to GAPDH or Ponceau S staining of the membrane as a loading control.
flouride) with protease inhibitor (Sigma-Aldrich) and were quantified using bicinchoninic acid protein assay (Thermo Fisher Scientific). Seventy-five micrograms of protein was resolved on a 12% Tris-glycine SDS-polyacrylamide electrophoresis gel and transferred to a polyvinylidene fluoride membrane (EMD Millipore). Membranes were blocked with 5% nonfat dry milk in PBS with Tween for 1 hour and then incubated with rabbit anti-HO-1 (1:2000; Enzo Life Sciences), anti-fibronectin (1:10,000; Sigma-Aldrich), or mouse anti-smooth muscle actin (1:5000; Sigma-Aldrich) antibodies, followed by a peroxidase-conjugated goat anti-rabbit (or mouse) IgG antibody (1:10,000; Jackson ImmunoResearch Laboratories). Horseradish peroxidase activity was detected using the enhanced chemiluminescence detection system (GE Healthcare). The membrane was stripped and probed with anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH) antibody (1:5000; Sigma-Aldrich) to confirm loading and transfer. Densitometry analysis was performed and results were normalized to GAPDH expression.

Light Microscopy
For histologic analysis, kidneys were cut transversely, fixed in 10% neutral buffered formalin overnight (Fisher Fisher Scientific, Pittsburgh, PA), and stored in 70% ethanol. After paraffin embedding, 5-μm sections were stained with picrosirius red or periodic acid–Schiff, as previously described. For the measurement of collagen deposition using picrosirius red, kidney sections were deparaffinized with xylene and then rehydrated in water through graded ethanol. The sections were incubated in picrosirius red for 1 hour followed by two washes in acidified water. The sections were then dehydrated, cleared, and mounted in a resinous medium. The area of collagen deposition, stained red by picrosirius red, was measured by color image analysis software (Image-Pro Plus; Media Cybernetics). To assess tubule injury and cast formation, kidney sections were stained with periodic acid–Schiff. Casts, loss of the brush border, and necrotic tubules were counted by a reviewer blinded to the different groups in proximal tubules of the outer medulla and are expressed as a total number per field. All proximal tubules (>80) per high-power field (×200 total magnification) were counted. At least five images were analyzed per mouse (n=4 per group).

Isolation and Enrichment of Renal Leukocytes for Flow Cytometry
Leukocytes were isolated as previously described with minor modifications, and details are included in the Supplemental Methods. Kidney preparations that were electronically sorted for quantification of gene expression by real-time PCR were enriched by density gradient centrifugation using NycoPrep 1.077 density gradient solution (Axis-Shield PoC, Oslo, Norway). Cells were then stained with fluorochrome-conjugated antibodies for 30 minutes on ice for analysis by flow cytometry. Isotype-matched, fluorescently conjugated antibodies of irrelevant specificity were used as controls. Results were analyzed using FlowJo Software (Tree Star Inc., Ashland, OR). The absolute cell numbers were determined using AccuCheck Counting Beads.
Table 2. Primer sequences used in real-time PCR analysis

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<td>TNFa Rev</td>
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</tr>
<tr>
<td>IL-10 Rev</td>
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</tr>
</tbody>
</table>

Fwd, forward; Rev, reverse.

Immunofluorescence Microscopy

Tissues harvested from mice transplanted with GFP⁺ kidneys that were used for immunofluorescence studies were cut in transverse section, fixed in 2% paraformaldehyde for 2 hours at 4°C, and then cryopreserved in 20% sucrose solution at 4°C for 2 days. The tissues were then embedded in Optimal Cutting Temperature freezing media and stored at −80°C. Five-micrometer sections were cut, mounted on slides, washed in PBS at room temperature, mounted with media that contained 4’,6-diamidino-2-phenylindole, and imaged. The stained sections were imaged on an epifluorescence microscope (Leica DMIRB) with Image-Pro 5.1 software (Media Cybernetics). Confocal images were captured using a Leica TCS-SP5 confocal microscope.

Real-Time PCR Analysis

RMNCs (CD45⁺CD11b⁺MHCII⁺Gr-1⁻) from mice subjected to IRI or sham surgery were isolated and collected on a FACSAria cell sorter (BD Biosciences) as described above. Total RNA was isolated from the sorted cells using TRIzol reagent (Invitrogen) according to the manufacturer’s protocol. One microgram of total RNA was converted to cDNA using the QuantiTect Reverse Transcription Kit (Qiagen). Quantitative real-time PCR was performed with SYBR Green Master Mix (Invitrogen) and 10 pmol of primers. Primer sequences (5′–3′) are provided in Table 2 as previously described. Relative gene expression was normalized to mouse GAPDH as an internal control and quantified using the ΔΔ threshold cycle method. Samples were performed in triplicate.

Statistical Analyses

Data are presented as the mean±SEM. The t test was used for comparison between two groups. Changes in serum creatinine values at days 1, 3, 5, and 7 after 25 minutes of bilateral IRI were compared with baseline levels using a paired t test of log-transformed values. ANOVA and the Newman–Keuls post test were used for analyses comparing more than two groups. Differences were considered statistically significant at P<0.05.

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DISCLOSURES

None.

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