Ischemia Reperfusion Induces IFN Regulatory Factor 4 in Renal Dendritic Cells, which Suppresses Postischemic Inflammation and Prevents Acute Renal Failure

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Ischemia Reperfusion Induces IFN Regulatory Factor 4 in Renal Dendritic Cells, which Suppresses Postischemic Inflammation and Prevents Acute Renal Failure

Saraswati Lassen,*1 Maciej Lech,*1 Christoph Röemmele,* Hans-Willi Mittruecker,†‡ Tak W. Mak,‡ and Hans-Joachim Anders*†

Ischemia reperfusion (IR) activates TLRs causing subsequent sterile inflammation, for example in postischemic acute renal failure. Unexpectedly, TLR signaling predominates in intrinsic renal cells and not in intrarenal APCs in the postischemic kidney. We hypothesized that certain factors suppress APC activation and thereby limit sterile renal inflammation, for example, IFN regulatory factor 4 (IRF-4), an inducible inhibitor of LPS signaling. Oxidative stress was a trigger for IRF4 induction in myeloid cells in vitro as well as in CD45CD11c+ cells in the postischemic kidney. Lack of IRF4 aggravated acute renal failure 24 h after renal artery clamping together with increased intrarenal expression of TNF-α, IL-6, CXCL2, and CCL2 as well as excessive tubular necrosis and peritubular neutrophil influx as compared with wild-type IR kidneys. This effect almost entirely depended on the role of IRF4 to suppress TNF-α release by intrarenal APCs because either clodronate liposome depletion of these cells or TNF-α blockade with etanercept entirely abrogated the aggravation of cytokine expression and acute renal failure in If4-deficient mice. Thus, loss-of-function mutations in the IRF4 gene predispose to IR injury because the postischemic induction of IRF4 in resident APCs like CD11c+ dendritic cells, suppresses them to secrete TNF-α, and thereby limits inappropriate immunopathology. The Journal of Immunology, 2010, 185: 000–000.

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Ischemia/reperfusion (IR)-induced tissue injuries (IRIs) like stroke, myocardial infarction, limb ischemia or postischemic acute renal failure commonly cause disability requiring costly medical care. Beyond direct hypoxic cell damage, IR triggers innate immune responses, which, for example, in the kidney, contribute indirectly to tubular cell necrosis and renal failure (1). How does IR cause innate immune activation? Several studies have documented that this process involves TLRs, a group of germ-line-encoded pathogen-recognition receptors that are highly conserved among species from Drosophila to humans (2). TLRs are transmembrane receptors in outer or endosomal membranes of macrophages, dendritic cells (DCs), neutrophils, B cells, and NK cells. Several TLRs are also expressed in nonimmune cells, including TLR2 and TLR4 on renal tubular epithelial cells (3). TLR signaling involves either of the two cytoplasmic adaptor molecules MyD88 or Toll/IL-1R domain-containing adaptor inducing IFN-β that facilitate the activation of MAPK, NF-κB or IFN regulatory factor (IRF) (4). In renal infection TLR activation drives the local production of proinflammatory cytokines, chemokines, and other soluble mediators that orchestrate inflammation and host defense (5, 6). Remarkably, damaged tissues release endogenous molecules that have the potential to activate TLR2/MyD88 and TLR4/MyD88 just like microbes (7, 8). This mechanism, referred to as danger signaling, also contributes to the sterile inflammation during IRIs. For example, Tlr4-, Tlr2-, and/or Myd88-deficient mice are protected from renal IRIs (9–11) similar to that of the liver (12), the heart (13, 14), and the brain (15). The network of intrarenal DC is considered to significantly contribute to danger signaling in the kidney (16). However, transfer of Tlr2-deficient bone marrow into wild-type recipient mice did not affect the extent of acute renal failure, whereas Tlr2-deficient recipients of wild-type bone marrow were protected from acute renal failure (9, 10). Similarly, transfer of Tlr4-deficient bone marrow into wild-type recipient mice had only a minor affect the extent of acute renal failure, whereas Tlr4-deficient recipients of wild-type bone marrow were protected from acute renal failure like Tlr4-deficient recipients of Tlr4-deficient bone marrow (9, 10). Obviously, TLR2 and TLR4 signaling in intrarenal myeloid cells is less important as compared with that in parenchymal cells of the postischemic kidney. We speculated that certain factors suppress TLR signaling in intrarenal myeloid cells during IR. Several endogenous molecules counterbalance inappropriate TLR2 and TLR4 signaling (17). For example, single Ig/IL-1–related receptor (SIGIRR) is a member of the transmembrane TLR/IL-1R family that inhibits TLR2/MyD88 and TLR4/MyD88 signaling via its intracellular domain (18–20). The constitutive expression of SIGIRR in intrarenal DCs (21) suppresses renal DC activation during IR and thereby limits postischemic sterile inflammation and protects from acute renal failure (22, 23). We speculated that beyond such constitutively expressed inhibitors the control of IRIs may also involve inducible inhibitors of TLR signaling in renal DCs.
We considered Irf4 as a candidate gene for such an inducible suppressor of postischemic innate immune activation. Irf4 is a member of the IRF transcription factor family but unlike other IRFs, Irf4 is not regulated by IFNs and its expression is restricted to immune cells (24). By contrast, Irf4 has multiple regulatory functions in adaptive immunity (24, 25). For example, Irf4 is required for the maturation of B and T cells (26), isotype switching (27), the ability of T regulatory cells to suppress Th2 responses (28), and the induction of Th17 T cells (29, 30). Irf4 also regulates innate immunity as it can suppress TLR2 and TLR4 signaling by binding to MyD88, which impairs its interaction with downstream signaling elements (31). For example, bacterial products specifically induce Irf4 in DCs of the intestinal wall, a mechanism that protects mice from experimental colitis (32). We therefore hypothesized that IR can also induce Irf4 in renal APCs that may suppress postischemic renal inflammation and acute renal failure, a hypothesis supported by our results.

Materials and Methods

Animal studies

Irf4-deficient mice were generated, genotyped, and backcrossed to the C57BL/6J strain for 10 generations as previously described (26). Mice were housed in groups of five in filter top cages with unlimited access to food and water. Cages, nestlets, food, and water were sterilized by autoclaving before use. All experimental procedures were approved by the local government authorities. C12MDP (clodronate) was a gift from Roche Diagnostics (Mannheim, Germany) and incorporated into liposomes as previously described (33). Baseline mRNA expression of TNF-α, IL-6, IL-12, MIP2, TGF-β, and IFN-γ was identical in wild-type and Irf4−/− mice (data not shown). In one experiment wild-type mice were injected with 5 mg/kg of the cell-permeant superoxide-dismutase mimetic manganese tetrakis (1-methyl-4-pyridyl) porphyrin (MnTMPyP, Sigma-Aldrich, Steinheim, Germany) 12 h before surgery, right after surgery, and 12 h after surgery. In other experiments wild-type mice were injected i.v. with 200 μl clodronate- or control-liposomes on days −3 and −2 before the IR procedure as described (34). The TNF-α inhibitor etanercept (Wyeth Pharma, Madison, NJ) was given into the abdominal cavity after clamp removal at a single dose of 10 mg/kg.

Induction of renal IRIs

Groups of mice (n = 6) were anesthetized as described (22) before both renal pedicles or only the left renal pedicle were clamped for 30 or 45 min with a microaneurysm clamp via 1 cm flank incisions (Medicon, Tutlingen, Germany). Body temperature was continuously measured with a rectal probe and maintained at 36˚C–37˚C throughout the procedure by placing the mice on a heating pad. After clamp removal, the kidney was inspected for restoration of blood flow as evidenced by returning to its original color before closing the wound with standard sutures (35). To maintain fluid balance, all mice were supplemented with 0.5 ml saline. Mice were sacrificed 1, 5, and 10 d after surgery, and pieces from IRIs and contralateral (sham) kidneys were either snap frozen in liquid nitrogen or fixed in 10% buffered formalin.
Kidneys were obtained from Irf4-deficient and wild-type mice at various time intervals as indicated. A. Periodic acid-Schiff stains from paraffin-embedded kidneys are shown at a magnification of ×100. B. Semiquantitative morphometry of tubular injury is shown as means ± SEM from five to six mice of each group. *p < 0.05 versus wild-type of the respective time point.

Histological evaluation

Kidneys were embedded in paraffin and 2 μm sections were used for periodic acid-Schiff stains and immunostaining as described (36). Postischemic tubular injury was scored by assessing the percentage of tubules in the corticomedullary junction that displayed cell necrosis, loss of brush border, cast formation, and tubular dilatation as follows: 0, none; 1, ≤10%; 2, 11–25%; 3, 26–45%; 4, 46–75%; and 5, >76% (35). For immunostaining the following primary Abs were used: rat anti-Mac2 (Cederlane, Ontario, Canada, 1:50), rat anti-mouse neutrophils (Serotec, Oxford, U.K., 1:50). To count interstitial cell isolates from postischemic kidneys 24 h after reperfusion (Fig. 1D), 1E). IRF4 mRNA mainly originated from intrarenal CD45+/CD11c+ cells when we compared IRF4 mRNA expression in kidney as compared with the high levels in spleen, thymus, and bone marrow (Fig. 1C). Therefore, we next determined whether IRF4 and IRF5 are induced during renal IRIs by measuring IRF4 and IRF5 mRNA levels in IRIs and sham kidneys by real-time PCR. Groups of C57BL/6J mice underwent unilateral clamping of the renal artery for 45 min, and both kidneys were harvested at 1, 5, and 10 d after surgery. Surgery itself did not significantly induce IRF4 or IRF5 mRNA levels in sham kidneys over time, whereas IRF4 (but not IRF5) mRNA levels were significantly elevated from day 5 compared with baseline (Fig. 1D, 1E). IRF4 mRNA mainly originated from intrarenal CD45+/CD11c+ cells when we compared IRF4 mRNA expression of CD45+/CD11c-depleted with CD45+/CD11c+ cell isolated postischemic kidneys 24 h after reperfusion (Fig. 1F). Pretreating mice with the superoxide dismutase agonist and antioxidant MnTMPyP entirely blunted the postischemic IRF4 induction in renal CD45+/CD11c+ cells (Fig. 1F). Therefore, we conclude that mouse kidneys express low levels of IRF4 but oxidative stress induces IRF4 expression in resident CD45+/CD11c+ cells.

Lack of IRF4 aggravates postischemic acute renal failure and renal IRIs

To determine the functional role of IRF4 induction in renal IRIs, we first induced bilateral renal IR for 30 min in wild-type and Irf4−/− mice (n = 5–6), respectively. In wild-type mice, serum creatinine levels increased from 0.4 ± 0.2 mg/dl at baseline to 1.6 ± 0.3 mg/dl within 24 h. In Irf4−/− mice, serum creatinine levels increased from 0.4 ± 0.1 mg/dl to 2.9 ± 0.1 mg/dl (p = 0.01 versus wild-type). Thus, lack of IRF4 aggravates acute renal failure after bilateral renal artery clamping in mice. The impact of the IRF4 genotypes on renal excretory function correlated with the extent of renal IRI after unilateral renal artery clamping. IRF4-deficiency enhanced IRI as evidenced by the composite score of widespread tubular necrosis, loss of brush border, cast formation, and tubular dilatation at the corticomedullary junction in the IR kidney (Fig. 2). The difference of IRI between Irf4−/− and Irf4+/+ mice was evident at day 1, 5, and 10 postsurgery. Contralateral kidneys were neither significantly affected by the surgical procedure nor by the Irf4 genotype. Together, lack of IRF4 aggravates postischemic acute renal failure and renal IRI.

Results

Renal IR induces IRF4 in renal CD45+/CD11c+ cells

We first studied whether oxidative stress is a trigger for IRF4 induction by exposing C57BL/6 mouse monocytes and tubular epithelial cells to a mixture of hypoxanthine and xanthine oxidase, which served as a ROS donor. Monocytes significantly induced IRF4 and HSP70 mRNA (Fig. 1A), whereas tubular epithelial cells induced HSP70 but not IRF4 (Fig. 1B). Thus, oxidative stress is a previously unknown trigger for IRF4 expression in monocytes. An IRF4 mRNA expression screen in solid organs of healthy adult C57BL/6 mice revealed low IRF4 mRNA expression levels in kidney as compared with the high levels in spleen, thymus, and bone marrow (Fig. 1C). Therefore, we next determined whether IRF4 and IRF5 are induced during renal IRIs by measuring IRF4 and IRF5 mRNA levels in IRIs and sham kidneys by real-time PCR. Groups of C57BL/6J mice underwent unilateral clamping of the renal artery for 45 min, and both kidneys were harvested at 1, 5, and 10 d after surgery. Surgery itself did not significantly induce IRF4 or IRF5 mRNA levels in sham kidneys over time, whereas IRF4 (but not IRF5) mRNA levels were significantly elevated from day 5 compared with baseline (Fig. 1D, 1E). IRF4 mRNA mainly originated from intrarenal CD45+/CD11c+ cells when we compared IRF4 mRNA expression of CD45+/CD11c-depleted with CD45+/CD11c+ cell isolated postischemic kidneys 24 h after reperfusion (Fig. 1F). Pretreating mice with the superoxide dismutase agonist and antioxidant MnTMPyP entirely blunted the postischemic IRF4 induction in renal CD45+/CD11c+ cells (Fig. 1F). Therefore, we conclude that mouse kidneys express low levels of IRF4 but oxidative stress induces IRF4 expression in resident CD45+/CD11c+ cells.
IRF4 suppresses TNF-α, IL-6, CCL2/MCP-1, and CXCL2/MIP2 expression after IR

It is known that IRF4 inhibits TLR-mediated expression of proinflammatory cytokines and chemokines (31), hence, lack of IRF4 may aggravate renal IRIs via increasing local cytokine and chemokine expression. We therefore determined the renal mRNA levels of IL-6, TNF-α, CXCL2/MIP2, and CCL2/MCP-1 (Table I). Lack of IRF4 was associated with significant higher mRNA levels of all of these factors at day 1 after renal artery clamping (Fig. 3A). The increase of IL-6 and TNF-α exclusively originated from intrarenal CD45/CD11c positive DCs, whereas CXCL2/MIP2 and CCL2/MCP-1 also originated from other CD45+ leukocytes that had been isolated from kidneys of wild-type and Irf4-deficient mice 24 h after IR (Fig. 3B). However, lack of IRF4 had no effect on cytokine and chemokine expression in CD45 negative renal parenchymal cells (Fig. 3B). Thus, IRF4 prevents postischemic acute renal failure by suppressing the early activation of intrarenal leukocytes, thereby preventing the expression of those proinflammatory cytokines and chemokines, which are known to trigger postischemic neutrophil recruitment and renal inflammation (34, 37).

Lack of IRF4 enhances interstitial neutrophil recruitment after IRIs

We suspected the increased intrarenal expression seen in Irf4-deficient mice would be associated with additional leukocyte influx. In fact, immunostaining documented increased neutrophil numbers at the corticomedullary junction adjacent to necrotic tubuli and in intratubular casts at 1 or 5 d after IR in Irf4-deficient versus wild-type mice (Fig. 4A,4B). By contrast, the Irf4 genotype did not affect the amount of interstitial macrophages or T cells as determined by

![FIGURE 3. Renal cytokine and chemokine mRNA expression after IRI. A, Total RNA was extracted from ischemic (designated as IR) and contralateral (designated as Co) kidneys of IRF4-deficient (white bars) or wild-type C57BL/6 mice (black bars) at different time intervals after IR as indicated. B, Renal cell suspensions from mice of both genotypes 24 h after surgery underwent magnetic bead isolation to separate CD45/CD11c double positive renal DCs, CD45+/CD11c+ renal leukocytes, and CD45 negative intrinsic renal cells. In A and B, mRNA expression levels were determined for the indicated cytokines and chemokines by real-time RT-PCR. Data are expressed as mean of the ratio versus the respective 18s rRNA level ± SEM. *p < 0.05 versus wild-type.]
the F4/80 positive interstitial area or by counting CD3 positive cells, respectively (Fig. 4B). Together, IRF4 suppresses postischemic acute renal failure and IRI in association with a lower influx of neutrophils early after IR.

Clodronate liposomes abrogate the IRI aggravation in Irf4-deficient mice

As we found TNF-α to be much induced in postischemic kidneys of Irf4-deficient mice and because intrarenal resident DCs had been reported to be the major source of TNF-α in postischemic kidneys (34), we speculated a dominant role of IRF4 to suppress TNF-α release in these cells. To address this question, we used i.p. injections of clodronate liposomes, a standard procedure to deplete intrarenal APCs (34). Clodronate injected 2 and 3 d before bilateral IR entirely prevented the aggravation of acute renal failure in Irf4-deficient mice (Fig. 5A). Clodronate also prevented the intrarenal induction of TNF-α, IL-6, CXCL2/MIP2, and CCL2/MCP-1 mRNA expression in Irf4-deficient mice (Fig. 5B). Together, renal IR specifically induces IRF4 in resident CD45+/CD11c+ cells, which suppresses their potential to massively induce TNF-α and other proinflammatory mediators of postischemic renal inflammation and acute renal failure.

The aggravation of postischemic renal failure in Irf4-deficient mice is mediated by TNF-α

Is the enhanced renal cytokine expression in Irf4-deficient mice causative for IRI and acute renal failure? To answer this question we blocked the biological effects of IR-induced TNF-α by a single dose of the functional TNF-α antagonist etanercept in both wild-type and Irf4-deficient mice. Etanercept, given directly after reperfusion completely abrogated the effect of the Irf4 genotype on serum creatinine levels at 24 h after IR (Fig. 5A). Etanercept also prevented the induction of TNF-α, IL-6, CXCL2/MIP2, and CCL2/MCP-1 mRNA expression in Irf4-deficient mice (Fig. 5B). Thus, unblocking of TNF-α induction in intrarenal CD45+/CD11c+ cells is the dominant mechanism by which IRF4-deficiency aggravates acute renal failure.

Discussion

IRF4 was recently reported to suppress TLR signaling in DC by preventing the interaction of MyD88 with IRF5 (31). Because postischemic inflammation involves MyD88 signaling we hypothesized that IRF4 may act as an endogenous inhibitor of postischemic DC activation and thereby limit sterile inflammation and organ failure. Our data confirm this hypothesis and illustrate that the activation of postischemic inflammation is balanced by IRF4 induction, which prevents overshooting immunopathology by suppressing the expression of proinflammatory mediators like TNF-α in resident APCs.

IRF4 is an immune cell-specific transcription factor that regulates B and T cell development as well as multiple aspects of adaptive immunity (26). Previous studies have mostly focused on the role of IRF4 in driving lymphoid malignancies (25), but IRF4 also controls the maturation of DCs in other settings (31, 32, 38–40), for example, on exposure to TLR or NOD-like receptor agonists. Our data document that oxidative stress adds onto the list of triggers for IRF4 induction, which suggests IRF4 regulates the activation of resident APCs in ischemic tissues. This was evidenced by exposing monocytes to hypoxanthine and xanthine oxidase, which generate ROS and induce stress response genes like HSP70 in a controllable manner in vitro (41). IRF4 was found to be induced not before 24 h after ROS exposure in spleen monocytes.
which was consistent with increased renal IRF4 expression 24 h after renal artery clamping, a phenomenon entirely prevented by antioxidant treatment. As such, oxidative stress does not induce IRF4 as an early response gene like IRF1, which was previously shown to respond within 15 min of oxidative stress (41). The delayed IRF4 response versus the rapid induction of IRF1 is interesting because IRF-1 promotes postischemic renal inflammation and acute renal failure (41), whereas our data documents the opposite for IRF4. Obviously, immediate induction of IRF1 fosters rapid onset of inflammation, namely, cytokine release and neutrophil influx, whereas the somewhat delayed IRF4 induction counterbalances and thereby limits the rapid postischemic immune response. As another aspect oxidative stress induces IRF4 only in myeloid cells, whereas IRF1 is also induced in tubular epithelial cells (41). Obviously, controlling the activation of resident APCs is sufficient to prevent overshooting postischemic renal inflammation. This concept is supported by the role of SIGIRR in IRI, a constitutively expressed molecule that also specifically inhibits the activation of resident DCs in the postischemic kidney (22, 23).

How does IRF4 induction suppress IRI? Renal IRI was recently reported to involve the activation of TLR2 and TLR4 (9–11, 42), potentially because dying tubular epithelial cells release intracellular molecules that can ligate TLR2 and TLR4 on adjacent cells and thereby act as so-called “damage-associated molecular patterns” (7). TLR2 and TLR4 both signal via the intracellular adapter MyD88, as such, MyD88-deficient mice are also protected from IRI (12, 43) and postischemic acute renal failure (10, 11). IRF4 induction inhibits outside-in TLR/MyD88 signaling by binding to MyD88 and blocking the interaction sites for downstream signaling molecules (31). Therefore, Irf4-deficient APCs displayed enhanced NF-κB activation and subsequent expression of proinflammatory mediators in vitro and in vivo (31, 32). Our data are consistent with this concept as lack of IRF4 was associated with increased expression of TNF-α and other NF-κB–dependent proinflammatory mediators in postischemic kidneys. Myeloid cell-derived cytokines were responsible for IRI aggravation in Irf4-deficient mice as depletion of monocytic APCs with clodronate liposomes entirely abrogated this phenotype. Furthermore, TNF-α
was the dominant proinflammatory mediator that accounted for disease aggravation as TNF-α blockade almost completely prevented the phenotype of Irf4-deficient mice. This finding is consistent with the known role of myeloid cell-derived TNF-α in renal IRs (34, 44) and our data document that Irf4 suppresses the induction of much more TNF-α secretion in these cells. Irf4-deficient mice display a number of baseline immune abnormalities, which potentially might affect outcomes of our experiments. Irf4 has nonredundant roles for the maturation of B and T cells (26), isotype switching (27), the ability of T regulatory cells to suppress Th2 responses (28), and the induction of Th17 T cells (29, 30). The immunoregulatory roles were shown to impair adaptive immune responses, for example, after immunization. Postischemic acute renal failure is largely devoid of adaptive immunity affecting cytokines or deficiency of the entire IRF4 gene results in enhanced TLR signaling in APCs and thereby increases intrarenal cytokine and chemokine production. This phenomenon increases postischemic intrarenal inflammation and renal dysfunction. DAMP, damage-associated molecular pattern.

Disclosures
The authors have no financial conflicts of interest.

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In summary, our findings allow three conclusions. First, resident CD45−/CD11c+ APCs are central regulators of sterile inflammation in the postischemic kidney and their activation needs to be counterbalanced to avoid inappropriate immunopathology (Fig. 6). Second, Irf4 is an inducible inhibitor of monocytes and DC activation by oxidative stress in vitro and in vivo. Third, our data identify loss-of-function mutations in the Irf4 gene as a genetic risk factor for IR injuries such as postischemic acute renal failure.

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FIGURE 6. Model for the role of IRF4 in postischemic acute renal failure. Left panel, Ischemia-reperfusion-induced ROS activate tubular epithelial cells to release DAMPs that active TLRs signaling and the subsequent production of proinflammatory cytokines and chemokines in intrinsic renal cells (white) as well as intrarenal APCs (light gray). ROS-induced induction of Irf4 suppresses TLR signaling in APCs and thereby limits postischemic inflammation and renal dysfunction. Right panel, Loss-of-function mutations or deficiency of the entire Irf4 gene results in enhanced TLR signaling in APCs and thereby increases intrarenal cytokine and chemokine production. This phenomenon increases postischemic intrarenal inflammation and renal dysfunction. DAMP, damage-associated molecular pattern.


