IL-6/IL-6R Axis Plays a Critical Role in Acute Kidney Injury

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ABSTRACT
The response to tissue injury involves the coordination of inflammatory and repair processes. IL-6 expression correlates with the onset and severity of acute kidney injury (AKI), but its contribution to pathogenesis remains unclear. This study established a critical role for IL-6 in both the inflammatory response and the resolution of AKI. IL-6–deficient mice were resistant to HgCl2-induced AKI compared with wild-type mice. The accumulation of peritubular neutrophils was lower in IL-6–deficient mice than in wild-type mice, and neutrophil depletion before HgCl2 administration in wild-type mice significantly reduced AKI; these results demonstrate the critical role of IL-6 signaling in the injurious inflammatory process in AKI. Renal IL-6 expression and STAT3 activation in renal tubular epithelial cells significantly increased during the development of injury, suggesting active IL-6 signaling. Although a lack of renal IL-6 receptors (IL-6R) precludes the activation of classical signaling pathways, IL-6 can stimulate target cells together with a soluble form of the IL-6R (sIL-6R) in a process termed trans-signaling. During injury, serum sIL-6R levels increased three-fold, suggesting a possible role for IL-6 trans-signaling in AKI. Stimulation of IL-6 trans-signaling with an IL-6/sIL-6R fusion protein activated STAT3 in renal tubular epithelium and prevented AKI. IL-6/sIL-6R reduced lipid peroxidation after injury, suggesting that its protective effect may be largely mediated through amelioration of oxidative stress. In summary, IL-6 simultaneously promotes an injurious inflammatory response and, through a mechanism of trans-signaling, protects the kidney from further injury.


Acute kidney injury (AKI) is a major source of morbidity and mortality in hospitalized patients, complicating the course of 5% of hospital admissions and 30% of intensive care unit admissions.1 Approximately 40% of patients with renal disease present with AKI mostly as a result of development of acute tubular necrosis (ATN).1 ATN is also the final common pathway of severe renal dysfunction in patients who have diseases of nonrenal origin; therefore, an understanding of the pathophysiologic principles and pathways of ATN is central to the development of potential means of prevention or treatment. The induction of IL-6 expression has been observed during the development of AKI both in humans2 and in experimental animal models3,4; however, the role of IL-6 in AKI has not been clearly established.

IL-6 is a pleiotropic cytokine that has long been described as having both pro- and anti-inflammatory properties.5,6 IL-6 is produced in copious amounts by endothelial cells in response to proinflammatory signals including TNF-α7 and hypoxia,8 and is also a common response to tissue injury and organ failure.9,10 On target cells, IL-6 acts by
binding to a specific cognate receptor (IL-6R), which triggers gp130 and leads to the activation of the Jak/STAT signaling pathway and in particular the activation of STAT3. Unlike the ubiquitously expressed gp130, the cellular distribution of IL-6R is limited to a few cell types, including hepatocytes, and some leukocyte subpopulations, including monocytes, neutrophils, and some T cells and B cells; however, IL-6R also exists in a soluble form (sIL-6R) that upon binding to IL-6 stimulates cells via direct interaction with gp130. Of importance, then, in a process called IL-6 trans-signaling, the IL-6/sIL-6R complex acts as an agonist on cell types that, although expressing gp130, would not inherently respond to IL-6 alone.

This study was designed to elucidate the role of IL-6 in AKI. The results of this study reveal that an IL-6–mediated inflammatory response contributes in part toward the generation of renal injury; however, the results also demonstrate that IL-6 trans-signaling mediates the induction of a protective response to renal injury. Moreover, we demonstrate that activation of gp130 by the administration of an IL-6/sIL-6R fusion protein, called Hyper-IL-6, prevents the onset of AKI and significantly enhances survival.

RESULTS

IL-6 and STAT3 Signaling Increase after HgCl₂-Induced Renal Injury

Administration of mercury-containing compounds such as mercuric chloride (HgCl₂) is a well-established model for the study of nephrotoxin-induced AKI. Administration of HgCl₂ to mice resulted in a rapid and substantial elevation of serum IL-6 protein levels that rose to peak levels by 6 h (Figure 1A), concurrent with the development of AKI. Quantitative analysis of IL-6 mRNA in the kidney showed that IL-6 expression was strongly induced in the kidney (113-fold) and correlated with the manifestation of renal injury, reaching peak levels at 15 h and persisting more than 24 h, when serum IL-6 levels had largely diminished. Increased levels of IL-6 mRNA were also observed in the liver and moderately so in the spleen (Supplemental Figure 1). Together with the rise in IL-6 levels, we also observed a five-fold increase in pSTAT3 (Tyr705) levels (0.23 ± 0.07 versus 0.04 ± 0.06 OD units for HgCl₂ versus control mice; P = 0.05) and upregulation of SOCS3 mRNA in the kidney, consistent with an induction of IL-6 signaling after HgCl₂-induced injury (Figure 1, B and C). Immunohistochemical staining showed that activated STAT3 was present in a variety of cell types and predominantly so in renal tubular epithelial cells (pSTAT3 positive tubular epithelial cells = 144.3 ± 21 versus 1.2 ± 0.9 per high-power field for HgCl₂ and untreated mice, respectively; P < 0.001; Figure 1D).

IL-6 Mediates a Proinflammatory Response that Exacerbates Renal Injury

To determine the physiologic role of IL-6 in the development of renal injury, we compared the sensitivity of IL-6–deficient (IL-6⁻/⁻) and wild-type (IL-6⁺/+ ) mice with HgCl₂-induced AKI. IL-6⁺/+ mice treated with HgCl₂ developed AKI manifested by an elevation of blood urea levels culminating in mortality of up to 70% of treated animals. In contrast, IL-6⁻/⁻ mice showed remarkable resistance to HgCl₂ toxicity (Figure 2A) and better survival (Figure 2B). A dose-response analysis of HgCl₂ toxicity showed that whereas IL-6⁻/⁻ mice were significantly more resistant to HgCl₂ toxicity than IL-6⁺/+ mice at dosages ranging from 5 to 7 mg/kg, higher dosages of HgCl₂ (≥8 mg/kg) led to levels of renal failure that were indistinguishable between the two strains (blood urea nitrogen [BUN] at 24 h = 210 ± 160 (n = 5) versus 281 ± 188 mg/dl (n = 6) in IL-6⁻/⁻ and IL-6⁺/+ mice, respectively; NS). These results indicated that IL-6 participates as a proinflammatory agent contributing toward the development of AKI.

To determine whether other inflammatory pathways mediated HgCl₂-induced AKI, we examined the renal toxicity of HgCl₂ in TNF-α–deficient (TNFα⁻/⁻) mice. Similar to the IL-6⁻/⁻ mice, TNF-α⁻/⁻ mice displayed relative resistance to...
Comparing HgCl₂-induced AKI in SCID/bg versus wild-type BALB/c mice (BUN at 48 h = 41.8 ± 3.5 versus 437.5 ± 216.2 mg/dl, in SCID/bg [n = 6] versus wild-type [n = 8] mice, respectively; P < 0.003; mortality at 120 h 25 versus 66%, respectively).

Neutrophils participate in the development of AKI and are considered to be a source of exacerbation of injury in ischemic- and sepsis-induced AKI. To determine whether neutrophil accumulation was related to IL-6 expression in HgCl₂-induced AKI, we compared neutrophil infiltration to kidneys in wild-type and IL-6⁻/⁻ mice after HgCl₂-induced injury. Twenty-four hours after HgCl₂ administration, robust accumulation of neutrophils within the peritubular capillaries and interstitium was observed in wild-type mice but was significantly less in IL-6⁻/⁻ mice (Figure 3A). This difference was also apparent in the few IL-6⁻/⁻ mice displaying more extensive morphologic injury. Substantial macrophage infiltration to the inner cortex was also observed after HgCl₂-induced injury but did not seem to be IL-6 dependent (Figure 3B). A corresponding infiltration of CD3⁺ T cells after HgCl₂-induced injury was not observed in either wild-type or IL-6⁻/⁻ mice (data not shown). Depletion of neutrophils before HgCl₂ administration dramatically prevented the development of AKI (creatinine at 24 h 1.10 ± 0.21 versus <0.5 mg/dl in control [n = 7] and antineutrophil serum–treated [n = 5] mice, respectively; P = 0.01; Figure 3C). Thus, IL-6 intrinsically links the cellular inflammatory response to the development of AKI.

In the Absence of IL-6R, IL-6 Trans-Signaling but not Classical Signaling Mediates a Protective Response to Kidney Injury

IL-6 is known to have pleiotropic effects, participating in both inflammatory processes and tissue protection. Therefore we questioned whether pretreatment of mice with IL-6 would affect HgCl₂-induced AKI. Mice treated with IL-6 before HgCl₂ administration displayed BUN levels and a mortality rate similar to those of saline-treated control mice (Figure 4, A and B). Prolonged exposure to IL-6 by hydrodynamics-based in vivo transfection of the IL-6 expression plasmid phAAT-IL-6 did not affect HgCl₂-induced AKI (data not shown).

Because responsiveness to IL-6 via the classical IL-6 signaling pathway depends on the expression of IL-6R on the target...
cell, we examined IL-6R expression in the renal parenchyma. Western blot and reverse transcriptase–PCR (RT-PCR) analyses of tissue extracts showed that the IL-6R protein and mRNA are expressed at very low levels in the normal mouse kidney in comparison with the liver, which was used as a positive control (Figure 4C). Six hours after treatment with HgCl₂, a slight but clear increase in IL-6R mRNA was evident; however, no change in the level of IL-6R protein was apparent at this or later times (Figure 4, C and D). Thus, the absence of IL-6R in the kidney precludes the activation of gp130 in the renal parenchyma via the classical IL-6 signaling pathway.

To account for the remarkable increase in STAT3 activation after renal injury (Figure 1, B and D), we postulated that gp130 signaling through a mechanism of sIL-6R–mediated trans-signaling may occur. Analysis of serum sIL-6R levels revealed a three-fold increase in sIL-6R during the 48 h after HgCl₂ administration (Figure 4E), which, together with the concurrent rise in IL-6 expression (Figure 1A), strongly points to the presence of IL-6 trans-signaling after kidney injury. Receptor shedding by activated neutrophils has recently been attributed as being a significant source of sIL-6R production. Neutrophil depletion before HgCl₂ administration significantly diminished the sIL-6R levels (Figure 4F), suggesting that the rise in sIL-6R levels during HgCl₂-induced injury is substantially due to IL-6R shedding by neutrophils.

To determine the effect of IL-6 trans-signaling on the kidney in the absence of injury, we administered to mice an injection of the IL-6/sIL-6R fusion protein Hyper-IL-6 (HIL-6). Analysis of renal STAT3 activation as a marker of IL-6 signaling clearly demonstrated that treatment with HIL-6, as opposed to IL-6, strongly stimulates gp130 signaling in the kidney (Figure 5A). pSTAT3 immunostaining after HIL-6 treatment revealed substantial nuclear staining in epithelial cells of the distal and proximal renal tubules (Figure 5B).

We next assessed the effect of HIL-6 on the development of AKI. Mice treated with HIL-6 protein before HgCl₂ administration were dramatically resistant to AKI (serum creatinine at 48 h 1.55 ± 0.684 mg/dl [n = 13] versus <0.5 mg/dl in saline- and HIL-6–treated mice, respectively; P = 0.001; Figure 5C). Moreover, whereas HgCl₂–treated mice displayed significant mortality, all mice treated with HIL-6 survived (Figure 5D). Morphologic analysis of renal tissue 24 h after HgCl₂ induction revealed extensive necrosis of proximal tubules. Quantification of renal neutrophilic infiltration in IL-6−/− and IL-6+/+ mice 24 h after HgCl₂ administration is shown. Data are means ± SEM. *P = 0.04 (n = 7) versus other groups. (B) Macrophage infiltration after AKI is not IL-6 dependent. Staining and quantification of renal macrophages in the inner cortex in IL-6−/− and IL-6+/+ mice 24 h after HgCl₂ administration. Data are mean ± SEM. *P < 0.05 (n = 7) versus other groups. (C) Effect of neutrophil depletion on HgCl₂-induced AKI. BUN levels in antineutrophil serum (□), control serum (■), and untreated (baseline; □) are shown. Data are means ± SEM. *P = 0.003 and 0.01 versus control serum + HgCl₂–treated and untreated mice, respectively. HPF, high-power field. Magnification, ×200.

Figure 3. Neutrophilic infiltration to the renal parenchyma after injury is IL-6 dependent and promotes renal injury. (A) Few peri-tubular neutrophils are present at baseline in IL-6+/+ mice. Accumulation of neutrophils in peritubular capillaries at the inner cortex and outer medulla accompanied by neutrophilic extravasation into the renal interstitium is evident 24 h after HgCl₂ administration. Peritubular accumulation of neutrophils in IL-6−/− mice after HgCl₂ administration was significantly diminished compared with IL-6+/+ mice but not significantly different than in naïve IL-6+/+ mice.
HIL-6 Induces Antioxidant Factors in the Kidney

The mechanism of HgCl₂-induced ATN involves oxidative stress, leading to apoptosis and necrosis of the proximal tubular epithelial cells. To elucidate the mechanism of HIL-6 –induced protection, we analyzed the effect of HIL-6 in the presence of the specific competitive inhibitor of HO-1 activity, tin mesoporphyrin (SnMP). Administration of SnMP, however, did not prevent the HIL-6–generated resistance to HgCl₂-induced injury (BUN at 24 h 103 ± 43, 47 ± 10, and 44 ± 10 mg/dl [n = 8] in salinetreated, HIL-6–treated, and HIL-6+SnMP-treated mice, respectively; P < 0.003 for HIL-6 versus saline P = 0.54 for HIL-6 versus HIL-6+SnMP). As a positive control for the effect of SnMP, we tested whether SnMP would block HIL-6 protection of glycerol-induced abrupt rhabdomyolysis, in which HO-1 is essential to renal protection.29 Mice transfected with the HIL-6 expression plasmid pHAT-HIL-6 were dramatically resistant to glycerol-induced AKI, and SnMP significantly blocked the protective effect of HIL-6 (Supplemental Figure 2). SmMP also strongly exacerbated renal injury in control transfected mice, although SmMP per se was not nephrotoxic (data not shown). Thus, HIL-6–induced resistance to glycerol– but not HgCl₂–induced AKI is HO-1 dependent.

These results suggested that HIL-6–induced protection against AKI is largely mediated by mechanisms that reduce oxidative stress. Measurement of the lipid peroxidation end product malondialdehyde (MDA) by thiobarbituric acid reac-
tive substances analysis did not show a significant increase in MDA levels in kidney extracts after HgCl$_2$-induced injury (data not shown), in agreement with previous studies performed on mice.$^{30,31}$ In contrast, HgCl$_2$ significantly elevated serum MDA levels (Figure 7), which strongly correlated with levels of AKI (correlation coefficient $r = 0.9521$, $P < 0.0001$). HIL-6 treatment maintained serum MDA at nearly baseline levels after HgCl$_2$-induced injury, indicating that prevention of oxidative stress may be an important mechanism through which HIL-6 ameliorates renal injury.

**DISCUSSION**

Recent studies have shown a strong correlation between IL-6 expression and AKI. The novel findings of this study indicate that IL-6 mediates two functions during the induction of AKI: A cytokine-dependent cell-mediated immune response that exacerbates renal injury and a protective response in tubular epithelial cells that ameliorates injury and maintains renal function.

The results of this study demonstrate that IL-6 is critical to the inflammatory response to renal injury. The salient observations supporting this conclusion are, first, that IL-6 deficiency diminishes neutrophil accumulation after injury and renders mice relatively resistant to injury, and, second, that neutrophil depletion in wild-type mice significantly reduced HgCl$_2$-induced injury. In this respect, ischemia-induced AKI is similar to both HgCl$_2$- and endotoxemia-induced AKI regarding neutrophil infiltration but dissimilar regarding the contribution of neutrophils to injury.$^{16,17,32,33}$ Together, these observations support the notion that local IL-6 expression is an intrinsic element in the cell-mediated inflammatory response that contributes significantly to HgCl$_2$-induced AKI (Figure 8A).

Whereas in other examples of organ failure IL-6 induces resistance to injury,$^{20,34–36}$ this study and previous reports$^{37}$ showed that IL-6 treatment does not prevent AKI. As shown here, the lack of IL-6R expression in the kidney precludes an IL-6-mediated protective response through the classical signaling pathway. We postulated that STAT3 activation observed after injury in the absence of IL-6R expression occurs through a mechanism of IL-6 trans-signaling. Support for this notion is found in the threefold increase in serum sIL-6R levels after renal injury, which may result through receptor shedding by infiltrating neutrophils, as shown previously$^{6,24}$ and as indicated by neutrophil depletion studies performed here. Stimulation of trans-signaling using HIL-6 dramatically reduced renal injury and maintained

![Figure 5](https://www.jasn.org)
renal function, demonstrating that IL-6 \textit{trans}-signaling functions to induce protection in response to renal injury. Multiple factors may participate simultaneously to induce gp130 signaling and activate STAT3 after renal injury, including IL-11 and leukemia inhibitory factor, which are also induced after renal injury.\(^4\) This study provides the first demonstration that gp130 signaling and STAT3 activation in the kidney function to ameliorate AKI. That similar findings were observed in two independent and dissimilar models of AKI suggest that IL-6 \textit{trans}-signaling produces a broad protective response to various types of injury.

To understand the mechanism(s) by which gp130 activation prevents renal injury, we examined two molecular pathways associated with \textit{HgCl}_2-induced renal injury: Apoptosis and oxidative stress. The notion that amelioration of renal injury may be controlled through antiapoptotic factors is appealing because it is known that IL-6 and STAT3 upregulate antioxidative stress–related factors; however, their importance in the protective effect has only been partially elucidated. Our results show that \textit{HIL-6}–mediated protection to glycerol-induced AKI but not \textit{HgCl}_2–induced AKI largely depends on HO-1 activity. This is consistent with the importance of HO-1 in AKI, as previously reported,\(^1,3,29\) and indicates that alternative factors must be responsible for the \textit{HIL-6}–mediated protection against \textit{HgCl}_2–induced injury. Whether Ref-1 is essential for the \textit{HIL-6}–induced protective response has yet to be determined. \textit{HIL-6} treatment also dramatically reduced the levels of lipid peroxidation, indicating that induction of antioxidative stress–related factors may be of particular importance in the protective mechanism. Taken together, these results suggest that gp130-mediated protection against AKI is largely mediated by mechanisms that ameliorate oxidative stress.

This study shows that IL-6 and gp130 signaling is an important physiologic response to renal injury. We propose a general model (Figure 8B) in which renal injury induces local and systemic elevation of IL-6 that promotes neutrophil infiltration and exacerbates renal injury. The neutrophils can release their membrane-bound \textit{IL-6R},\(^24\) which, via IL-6 \textit{trans}-signaling, activates STAT3 in the renal epithelial cells. \textit{Trans}-signaling functions to render protection against oxidative stress and further injury in the surrounding tissue and promotes resolution of injury. This may represent a universal dual role of IL-6 classical and \textit{trans}-signaling in other types of tissue injury as well. Because in many cases AKI is a consequence of an unrelated therapeutic intervention or the result of a clinically related episode, it can, in practice, be anticipated and is therefore potentially preventable. This study directly supports the conclusion that an \textit{HIL-6}–based therapeutic strategy protects the kidney from injury and can facilitate maintenance of renal function during stress.

### Conclusive Methods

**Reagents**

All chemicals were from Sigma-Aldrich Chemicals (St. Louis, MO), unless otherwise stated. Recombinant human IL-6 was from PeproTech (Rocky Hill, NJ). \textit{HIL-6} produced by a stably transfected CHO cell line was purified as described previously.\(^25\) A fresh aliquot was used for each experiment. SnMP was purchased from Frontier Scientific (Logan, UT).

**Animals**

Male BALB/c and C57BL/6 mice (19 to 21 g) were purchased from Harlan Laboratories (Jerusalem, Israel). IL-6\(^{-/-}\) mice and TNF\(^{-/-}\) mice, both on a C57BL/6 background, were bred in-house from breeding pairs originally purchased from the Jackson Laboratory (Bar Harbor, ME).\(^45,46\) Weight-matched C57BL/6 control mice were used in all experiments involving IL-6\(^{-/-}\) or TNF\(\alpha\)-deficient mice. BALB/c nu/nu mice and SCID/bg were purchased from Harlan UK (Blackthorn, Bicester, Oxon, England). Procedures and maintenance (see Supplemental Concise Methods) were performed in accordance with the Institutional Animal

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**Table 1.** Histological analysis of control and HIL-6–treated mice

<table>
<thead>
<tr>
<th>Parameter</th>
<th>\textit{HgCl}_2 (n = 5)</th>
<th>HIL-6 + \textit{HgCl}_2 (n = 5)</th>
<th>P</th>
</tr>
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<tbody>
<tr>
<td>No injury (%)</td>
<td>23.6 ± 6.4</td>
<td>55.3 ± 20.7</td>
<td>&lt;0.010</td>
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<tr>
<td>Reversible injury (%)</td>
<td>42.5 ± 7.0</td>
<td>33.8 ± 19.1</td>
<td>NS</td>
</tr>
<tr>
<td>Necrosis (%)</td>
<td>34.0 ± 10.3</td>
<td>11.3 ± 2.4</td>
<td>&lt;0.001</td>
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\(^*\) Renal histology is expressed as percentage area examined presenting the corresponding finding. Data are means ± SD.

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**Figure 6.** Effect of \textit{HIL-6} and IL-6 on the expression of oxidative stress response genes in normal and \textit{HgCl}_2–treated mice. (A) Real-time PCR analysis of HO-1 mRNA. \(*P = 0.0006\) versus control, \(\textit{***P} = 0.002\); versus control, \(\textit{***P} = 0.0001\) versus control and \(P = 0.03\) versus \textit{HgCl}_2, (n = 4 to 7). (B) Real-time PCR analysis of Ref-1 mRNA. \(*P = 0.0002\) versus other groups (n = 4 to 7).
Care and Use Committee–approved animal treatment protocol (license no. OPRR-A01-5011).

Mercury-induced AKI was induced by intraperitoneal injection of a freshly prepared solution of HgCl₂ (6 mg/kg) dissolved in PBS.41 Glycerol-induced AKI was induced in anesthetized (Isoflurane) BALB/c mice by injection of 50% glycerol, at a total dosage of 8 ml/kg body wt, one-half dose injected into the anterior thigh muscle of each hind leg. HO-1 was inhibited by administration of SnMP,13 as described in the Supplemental Concise Methods.

Neutrophil Depletion

Circulating neutrophils were depleted by intraperitoneal injection of 0.2 ml of rabbit antineutrophil serum (Accurate Chemical and Scientific Corp, Westbury, NY) 24 h before HgCl₂ administration.47 This procedure removed 83±17% of polymorphonuclear cells from the peripheral circulation. Previous studies reported that treatment with this antibody does not have a significant effect on other leukocyte subpopulations in the peripheral circulation.47,48

In Vivo DNA Transfection

Animals were treated by hydrodynamics-based in vivo plasmid DNA transfection49,50 with phAAT-IL-6 (10 μg), phAAT-HIL-6 (2.5 μg), or a control plasmid, pGEM-7 (20 μg). Details of plasmid DNA construction and preparation are in the Supplemental Concise Methods.

Determination of BUN and Creatinine

Blood samples were obtained by tail-vein bleeding. BUN levels and creatinine were determined in heparinized serum using the Reflotron system and Urea or Creatinine test strips (Roche Diagnostics, Basel, Switzerland).

Histologic and Immunohistochemical Analysis

Kidneys were removed 24 to 48 h after HgCl₂ administration and fixed in 4% buffered formaldehyde, followed by 80% ethanol, and embedded in paraffin blocks. Tissue sections were stained with hematoxylin and eosin. Neutrophils were stained with rat anti-mouse neutrophil antibody (Serotec, Oxford, England) diluted 1:3000 followed by biotinylated rabbit anti-rat (Dako, Glostrup, Denmark), and developed with horseradish peroxidase (HRP)-streptavidin (Invitrogen, Carlsbad, CA) using 3-aminoo-9-ethyl-carbazol (AEC) (Dako). Phospho-STAT3 was stained using monoclonal rabbit anti-mouse pSTAT3 (Tyr 705; Cell Signaling, Danvers, MA) diluted 1:500, followed by biotinylated goat anti-rabbit (Jackson Laboratory) diluted 1:5000, amplified using Tyramide Signal Amplification kit (PerkinElmer, Boston, MA) and developed with AEC. CD3⁺ cells were stained using rat anti-mouse F4/80 antigen (Serotec) diluted 1:200, followed by anti-rat HRP Histofine (Nichirei, Tokyo, Japan) and developed with AEC. CD3⁺ cells were stained using rat anti-human CD3 antigen (Serotec) diluted 1:200, followed by biotinylated rabbit anti-rat diluted 1:50 and developed with HRP-streptavidin using AEC. The number of positively stained cells per high-power field (Magnification, ×400) by immunohistochemistry was counted in 20 fields for each sample from
coded specimens. Morphologic analysis of tubular injury was performed as described in the Supplemental Concise Methods.

**Western Blot Analysis**

Protein extracts were prepared from tissue samples (approximately 100 mg) by homogenization in 1 ml of whole-cell lysis buffer (1% NP-40, 10 mM Tris [pH 7.8], 150 mM NaCl, 40 mM EDTA, 10 mM Na-pyrophosphate, 10 mM NaF, 1 mM PMSF, 4 mM orthovanadate, 1 μg/ml pepstatin A, and 2 μg/ml leupeptin). Protein extracts for analysis of IL-6R were prepared by homogenization in m-PER buffer (Pierce, Rockford, IL). Protein extracts (50 μg) were separated by PAGE and subjected to Western blot analysis. For analysis of murine IL-6R, Western blots were probed with goat anti-mouse IL-6R antibody (R&D Systems, Minneapolis, MN) followed by rabbit anti-goat antibody (Zymed) and anti-rabbit HRP polymer (Dako) and developed using the ECL-Plus Western blotting Detection System (GE Healthcare, Uppsala, Sweden). For analysis of pSTAT3 and STAT3, Western blots were probed with mouse monoclonal anti-phosphorylated STAT3 (sc-8059) and mouse monoclonal anti-STAT3 (sc-8019; Santa Cruz Biotechnology, Santa Cruz, CA), respectively. As a loading control, the blots were stripped with 0.1 M glycine (pH 2.8) and re-probed with a monoclonal anti-β-actin antibody, clone AC-74 (Sigma), and developed with HRP Envision (Dako).

**IL-6 and sIL-6R ELISA**

IL-6 and sIL-6R levels were determined using a mouse IL-6 and a mouse sIL-6R DuoSet ELISA kit (R&D Systems) according to the manufacturer’s instructions on serum samples that were collected and frozen at −20°C until analysis.

**RNA Extraction, RT-PCR, and Real-Time PCR Analysis**

RNA samples were prepared from approximately 100 mg of snap-frozen tissue by homogenization in Trizol Reagent (Invitrogen, Carlsbad, CA) according to the manufacturer’s instructions using a Polytron high-speed homogenizer, followed by treatment with DNA-free DNase (Ambion, Austin, TX). The primer sequences and details for RT-PCR and real-time PCR analysis are provided in the Supplemental Concise Methods.

**Lipid Peroxidation Analysis**

MDA, an end product of lipid peroxidation, was analyzed in serum and tissue samples by measurement of thiobarbituric acid reactive substances as described previously. A detailed description is provided in the Supplemental Concise Methods.

**Statistical Analyses**

Mortality data were compared using the Kaplan-Meier survival procedure and the log rank (Mantel-Cox) test to compare equality of survival distributions, with P ≤ 0.05 considered statistically significant. Comparison of mean serum creatinine levels was performed using a nonparametric Mann-Whitney test, in which all values less than the level of detection (<=0.5 mg/dl) were arbitrarily set at 0.5 mg/dl. All other comparisons were subjected to the t test, with P ≤ 0.05 considered statistically significant.

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**DISCLOSURES**

None.

**REFERENCES**