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Both PD-1 Ligands Protect the Kidney from Ischemia Reperfusion Injury

Katarzyna Jaworska, Joanna Ratajczak, Liping Huang, Kristen Whalen, Mana Yang, Brian K. Stevens, and Gilbert R. Kinsey

Acute kidney injury (AKI) is a common problem in hospitalized patients that enhances morbidity and mortality and promotes the development of chronic and end-stage renal disease. Ischemia reperfusion injury (IRI) is one of the major causes of AKI and is characterized by uncontrolled renal inflammation and tubular epithelial cell death. Our recent studies demonstrated that regulatory T cells (Tregs) protect the kidney from ischemia reperfusion–induced inflammation and injury. Blockade of programmed death-1 (PD-1) on the surface of Tregs, prior to adoptive transfer, negates their ability to protect against ischemic kidney injury. The present study was designed to investigate the role of the known PD-1 ligands, PD-L1 and PD-L2, in kidney IRI. Administration of PD-L1 or PD-L2 blocking Abs prior to mild or moderate kidney IRI significantly exacerbated the loss of renal function, renal inflammation, and acute tubular necrosis compared with mice receiving isotype control Abs. Interestingly, blockade of both PD-1 ligands resulted in worse injury, dysfunction, and inflammation than did blocking either ligand alone. Genetic deficiency of either PD-1 ligand also exacerbated kidney dysfunction and acute tubular necrosis after subthreshold ischemia. Bone marrow chimeric studies revealed that PD-L1 expressed on non-bone marrow–derived cells is critical for this resistance to IRI. Finally, blockade of either PD-1 ligand negated the protective ability of adoptively transferred Tregs in IRI. These findings suggest that PD-L1 and PD-L2 are nonredundant aspects of the natural protective response to ischemic injury and may be novel therapeutic targets for AKI. The Journal of Immunology, 2015, 194: 000–000.

A

 acute kidney injury (AKI) occurs in ∼5% of hospitalized patients with detrimental consequences in terms of morbidity and mortality (1, 2). Additionally, AKI increases the likelihood of developing chronic kidney disease and end-stage renal disease (3, 4). Kidney ischemia reperfusion injury (IRI) is a common cause of AKI (5, 6). Animal models have revealed that inflammation begins as early as 30 min of reperfusion, and inhibition of the immune response to IRI by various strategies dramatically improves renal function and histological integrity after ischemia (7–13). The innate inflammatory response, consisting of neutrophils and macrophages, is an important component of kidney IRI (8, 12, 14–17). Our recent studies have demonstrated that regulatory T cells (Tregs) 1) make up a critical component of the natural intrinsic protective response to kidney IRI (18), and 2) can be used therapeutically (by adoptive transfer) to protect against kidney IRI in naive mice (18–20). Other groups have demonstrated that Tregs protect against nephrotoxic AKI (21) and promote recovery from established AKI (22, 23) in mouse models. Tregs use many different mechanisms to reduce inflammation, including TGF-β, IL-10, extracellular adenosine, CTLA-4, and programmed death-1 (PD-1) (19, 24–28).

PD-1 is a negative costimulatory molecule expressed by T lymphocytes, monocytes, dendritic cells, and B cells (29, 30). PD-1 has two ligands, PD-L1 and PD-L2. PD-L1 is expressed by numerous immune and nonimmune cells, whereas PD-L2 expression is limited primarily to APCs (29, 30). PD-1 stimulation leads to inhibition of TCR signaling in CD4+ and CD8+ T cells (29, 30). Nonetheless, PD-1 is indispensable for Treg function, as recent studies have shown that Tregs lacking PD-1, or Tregs in the presence of PD-1 blocking Abs, display impaired suppressive activity in vitro and in vivo (19, 25, 28, 31, 32).

Given that PD-1 expression on Tregs is vital for their ability to suppress kidney IRI (19) and the increasing use of PD-1 and PD-L1 ligand blocking Abs in clinical practice (33–35), we sought to determine the role of PD-L1 and PD-L2 in the natural course of kidney IRI and in Treg-mediated protection from IRI.

Materials and Methods

Mice

Six- to 10-wk-old male C57BL/6 mice were obtained from Charles River Laboratories (Wilmington, MA) or The Jackson Laboratory (Bar Harbor, ME). B7-H1 knockout (KO; PD-L1 KO) mice on the C57BL/6 background have been described previously (36) and were a gift from Lieping Chen (Yale University, New Haven, CT) via Victor Engelhard (University of Virginia). B7-DC KO (PD-L2 KO) mice (37) and CD45.1 (B6.SJL-Pepc−/−Pepe+/Boij) mice on the C57BL/6 background were obtained from The Jackson Laboratory. Mice were housed in a specific pathogen-free facility at the University of Virginia. Routine testing confirmed the absence of murine norovirus, epizootic diarrhea in infant mice, Theiler’s murine encephalomyelitis virus, minute virus of mice, mouse parvovirus, hepatitis virus, fur mites, and pinworm.

Ab treatment and adoptive transfer

Mice were injected i.p. with 300 μg isotype control or blocking Abs purchased from Bio X Cell (West Lebanon, NH): rat IgG2a (clone 2A3), control for anti–PD-L2; rat IgG2b (clone LTF-2), control for anti–PD-L1;
rat anti–PD-L1 (clone 10F9G2), and rat anti–PD-L2 (clone TY25). The Abs were administered to the mice 24 h prior to IRI. Tregs were isolated from spleens of naive wild-type (WT) mice with a Dynal CD4 negative selection kit (Life Technologies, Carlsbad, CA) and a CD25 positive selection kit (Miltenyi Biotec, Auburn, CA) according to the manufacturers’ protocols as previously described (18–20). One hundred thousand freshly isolated Tregs (in 200 μl normal saline) were administered to the mice 18 h prior to IRI (6 h after injection of isotype or blocking Abs).

Renal IRI model

Bilateral renal ischemia was induced as described previously (18–20). Briefly, mice were anesthetized and bilateral flank incisions were made. For sham animals renal pedicles were isolated but not clamped, and in experimental animals both renal pedicles were clamped for the indicated number of minutes, after which the clamps were removed and kidneys observed to ensure reperfusion. Some kidney IRI experiments were performed using a Harvard Apparatus mouse temperature controller (Harvard Bioscience, Holliston, MA) (Figs. 6, 7) in the Old Medical School Building at the University of Virginia whereas others were performed using a mouse temperature controller from Fine Science Tools (Foster City, CA) (no longer available from the manufacturer) in Jordan Hall at the University of Virginia (Figs. 1–5, 8) due to a laboratory move. Our experience was that when using the Harvard Apparatus temperature controller, a shorter ischemic time was required for equivalent renal injury than with the Fine Science Tools controller, and thus 21 min was used with this setup to induce mild subthreshold kidney IRI in Figs. 6 and 7. Multiple factors are critical in the response to kidney IRI, and changing surgery room and mouse temperature controller has been shown to affect the response to kidney IRI in mice by others (38). All animal experiments were performed in compliance with the U.S. Department of Health and Human Services Guide for the Care and Use of Laboratory Animals and approved by the University of Virginia Institutional Animal Care and Use Committee.

Bone marrow chimera generation

WT (CD45.1) mice and PD-L1 KO (CD45.2) mice were irradiated with 650 rd, two times 4 h apart, and then reconstituted with ~5 million bone marrow cells isolated from either WT (CD45.1) or PD-L1 KO (CD45.2) mice on the same day, as described previously (12). Seven to 8 wk after irradiation and bone marrow cell transfer, the mice were subjected to mild IRI. Flow cytometry to determine the percentage CD45.1+ cells out of circulating CD11b+ cells confirmed the efficiency of the bone marrow transfer, as 90.3 ± 2.3% (n = 9) of circulating leukocytes were of bone marrow donor origin at the time of kidney IRI surgery.

Assessment of renal injury

Plasma creatinine was measured 24 h after IRI using a DetectX Ncar NIST-calibrated serum creatinine detection kit (Arbor Assays, Ann Arbor, MI) according to the manufacturer’s protocol. Tubular necrosis in outer medulla region of the kidney was assessed by H&E staining as reported (18–20). Briefly, kidney sections were observed under ×200 magnification and acute tubular necrosis score was assigned in a blinded manner based on the percentage of necrotic tubules in outer medulla (pink casts visible inside the tubules). The applied scoring system was as follows: 0, no damage; 1, <10%; 2, 10–25%; 3, 25–50%; 4, 50–75%; and 5, >75%.

Flow cytometry

AccuCheck counting heads from Invitrogen (Carlsbad, CA) were used to determine total CD45+ cell number per gram of collagenase-digested kidney as reported previously (18–20). Leukocyte number was used to transform the percentages of leukocytes of specific phenotype to the leukocyte count and used to compare different groups. Fc receptors were blocked with 2.4G2, and 7-aminactinomycin D (7-AAD) viability staining solution (BioLegend, San Diego, CA) was applied to allow gating on live cells. The following anti–PD-L1 and anti–PD-L2 Abs induced a significantly greater rise in plasma creatinine when compared with the treatment with PD-L1 or PD-L2 blocking Abs. Twenty-four hours after the treatment, mice underwent sham or mild (24-hm ischemia) bilateral renal ischemia surgery. Subsequently, the kidneys were allowed to reperfuse for 24 h. Whereas mild ischemia did not result in a significant increase in plasma creatinine in the isotype control Ab-treated mice, administration of PD-L1 or PD-L2 blocking Abs caused a significant increase in plasma creatinine, denoting significant renal dysfunction (Fig. 1). Importantly, simultaneous administration of both anti–PD-L1 and anti–PD-L2 Abs induced a significantly greater rise in plasma creatinine when compared with the treatment with PD-L1 or PD-L2 blocking Abs alone (Fig. 1). Acute tubular necrosis (ATN) in the outer medulla region of the kidney mirrored plasma creatinine levels, confirming exacerbated renal injury in the mice in which PD-1 ligand signaling was inhibited (Fig. 2, Table I). As an additional measure of the effect of PD-1 ligand blockade on tubular epithelial cell injury, mRNA for kidney injury molecule (KIM)-1 was compared between groups. KIM-1 is not expressed in healthy proximal tubular epithelial cells, but becomes upregulated shortly after injury (41). Sham KIM-1 mRNA abundance, normalized to GAPDH, was set as 1. Kidney KIM-1 expression was elevated in isotype control–treated mice after IRI (645 ± 332.7) and even further elevated in

Statistical analysis

Data were analyzed using one- or two-way ANOVA with post hoc analysis as appropriate using SigmaPlot 11.0 (SigmAPlot, San Jose, CA) or GraphPad InStat 3 (GraphPad Software, La Jolla, CA). A p value <0.05 was considered statistically significant.

PD-1 ligands in kidney IRI

Immediately after harvesting, kidney samples were frozen in liquid nitrogen and stored at −80°C until further analysis. The samples were placed in ultra-pure buffer consisting of Pierce RIPA buffer (Thermo Scientific, Rockford, IL), phenylmethanesulfonyl fluoride (Sigma-Aldrich), and protease inhibitor cocktail (Sigma-Aldrich) and homogenized using a TissueLyser LT (Qiagen). Subsequently, the samples were spun down and the supernatant was sonicated with an ultrasonic processor GEX130 (Coe-Parmer, Vernon Hills, IL) for 10 s set on 70% power. The samples were spun down once again and an aliquot of the supernatant was used to determine protein content using a Pierce BCA protein assay kit (Thermo Scientific). Absorances were measured with an Epoch microplate spectrophotometer (BioTek, Winooski, VT), and cytokine concentrations were normalized to total protein concentration in the kidney extract (pg/mg).

Immunofluorescence microscopy

Kidney samples were fixed as described previously (18). Subsequently, 7-μm frozen kidney sections were incubated 30 min in a PBS solution containing 0.3% Triton X-100 (Sigma-Aldrich, St. Louis, MO), 2.4G2 (BD Pharmingen), 1% BSA, and 1% horse serum. Afterward, the samples were labeled with a FITC-conjugated Ab (clone 7/4; Cedarlane Laboratories, Burlington, ON, Canada) to visualize neutrophils and recently emigrated monocytes/macrophages (39, 40). ProLong Gold antifade reagent with DAPI (Molecular Probes, Eugene, OR) was used to stain nuclei and to preserve the samples. The slides were observed and images captured under ×200 magnification using an Olympus BX51 fluorescence microscope (Olympus, Center Valley, PA) in the Advanced Microscopy Core Facility at the University of Virginia.
mice pretreated with the PD-L1 blocking Ab (6326 ± 1489, p < 0.05 versus isotype plus IRI). Administration of anti–PD-L1 blocking Ab also resulted in increased KIM-1 expression compared with administration of isotype control Abs (3662 ± 763.5), although this increase did not reach statistical significance.

**PD-L1 and PD-L2 blockade enhances the innate immune response in the kidney after IRI**

Flow cytometry was used to assess the accumulation of different immune cells in the kidney at 24 h of reperfusion. Mild ischemia (24 min) in the isotype control–treated mice led to an increase in the total CD45⁺ population in the kidney and an increase in the CD11b⁺GR-1int cells (Fig. 3, Supplemental Fig. 1) that have previously been identified by ImageStream analysis as kidney-infiltrating neutrophils (11). When either PD-1 ligand was blocked, the percentage of total CD45⁺ cells in the kidney was further increased and the percentage of CD45⁺ cells that were CD11b⁺GR-1int was markedly enhanced (Supplemental Fig. 1). The absolute number of CD11b⁺GR-1high cells in the kidney was significantly higher in all groups that were treated with PD-1 ligand blocking Abs (Fig. 3A). Similar to plasma creatinine levels and histological damage in the kidney, blockade of both PD-1 ligands resulted in additive neutrophil accumulation (Fig. 3A). We also compared the accumulation of CD11b⁻F4/80int leukocytes, which have properties of inflammatory monocytes/macrophages in the postischemic kidney (12). In contrast to the effect on neutrophils, blockade of PD-L1 had no effect on the accumulation of CD11b⁻F4/80int leukocytes (Fig. 3B). However, blockade of PD-L2 did cause a significant increase in the inflammatory monocyte/macrophage number in the postischemic kidney (Fig. 3B). Flow cytometry revealed that there is no increase in CD4⁺ or CD8⁺ T cell infiltration after IRI in mice with PD-1 ligand blockade (Fig. 3C, 3D). To confirm flow cytometry results, frozen kidney sections were stained with DAPI and the 7/4 Ab that recognizes neutrophils and monocytes/macrophages (39, 40). Immunofluorescence microscopy revealed increased innate leukocyte influx in the kidneys of mice treated with PD-L1 and PD-L2 blocking Abs (Fig. 4).

**PD-L1 and PD-L2 blockade leads to increased level of IL-6, CXCL1, and ICAM-1 in the postischemic kidney**

Taking into consideration studies linking PD-1 or PD-L1 blockade/deficiency to increased IL-6 production (42–44), we measured IL-6 mRNA level in the kidney of anti–PD-L1- and/or anti–PD-L2-treated mice. Sham mRNA expression of IL-6 normalized to GAPDH levels was set as 1. The values for the other groups are reported relative to sham (Table II). Blockade of both PD-1 ligands simultaneously resulted in a >1000-fold increase in IL-6 mRNA expression (Table II). To confirm our results, we performed ELISA on kidney homogenates and detected a significant increase in IL-6 protein abundance in kidneys from mice treated with the combination of PD-1 ligand blocking Abs (Supplemental Fig. 2). The qPCR analysis also revealed significant increases in CXCL1 and ICAM-1 mRNA expression in mice pretreated with both PD-1 ligand blocking Abs (Table II). Renal IFN-γ protein levels were not significantly different after IRI between groups (ng/mg total protein measured by ELISA: IRI plus isotype, 5.8 ± 1.2; IRI plus anti–PD-L1, 6.4 ± 0.8; IRI plus anti–PD-L2, 5.9 ± 0.7; IRI plus both blocking Abs, 5.2 ± 0.9; n = 7–9/group pooled from three independent experiments).

**Blockade of either PD-1 ligand exacerbates moderate renal IRI**

To investigate the role of PD-1 ligands in moderate kidney IRI, mice were treated with blocking Abs to PD-L1 or PD-L2 or isotype controls prior to 26-min bilateral IRI and analyzed after 24 h of

![Image](https://example.com/image1.png)

**FIGURE 1.** Blocking PD-1 ligands exacerbates renal dysfunction after mild ischemia reperfusion. Naive WT mice were treated with isotype control Abs, anti–PD-L1, anti–PD-L2, or anti–PD-L1 and anti–PD-L2 together. After 24 h, sham or mild bilateral kidney IR surgery (24 min ischemia) was performed. Plasma creatinine levels were assessed after 24 h of reperfusion; n = 7–11/group, pooled from three independent experiments. Data are presented as means ± SEM. **p < 0.01.

![Image](https://example.com/image2.png)

**FIGURE 2.** Blocking PD-1 ligands increases ATN after kidney IRI. Naive WT mice were treated with isotype control Abs (B), anti–PD-L1 (C), anti–PD-L2 (D), or anti–PD-L1 and anti–PD-L2 together (E). Twenty-four hours later sham (A) or mild bilateral kidney IR surgery (24 min ischemia) was performed. After 24 h of reperfusion, kidney sections were stained with H&E to assess outer medulla tubular necrosis. Images are representative of n = 5 for the sham group and n = 9–11 for IRI groups.
reperfusion for the degree of renal injury and dysfunction. Twenty-six minutes of ischemia resulted in a significant increase in plasma creatinine and ATN at 24 h of reperfusion compared with the sham-operated control mice (Fig. 5). Blockade of either PD-1 ligand significantly exacerbated the IRI-induced renal dysfunction and histological damage as measured by plasma creatinine levels and ATN, respectively (Fig. 5).

Genetic deficiency of PD-L1 or PD-L2 increases ischemic kidney injury in mice

To investigate the effect of global deficiency of PD-L1 or PD-L2, KO mice were compared with WT controls (C57BL/6) after mild kidney IR surgery (21 min ischemia) (Fig. 6). Compared to WT controls, both PD-L1 KO and PD-L2 KO mice had significant renal dysfunction and ATN when exposed to mild kidney IR (Fig. 6).

Role of PD-L1 ligand expression on bone marrow–derived versus non-bone marrow–derived cells in protection from kidney IRI

Because PD-L1 is expressed on many immune cells and nonimmune cells such as kidney tubular epithelial cells and vascular endothelial cells, we investigated the contribution of PD-L1 in the immune and nonimmune compartments by generating bone marrow chimeric mice as described in Materials and Methods. Seven to 8 wk after irradiation and bone marrow cell transfer, the mice were subjected to mild kidney IR surgery (21 min ischemia) that was not sufficient to cause renal dysfunction or inflammation in the WT→WT control chimeras or in chimeric WT mice reconstituted with PD-L1 KO bone marrow (Fig. 7). However, mild ischemia did cause marked renal dysfunction and infiltration of CD11b^GR-1^{high} innate leukocytes in PD-L1 KO mice recon-
To study the role of PD-1 ligands in protection from kidney IRI, we investigated the role of the known PD-1 ligands in 1) the natural protective response to IRI in naive mice and 2) in the ability of adoptively transferred Tregs to protect the kidney during IRI. Using monoclonal blocking Abs to PD-L1 and/or PD-L2, we found that PD-L1 or PD-L2 blockade, prior to IRI, results in increased kidney damage and inflammation. Furthermore, simultaneous blockade of both PD-1 ligands caused a further increase in inflammation and renal damage when compared with blockade of only one ligand. KO mouse models confirmed the protective role of both PD-L1 and PD-L2 in kidney IRI. Bone marrow chimeric studies revealed that PD-L1 expressed on non-bone marrow–derived cells is critical for this resistance to IRI. Finally, by treating recipient mice with either PD-L1 or PD-L2 blocking Abs, prior to adoptive transfer, the protective action of Treg transfer was completely abolished. Thus, to confirm that the freshly isolated Tregs used in these experiments were functional, we adoptively transferred 100,000 WT Tregs to naive mice in the absence of PD-1 ligand blocking Abs (mice were pretreated with an isotype control Ab), and as described previously (18–20) the WT Tregs offered significant protection from kidney dysfunction induced by 26 min of ischemia (24 h after IRI plasma creatinine [mg/dl]: sham, 0.37 ± 0.06; IRI plus saline, 1.5 ± 0.27, p < 0.01 versus sham; IRI plus Tregs, 0.30 ± 0.02, p < 0.01 versus IRI plus saline). Twenty-six minutes of ischemia were used in this control experiment so that there would be measurable and significant renal dysfunction in the IRI plus saline group for the Tregs to protect from because 24 min of ischemia in the absence of PD-1 ligand blocking Abs does not result in a significant increase in plasma creatinine at 24 h of reperfusion (Fig. 1). In these control experiments, adoptive transfer of Tregs inhibited CD11b+GR-1high cell (neutrophil) accumulation in the kidneys of mice that underwent 26 min of ischemia, compared with the normal saline-treated group, similar to our previous reports (18–20). CD457−AAD−CD11b+GR-1high leukocyte counts per gram of kidney were as follows: sham, 105,691 ± 64,477; IRI plus saline, 487,080 ± 148,260, p < 0.05 versus sham; IRI plus Tregs, 108,486 ± 73,585, p < 0.05 versus IRI plus saline. However, Treg administration to the mice pretreated with Abs against PD-L1 or PD-L2 caused an increase in the neutrophil accumulation in ischemic kidneys compared with mice that did not receive Treg adoptive transfer (CD457−AAD−CD11b+GR-1high leukocyte count per gram of kidney: IRI plus anti–PD-L1 plus saline, 484,151 ± 41,982 versus 772,828 ± 141,740 in the IRI plus anti–PD-L1 plus Tregs group, p < 0.01; and IRI plus anti–PD-L2 plus saline, 382,915 ± 79,245 versus 651,583 ± 97,049 in the IRI plus anti–PD-L2 plus Tregs group, p < 0.01).

**Discussion**

Based on our previous studies that revealed PD-1 expression on Tregs is required for their ability to protect the kidney from IRI, we investigated the role of the known PD-1 ligands in 1) the natural protective response to IRI in naive mice and 2) in the ability of adoptively transferred Tregs to protect the kidney during IRI. Using monoclonal blocking Abs to PD-L1 and/or PD-L2, we found that PD-L1 or PD-L2 blockade, prior to IRI, results in increased kidney damage and inflammation. Furthermore, simultaneous blockade of both PD-1 ligands caused a further increase in inflammation and renal damage when compared with blockade of only one ligand. KO mouse models confirmed the protective role of both PD-L1 and PD-L2 in kidney IRI. Bone marrow chimeric studies revealed that PD-L1 expressed on non-bone marrow–derived cells is critical for this resistance to IRI. Finally, by treating recipient mice with either PD-L1 or PD-L2 blocking Abs, prior to adoptive transfer, the protective action of Treg transfer was completely abolished. Thus, we adoptively transferred 100,000 WT Tregs to naive mice in the absence of PD-1 ligand blocking Abs (mice were pretreated with an isotype control Ab), and as described previously (18–20) the WT Tregs offered significant protection from kidney dysfunction induced by 26 min of ischemia (24 h after IRI plasma creatinine [mg/dl]: sham, 0.37 ± 0.06; IRI plus saline, 1.5 ± 0.27, p < 0.01 versus sham; IRI plus Tregs, 0.30 ± 0.02, p < 0.01 versus IRI plus saline). Twenty-six minutes of ischemia were used in this control experiment so that there would be measurable and significant renal dysfunction in the IRI plus saline group for the Tregs to protect from because 24 min of ischemia in the absence of PD-1 ligand blocking Abs does not result in a significant increase in plasma creatinine at 24 h of reperfusion (Fig. 1). In these control experiments, adoptive transfer of Tregs inhibited CD11b+GR-1high cell (neutrophil) accumulation in the kidneys of mice that underwent 26 min of ischemia, compared with the normal saline-treated group, similar to our previous reports (18–20). CD457−AAD−CD11b+GR-1high leukocyte counts per gram of kidney were as follows: sham, 105,691 ± 64,477; IRI plus saline, 487,080 ± 148,260, p < 0.05 versus sham; IRI plus Tregs, 108,486 ± 73,585, p < 0.05 versus IRI plus saline. However, Treg administration to the mice pretreated with Abs against PD-L1 or PD-L2 caused an increase in the neutrophil accumulation in ischemic kidneys compared with mice that did not receive Treg adoptive transfer (CD457−AAD−CD11b+GR-1high leukocyte count per gram of kidney: IRI plus anti–PD-L1 plus saline, 484,151 ± 41,982 versus 772,828 ± 141,740 in the IRI plus anti–PD-L1 plus Tregs group, p < 0.01; and IRI plus anti–PD-L2 plus saline, 382,915 ± 79,245 versus 651,583 ± 97,049 in the IRI plus anti–PD-L2 plus Tregs group, p < 0.01).

**Table II. Changes in inflammatory mRNA abundance in the kidney 24 h after mild kidney IRI**

<table>
<thead>
<tr>
<th>Group</th>
<th>IL-6</th>
<th>CXCL1</th>
<th>ICAM-1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>IRI + isotype Ab</td>
<td>42 ± 18</td>
<td>21 ± 9</td>
<td>2 ± 1</td>
</tr>
<tr>
<td>IRI + anti–PD-L1</td>
<td>342 ± 85</td>
<td>71 ± 28</td>
<td>2.7 ± 0.5</td>
</tr>
<tr>
<td>IRI + anti–PD-L2</td>
<td>530 ± 220</td>
<td>89 ± 28</td>
<td>3 ± 0.4</td>
</tr>
<tr>
<td>IRI + both Abs</td>
<td>1853 ± 565*</td>
<td>147 ± 36*</td>
<td>4 ± 1*</td>
</tr>
</tbody>
</table>

*For each mRNA, expression levels in sham-operated mice were set to 1, and expression in each treatment group is expressed as relative arbitrary units.

*p < 0.05 versus IRI + isotype Ab group within each column.
both PD-1 ligands contribute in a nonredundant fashion to the body’s natural tissue-sparing and anti-inflammatory response to IRI and, additionally, both ligands contribute to the therapeutic mechanisms of adoptively transferred Tregs.

IRI (as examined in the present study) occurs during 24 h, which is a time frame most consistent with an innate immune response. Whereas most studies on PD-1 and the PD-1 ligands have focused on their role in Ag-specific adaptive immune responses (29, 45), some recent studies have demonstrated a role for this pathway in innate immunity (42, 46–50). In an acute liver IRI model, PD-L1, PD-L1, or PD-L2 blockade increased liver damage, and administration of a PD-L1 Ig fusion protein reduced liver inflammation and injury (48). In a murine model of tuberculosis, PD-1, PD-L1, or PD-L2 blockade augmented IFN-γ production by NK cells (46), and PD-1 KO mice are more resistant to Listeria monocytogenes infection than are WT controls in the absence of T and B cells (50). To further support the role of PD-1 in suppressing innate immunity, macrophages stimulated with a PD-L1 fusion protein produce less IL-12 after LPS administration than did the control group (47). In the present study, we found that blocking PD-1 ligands prior to injury only modulated the influx of innate leukocytes with properties of neutrophils and inflamed monocytes/macrophages with no effect on CD4+ or CD8+ T cells.

The finding that there is additive injury when both PD-1 ligands are blocked suggests that the two ligands play different and independent roles in protecting the kidney from IRI. Other laboratories have also found that the two PD-1 ligands operate independently in other models. Brown et al. (51) showed that blockade of PD-L1 and PD-L2 on human dendritic cells has an additive effect and causes enhanced CD4+ T cell proliferation. Other studies utilizing PD-L1 and PD-L2 small interfering RNA in dendritic cells showed that the lack of both ligands resulted in enhanced ability of dendritic cells to induce proliferation and cytokine production in Ag-specific CD4+ T cells (52). This may partially explain our results, as dendritic cells are important inducers of kidney IRI pathology on the one hand (53), but on the other hand, if stimulated properly, they can induce tolerance against kidney IRI (54) and offer protection against cisplatin-induced AKI in mice (55).

The finding that PD-1 ligands have independent roles in protection could be due to their differential expression patterns. For example, the highest expression of KIM-1 (a marker of direct tubular epithelial cell injury) was observed in the mice where PD-L1 was blocked, and PD-L1 is expressed on mouse and human kidney tubular epithelial cells (56–58). Although one group has shown that activating PD-L1 on isolated tubular epithelial cells promotes cell death under certain in vitro conditions (59), PD-L1 on other cell types promotes cell survival (60). Because PD-L1 is also expressed on a variety of immune cells, we generated bone marrow chimeric mice to investigate which types of cells must express PD-L1 for resistance to kidney IRI to occur. The results clearly show that PD-L1 expression on bone marrow–derived cells is not required for resistance to IRI, but that lack of PD-L1 on nonbone marrow–derived cells (possibly tubular epithelial cells or vascular endothelial cells or others) is required. Expression of PD-L2 in mice is limited exclusively to immune cells (61, 62), and PD-L2 blockade resulted in a more robust immune response than did PD-L1 blockade in our study. Based on the expression pattern of PD-L2 in mice and our current bone marrow chimera results, our findings suggest that PD-L1 on nonimmune cells and PD-L2 on immune cells mediate intrinsic resistance to kidney IRI.
Higher renal IL-6 mRNA and protein levels, after combined blocking Ab administration, may result from enhanced accumulation of IL-6–producing immune cells infiltrating the kidney. IL-6–producing monocytes/macrophages have been implicated by others as pathogenic in kidney IRI (63). Classically activated or M1 macrophages are the predominant macrophage phenotype in the kidney during the first several days of reperfusion (16) and are capable of producing IL-6 (64). Based on these studies and our results, the PD-1 ligands may restrain M1 macrophages that respond and contribute to the initial ischemic kidney injury. Whether the PD-1 ligands influence the conversion of M1 macrophages inside the kidney to M2 reparative macrophages several days after IRI (16, 65) has not yet been investigated.

The directionality of PD-1/PD-1 ligand signaling in kidney IRI is not known and could involve signaling into cells that express PD-1 could send anti-inflammatory signals into immune cells that express the PD-1 ligands as reported previously (66) or pro-survival signals through PD-1/PD-L1 interaction into PD-L1–expressing cells such as tubular epithelial cells (60). Finally, PD-L1 may interact with CD80 (67) expressed on immune cells in a manner that is anti-inflammatory and renal-protective during kidney IRI.

Our previous study showed that incubation of isolated Tregs with a blocking Ab to PD-1 (then washing), prior to adoptive transfer to WT recipients, prevented the ability of Tregs to protect the kidney. This suggested that Tregs interact with recipient cells via PD-1 and PD-1 ligand interactions that promote protection from kidney inflammation and injury. Administration of either PD-1 ligand blocking Ab prior to Treg adoptive transfer rendered the Tregs unable to protect the kidney. Surprisingly, we found that Treg adoptive transfer in the presence of PD-1 ligand blocking Abs significantly enhanced neutrophil accumulation in the postischemic kidney. The reasons for this are not currently known. Because we and others (18, 68) have not observed significant trafficking of Tregs into the kidney at 1–24 h of reperfusion, in the absence of pharmacological intervention, we hypothesize that Tregs either in the circulation or at some site outside the kidney provide renal protection. How and whether the PD-1 ligands modulate the trafficking of Tregs to the kidney and other locations have not been studied, but may offer some insight on how the PD-1 ligands promote Treg function. Because blocking Abs were injected before Treg administration, we cannot exclude the possibility that PD-L1 blocking Abs bound to PD-L1 on the adoptively transferred Tregs. In fact, PD-L1 was shown to be required for Treg-mediated suppression of IFN-γ production by CD4+ T cells (69).
and Treg-mediated protection from ischemic stroke (70). Although we have not been able to observe PD-L2 expression of Tregs by flow cytometry (data not shown), recent studies suggest that PD-L2 on Tregs may promote the ability of Tregs to suppress B cell activity (71). Thus PD-1 ligands expressed on Tregs (in addition to PD-1 on Tregs) may also be important for their function in kidney IRI. Additional studies are required to determine which cells interact with Tregs to mediate their protective function in kidney IRI. However, these findings demonstrate that to protect the kidney from acute ischemic kidney injury, Tregs require the availability of both PD-L1 and PD-L2.

In contrast to our present results and the studies from other laboratories referenced above (suggesting that PD-1 ligand signaling is anti-inflammatory), there are multiple reports of both PD-1 ligands promoting inflammation in other models (37, 72–75). Interestingly, the ability of PD-L1 and PD-L2 to promote inflammation does not require PD-1 expression on target cells, suggesting the existence of a currently unknown proinflammatory receptor for PD-L1 and PD-L2 (37, 75). Taken together with our previous results pointing to a key role for PD-1 on Tregs (additionally light on the PD-1 signaling pathway in innate immune cells), our results suggest that the anti-inflammatory and tissue-protective interaction between PD-L1/PD-L2 and PD-1 is more important than the interaction of PD-L1/PD-L2 with an immune-activating receptor in ischemic kidney injury.

In summary, our findings establish a protective role for both PD-1 ligands in a mouse model of ischemic AKI and shed additional light on the PD-1 signaling pathway in innate immune responses. It appears that PD-L1 on nonimmune cells and PD-L2 on immune cells are key elements of the natural resistance to kidney IRI. Additionally, our results indicate that PD-L1 and PD-L2 participate in Treg-mediated protection against kidney IRI. Therefore, induction of intrinsic PD-L1 and/or PD-L2 expression and signaling may constitute a novel strategy to protect the kidney from ischemic injury.

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Disclosures

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