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

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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 adoptive transfer, negates their ability to protect against ischemic kidney injury. These findings suggest that PD-L1 and PD-L2 are nonredundant aspects of the natural protective response to ischemia injury and may be novel therapeutic targets for AKI. The Journal of Immunology, 2015, 194: 000–000.

Abbreviations used in this article: 7-AAD, 7-aminoactinomycin D; AKI, acute kidney injury; ATN, acute tubular necrosis; IL-10, interleukin-10; PD-1, programmed death-1; PD-L, programmed death ligand; Treg, regulatory T cell; WT, wild-type.

The online version of this article contains supplemental material.
rat anti–PD-L1 (clone 10F.9G2), 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 manufacturer’s 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 rad, 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 irradiation and bone marrow cell transfer as previously described (19). No-template controls confirmed the lack of contamination and nonspecific amplification (data not shown).

Assessment of renal injury

Plasma creatinine was measured 24 h after IRI using a DetectX NcAL NIST-calibrated serum creatinine detection kit (Arbor Assays, Ann Arbor, MI) according to the manufacturer’s protocol. Tubular necrosis in outer medullary region of the kidney was assessed by H&E staining as reported before (18–20). Briefly, kidney sections were observed under 200 magnification and an aliquot of the supernatant was used to determine protein concentration in the kidney extract (pg/mg).

Flow cytometry

AccuCheck counting heads from Invitrogen (Carlsbad, CA) were used to determine total CD45+ cell number per gram of collagenase-digested kidney as previously reported (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-aminoactinomycin D (7-AAD) viability staining solution (BioLegend, San Diego, CA) was applied to gating on live cells. The following antibodies were used: GR-1–FITC (clone RB6-8C5), CD11b–PE (M1/70), CD45–PE-Cy7 (30–F11), and F4/80–Alexa Fluor 647 (BMA) from BioLegend; and CD45–PE (30–F11), CD4–PE-Cy7 (RM4–5), CD3–FITC (145–2C11), CD8–PE (53–67), and NK1.1–PerCP-Cy5.5 (PK136) from BD Pharmingen (San Jose, CA). Data acquisition was performed on a FACScalibur flow cytometer (BD Biosciences, San Jose, CA) with a Cytek 8 color upgrade (Cyt ek Development, Freemont, CA) using FlowJo collection’s edition software (Tree Star, Ashland, OR). Flow cytometry analysis was performed using FlowJo version X for Windows (Tree Star).

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 10% horse serum. Afterward, the samples were labeled with a FITC-conjugated Ab (clone 7/4; Cederlane 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.

Real-Time RT-PCR

After harvesting, kidney sections were placed in RNA Later (Ambion, Austin, TX). RNA and cDNA were prepared as previously reported (19). GAPDH, KIM-1, IL-6, CXCL1, and ICAM-1 QuantiTect primers were purchased from Quagen (Valencia, CA). Real-time RT-PCR was performed with a MyiQ single-color real-time PCR detection system (Bio-Rad, Hercules, CA) as previously described (19). No-template controls confirmed the lack of contamination and nonspecific amplification (data not shown).

ELISA on total kidney homogenate

Immediately after harvesting, kidney samples were frozen in liquid nitrogen and stored at −80°C until further analysis. The samples were placed in ysis buffer consisting of Pierce RIPA buffer (Thermo Scientific, Rockford, IL), phenylmethylsulfonyl 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 (Cole-Parmer, Vernon Hills, IL) for 10 s at 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) according to the manufacturer’s protocol. An Ab pair buffer kit and mouse IL-6, TNF-α, and IFN-γ Ab pairs were purchased from Life Technologies. Absorbances 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).

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.

Results

Blockade of PD-1 ligands exacerbates renal dysfunction and tissue injury after mild IRI

To investigate the role of PD-1 ligands in kidney IRI naive C57BL/6 (WT) mice, mice were treated with isotype control (rat IgG2a and/or rat IgG2b), anti–PD-L1, anti–PD-L2, or a combination of both blocking Abs. Twenty-four hours after the treatment, mice underwent sham or mild (24-min 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...
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-1high 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-1high 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+F4/80high 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+F4/80high macrophages in the postischemic kidney (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).

**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 reperfusion.

![FIGURE 1](http://www.jimmunol.org/)

**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.

![FIGURE 2](http://www.jimmunol.org/)

**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-
presence of PD-L1 or PD-L2 blocking Abs were not protected. Mice that were subjected to 24 min of ischemia in the ureter measurements and evaluation of outer medulla ATN scores were performed. After Treg or saline injection, the mice underwent sham or mild injury and inflamed mouse kidney. Images taken of the outer medulla region of each kidney are representative of at least five mice per treatment group. Original magnification ×200.

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.

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 prevent kidney damage induced by 24 min of ischemia. Using monoclonal blocking Abs to PD-L1 and/or PD-L2, we found that PD-L1 or PD-L2 blockade, prior to IRI, resulted 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,
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 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 CD45+ 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 non–bone 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 innate 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 restrain M1 macrophages that respond and contribute to the initial ischemic kidney injury, 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 to inhibit their inflammatory response to IRI. Alternatively, stimulating PD-1 on Tregs may enhance their anti-inflammatory properties in a currently unknown way. In support of this, we and several other laboratories have found that PD-1 KO Tregs or Tregs in the presence of Abs that interfere with PD-1 signaling do not suppress inflammation (19, 25, 28, 31, 32). Another possibility is reverse signaling, whereby Tregs (and other 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 expressed on Tregs in protection from kidney IRI (19), our current findings 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

The authors have no financial conflicts of interest.

References


Figure S1. Renal leukocyte accumulation is exacerbated by PD-1 ligand blockade. Naïve WT mice were treated with isotype control antibodies, anti-PD-L1 or anti-PD-L2. After 24 hours, sham or bilateral kidney IR surgery was performed. At 24 hours of reperfusion kidneys were harvested and digested with collagenase and cells were labeled for flow cytometry as described in the Materials and Methods section. The left side dot plots show the total leukocyte percentage (CD45+) in the kidneys from each group and the right side plots show the percentage of CD11b+ and GR-1high innate leukocytes (A-D). Dead cells (7-AAD+) were excluded from analysis (not depicted). N=7-11 per group, pooled from 3 independent experiments. Data are presented as mean + SEM, * denotes P<0.05; ** denotes P<0.01 (E).
Figure S2. Combined PD-1 ligand blockade increases renal IL-6 protein levels after ischemia. Naïve WT mice were treated with isotype control antibodies, anti-PD-L1 or anti-PD-L2 or the combination of both blocking antibodies. After 24 hours, sham or bilateral kidney IR surgery was performed. At 24 hours of reperfusion kidneys were homogenized and subjected to IL-6 ELISA as described in the Materials and Methods section. Data are presented as mean + SEM, ** denotes P<0.01. N=5 for sham, 9-11 for each IRI group, pooled from 3 independent experiments.
Supplemental Figure 3

Figure S3. PD-1 ligand blocking antibodies negate the protective effect of regulatory T cell adoptive transfer in kidney IRI. Naïve WT mice were treated with isotype control antibodies, anti-PD-L1 or anti-PD-L2. Six hours later mice were administered either 100,000 freshly-isolated WT Tregs in normal saline. After 18 hours mild kidney IR surgery (24 min ischemia) was performed, and the kidneys were allowed to reperfuse for 24 hours. To assess renal tissue injury ATN was assessed using H&E stained kidney sections.