Renal Ischemia-Reperfusion Injury and Adenosine 2A Receptor-Mediated Tissue Protection: The Role of CD4+ T Cells and IFN-γ

Yuan-Ji Day; et al

https://doi.org/10.4049/jimmunol.176.5.3108

Related Content

Adenosine A2A Receptor Agonist–Mediated Increase in Donor-Derived Regulatory T Cells Suppresses Development of Graft-versus-Host Disease

J Immunol (January, 2013)

Activation of adenosine 2a receptor (A2aR) blocks IL-23/IL-17 mediated neutrophil migration in kidney ischemia-reperfusion injury (37.9)

J Immunol (April, 2010)

A2A Adenosine Receptor Induction Inhibits IFN-γ Production in Murine CD4+ T Cells

J Immunol (January, 2005)
Renal Ischemia-Reperfusion Injury and Adenosine 2A Receptor-Mediated Tissue Protection: The Role of CD4+ T Cells and IFN-γ

Yuan-Ji Day, Liping Huang, Hong Ye, Li Li, Joel Linden, and Mark D. Okusa

A A2A adenosine receptor (A2A R)-expressing bone marrow (BM)-derived cells contribute to the renal protective effect of A2A agonists in renal ischemia-reperfusion injury (IRI). We performed IRI in mice lacking T and B cells to determine whether A2A R expressed in CD4+ cells mediate protection from IRI. Rag-1 knockout (KO) mice were protected in comparison to wild-type (WT) mice when subjected to IRI. ATL146e, a selective A2A agonist, did not confer additional protection. IFN-γ is an important early signal in IRI and is thought to contribute to reperfusion injury. Because IFN-γ is produced by kidney cells and T cells, we performed IRI in BM chimeras in which the BM of WT mice was reconstituted with BM from IFN-γ KO mice (IFN-γ KO→WT chimera). We observed marked reduction in IRI in comparison to WT→WT chimeras providing additional indirect support for the role of T cells. To confirm the role of CD4+ A2A R in mediating protection from IRI, Rag-1 KO mice were subjected to ischemia-reperfusion. The studies we performed in Rag-1 KO mice were reversed in Rag-1 KO mice that were adoptively transferred WT CD4+ cells (WT CD4+→Rag-1 KO) or A2A KO CD4+ cells (A2A KO CD4+→Rag-1 KO). ATL146e reduced injury in WT CD4+→Rag-1 KO mice but not in A2A KO CD4+→Rag-1 KO mice. Rag-1 KO mice reconstituted with CD4+ cells derived from IFN-γ KO mice (IFN-γ CD4+→Rag-1 KO) were protected from IRI; ATL146e conferred no additional protection. These studies demonstrate that CD4+ IFN-γ contributes to IRI and that A2A agonists mediate protection from IRI through action on CD4+ cells.

A2A R reconstituted injury, an effect that was attenuated with ATL146e. The protective effect of ATL146e, however, was independent of macrophage A2A R. Kidneys from macrophage-depleted mice reconstituted with macrophages lacking A2A R (small interfering RNA deletion of macrophages A2A R) were still protected by ATL146e. These results suggested that A2A R reduce renal IRI through action on A2A R-expressing BM-derived cells but not on A2A R-expressing macrophages.

A number of different studies either directly or indirectly support the role of T cells in IRI (8–13). Additionally, there is evidence that demonstrates an early role of T cells in mouse liver IRI (13) as well as renal IRI (14). IRI in CD4/CD8 double KO mice was associated with a reduction of neutrophil infiltration and injury (15). Furthermore, reconstitution with CD4+ cells but not CD8+ cells in T cell-deficient mice reconstituted injury, suggesting an early role for CD4+ cells and not CD8+ cells in renal IRI (14). Another study showed that blockade of the CD28-B7 costimulatory pathway reduces injury (16). Taken together, these studies provide strong evidence of a role for T cells in the early phase of IRI.

The purpose of the current studies was to demonstrate directly the role of A2A R expressed on CD4+ cells in A2A agonist-mediated protection from renal IRI. Furthermore, we sought to determine the potential role of IFN-γ in IRI. Our results indicate: 1) CD4+ cells are necessary to mediate the full extent of IRI, confirming previous studies; 2) IFN-γ released from CD4+ cells is a key cytokine necessary for induction of injury; and 3) A2A R expressed on CD4+ cells are the primary target of A2A agonist-mediated tissue protection in IRI.

Materials and Methods

Renal IRI and treatment with ATL146e

All animals were handled and procedures were performed in adherence to the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals. C57BL/6 mice (7–8 wk of age; Charles River Breeding.
Labs), congenic A<sub>2A</sub> KO mice (C57BL/6 background, 6–7 wk of age) previously described (7, 17) (a gift from J.-F. Chen, Boston University, Boston, MA), congenic Rag-1 KO mice (on C57BL/6 background), and IFN-γ KO mice (mouse strain 10 wk; The Jackson Laboratory) were used. Rag-1 KO mice were originally derived from B6.129S7-Rag1tm1Mom/J breeders (The Jackson Laboratory) and have been backcrossed 20 generations onto a C57BL/6 background (provided by M. McDuffie, University of Virginia, Charlottesville, VA). Mice were allowed free access to food and water until the day of surgery. Mice were anesthetized with ketamine (100 mg/kg, i.p.), xylazine (10 mg/kg, i.p.), and acepromazine (1 mg/kg, i.m.) and subjected to bilateral flank incisions as previously described (7, 18). Both renal pedicles were clamped for 32 min. Surgical wounds were closed with metal staples and mice were returned to cages for 24 h. Following 24 h of reperfusion, animals were reanesthetized, blood was obtained by cardiac puncture, and kidneys were removed for various analyses.

Mice were anesthetized with vaporized halothane (Halothan Vapor 19.1) before s.c. implantation of osmotic minipumps (model 1003D; ALZA). The pumps released either vehicle (0.01% DMSO in PBS) or ATL146e (10 ng/kg/min). A dose of ATL146e was chosen on the basis of previous dose-ranging studies in WT C57BL/6 mice that produced a ~70–80% reduction of plasma creatinine levels relative to vehicle treatment 24 h after blocking nonspecific Fc binding with anti-mouse CD16/CD32 (10 μg). Following 24 h reperfusion surgery, kidneys were excised and were snap frozen in OCT (Sieve; E-C Apparatus), and washed in PBS. RBC were removed by hypotonic lysis with NH<sub>4</sub>Cl (1.5 mM) for 5 min at room temperature and three washes with 10% FCS-containing medium. To facilitate CD4<sup>+</sup> T cell separation, we used commercially available magnetic negative selection reagents (Miltenyi Biotec). We routinely achieved a release separation. We used commercially available magnetic negative selection reagents (Miltenyi Biotec). We routinely achieved a release separation. We used commercially available magnetic negative selection reagents (Miltenyi Biotec). We routinely achieved a release separation. We used commercially available magnetic negative selection reagents (Miltenyi Biotec). We routinely achieved a release separation. We used commercially available magnetic negative selection reagents (Miltenyi Biotec). We routinely achieved a release separation.

**Plasma creatinine**

Plasma creatinine concentrations were determined using a colorimetric assay according to the manufacturer’s protocol (Sigma-Aldrich).

**Purification of T cells, T cell transfer, and flow cytometry**

Splenocytes were isolated by the following method (19). Freshly isolated spleen cells were placed in RPMI 1640/10% FCS. Cells were released by blunt dissection of spleen followed by incubation with collagenase D at 37°C (for 30 min), strained through a 40-μm nylon mesh (Collectors Tissue Siev; E-C Apparatus), and washed in PBS. TECs were removed by hypotonic lysis with NH<sub>4</sub>Cl (1.5 mM) for 5 min at room temperature and three washes with 10% FCS-containing medium. To facilitate CD4<sup>+</sup> T cell separation, we used commercially available magnetic negative selection cell sorting kits (AutoMax; Miltenyi Biotec). We routinely achieved a relative enrichment of CD4<sup>+</sup> cells of ~85% using the negative selection process. We have found this system to be rapid and reproducible and to yield highly purified CD4<sup>+</sup> T cells. CD4<sup>+</sup> cells from WT C57BL/6, A<sub>2A</sub> KO, or IFN-γ KO mice were isolated, and ~1–2 × 10<sup>6</sup> cells were injected into recipient mice via the internal jugular vein 7 days before ischemia-reperfusion surgery.

To confirm the efficiency and specificity of CD4<sup>+</sup> T cells reconstitution in Rag-1 KO mice, blood, spleen, and kidney suspension from cell-transferred Rag-1 KO mice were stained with PE anti-CD4<sup>+</sup> (5 μg/ml; RM-5; eBioscience), Alex647 anti-CD3 (5 μg/ml; 550C; Caltag Laboratories) after blocking nonspecific Fc binding with anti-mouse CD16/CD32 (10 μg/ml; eBioscience). Subsequent flow cytometry data acquisition was performed on FACSCalibur (BD Biosciences). Data were analyzed by FlowJo software (Tree Star).

**Generation of chimeric mice**

C57BL/6 and IFN-γ KO mice were used to generate BM chimeras as previously described (7). Donor mice (12 wk of age; 25–28 g) were anesthetized. Neutrophils (50 mg/ml) were harvested by cervical dislocation.

The marrow from the tibia and femur was harvested under sterile conditions. Bones were flushed with RPMI 1640 (Invitrogen Life Technologies) plus 10% FCS. The marrow was passed sequentially through a 22-gauge m nylon mesh (Collector Tissue Sieve; E-C Apparatus), and washed in PBS. RBC were removed by hypotonic lysis with NH<sub>4</sub>Cl (1.5 mM) for 5 min at room temperature and three washes with 10% FCS-containing medium. To facilitate CD4<sup>+</sup> T cell separation, we used commercially available magnetic negative selection cell sorting kits (AutoMax; Miltenyi Biotec). We routinely achieved a relative enrichment of CD4<sup>+</sup> cells of ~85% using the negative selection process. We have found this system to be rapid and reproducible and to yield highly purified CD4<sup>+</sup> T cells. CD4<sup>+</sup> cells from WT C57BL/6, A<sub>2A</sub> KO, or IFN-γ KO mice were isolated, and ~1–2 × 10<sup>6</sup> cells were injected into recipient mice via the internal jugular vein 7 days before ischemia-reperfusion surgery.

To confirm the efficiency and specificity of CD4<sup>+</sup> T cells reconstitution in Rag-1 KO mice, blood, spleen, and kidney suspension from cell-transferred Rag-1 KO mice were stained with PE anti-CD4<sup>+</sup> (5 μg/ml; RM-5; eBioscience), Alex647 anti-CD3 (5 μg/ml; 550C; Caltag Laboratories) after blocking nonspecific Fc binding with anti-mouse CD16/CD32 (10 μg/ml; eBioscience). Subsequent flow cytometry data acquisition was performed on FACSCalibur (BD Biosciences). Data were analyzed by FlowJo software (Tree Star).

**Histochecmistry and immunohistochemistry**

Kidneys and spleens were fixed in periodate-lysine-paraformaldehyde (4% paraformaldehyde) and embedded in paraffin, and 4-μm sections were cut. Sections were subjected to routine staining with HE and viewed by light microscopy (Zeiss AxiosSkop). Photographs were taken and brightness/contrast adjustment was made with a SPOT RT camera (software version 3.3; Diagnostic Instruments). We quantitated the degree of necrosis in a masked fashion by scoring the degree of renal injury based on the following scoring system: 0 = normal, 1 = loss of brush border and or tubule debris, 2 = loss of nuclei, 3 = partial tubule obstruction, and 4 = tubule obstruction and dilatation (6). Eight to 10 fields from outer medulla were evaluated scored and averaged.

For immunohistochemical studies, kidney sections were subjected to Ag retrieval according to the manufacturer’s protocol (Vector Laboratories) then sections were incubated with a well-characterized rat anti-mouse mAb to murine neutrophils (clone 7/4, catalog no. RM6500; Caltag Laboratories) (1/100 dilution) followed by a biotinylated goat anti-rat secondary Ab. Peroxidase reaction was performed according to the manufacturer’s protocol (Vectastain ABC Elite kit; Vector Laboratories). Tissue sections were covered with an aqueous-based mounting solution consisting of p-phenylendiamine (1 mg/ml) and 70% glycerol, and coverslips were applied and affixed with nail polish. Sections were viewed under a Zeiss AxioSkop fluorescent microscope. Photographs were taken with a SPOT RT camera (software version 3.3; Diagnostic Instruments). Due to the confuence of neutrophils, discrete neutrophils could not be counted. Thus a semi-quantitative scoring method (0 = no neutrophils to 4 = confluent neutrophils surrounding tubule) was used based on the degree of neutrophil infiltration observed in a ×400 magnification field. Eight to 10 fields of a kidney section were scored in a masked fashion from kidneys of three to four mice selected randomly.

**Statistical analysis**

Unpaired Student’s t test or one-way ANOVA followed by Tukey’s post hoc analysis was used for all comparisons. A value of p < 0.05 was used to define statistical significance.

**Results**

A<sub>2A</sub> agonists do not confer additional protection in the absence of T and B cells

Kidneys subjected to IRI in chimeric mice whose BM was replaced with BM from A<sub>2A</sub> KO mice were resistant to protection usually seen with A<sub>2A</sub> agonist administration (7). Furthermore in mice whose macrophages were deficient of A<sub>2A</sub>R and subjected to IRI, A<sub>2A</sub> agonists were still able to reduce tissue injury (4). These results implicate nonmacrophage BM cells as the target of tissue protection. Given the growing evidence for the role of T cells in IRI we sought to determine whether T cells were necessary for A<sub>2A</sub> agonist-mediated tissue protection. The kidneys of WT mice or Rag-1 KO mice were subjected to 32 min of ischemia followed by 24 h of reperfusion (Fig. 1). Ischemia-reperfusion produced markedly elevated plasma creatinine of 2.17 ± 0.15 mg/dl (n = 4) in WT mice (see Ref. 18 and dose of ATL146e in Materials and Methods), but a much smaller increase of 0.52 ± 0.08 mg/dl (n = 5) in Rag-1 KO mice treated with vehicle (p < 0.001 compared with WT mice) or 0.57 ± 0.09 mg/dl (n = 5) in Rag-1 KO mice treated with ATL146e (p < 0.001 compared with WT mice) (Fig. 1c). There was no significant difference in plasma creatinine between Rag-1 KO mice treated with vehicle or ATL146e. Histologically, there was evidence of tissue necrosis in the outer medulla in WT mice (Fig. 1b), an effect that was reduced in Rag-1 KO mice (Fig. 1c). No additional histological evidence of tissue protective effect was observed in the outer medulla of Rag-1 KO treated with ATL146e (Fig. 1d). Quantitative analysis of histological changes yielded injury scores of 3.25 ± 0.14 (n = 10), 1.79 ± 0.26 (n = 7), and 1.42 ± 0.21 (n = 6) for WT, Rag-1 KO treated with vehicle, and Rag-1 KO treated with ATL146e, respectively (p < 0.001, for vehicle or ATL146e treated Rag-1 KO compared with WT mice). There was no statistically significant difference in the histological scores between Rag-1 KO mice treated with vehicle and those treated with ATL146e. These results indicate that kidneys from T and B cell-deficient mice were protected from IRI and that ATL146e did not provide additional tissue protection.
suggesting that the protective effect of ATL146e could be mediated through T or B cells.

**BM-derived IFN-γ mediates tissue injury in renal ischemia-reperfusion**

T cells are the primary source of BM-derived IFN-γ. Thus we reasoned that the absence of BM-derived IFN-γ would render kidneys resistant to IRI as well as to the normally protective effect of A2A agonists. Because IFN-γ is secreted from BM-derived cells and kidney resident cells, we performed IRI in which the BM of WT mice was ablated and reconstituted with BM from IFN-γ KO mice (IFN-γ KO→WT) (Fig. 2). In control WT→WT chimeric mice, the level of plasma creatinine rose following IRI to 1.98 ± 0.18 mg/dl (n = 4) but was reduced significantly to 0.68 ± 0.10 mg/dl (n = 5) in vehicle (p < 0.001 compared with WT→WT chimera) or to 0.56 ± 0.15 mg/dl (n = 5) in ATL146e-treated IFN-γ KO→WT chimeric mice (p < 0.001 compared with WT→WT chimera) (Fig. 2a). There was no significant difference between vehicle and ATL146e-treated IFN-γ KO→WT chimera. Histological analysis of the outer medulla (Fig. 2, b–d) revealed an injury score of 3.60 ± 0.25 (n = 5), 2.00 ± 0.00 (n = 4), and 1.38 ± 0.234 (n = 4) for WT→WT, IFN-γ KO→WT treated with vehicle, and IFN-γ KO→WT treated with ATL146, respectively (p < 0.001, IFN-γ KO→WT treated with vehicle or ATL146 compared with WT mice). These results suggest that BM-derived IFN-γ contributes significantly to renal IRI; in its absence, kidneys are protected from IRI and ATL146e does not add any further protection. These results indirectly implicate CD4 A2AR cells and IFN-γ derived from BM-derived cells in mediating renal IRI and tissue protection by A2A agonists.

**CD4+ reconstitution in Rag-1 KO mice**

We sought to determine the specific role of CD4+ A2AR and IFN-γ in IRI. Using a negative selection process we were able to purify CD4+ T lymphocytes for adoptive transfer into Rag-1 KO mice. We injected CD4+ cells into Rag-1 KO mice via jugular

---

**FIGURE 1.** Effect of IRI in Rag-1 KO mice in the absence and presence of ATL146e. a, Plasma creatinine level is shown for mice treated with vehicle or ATL146e (10 ng/kg/min) beginning 5 h before 32 min of ischemia and continuing for 24 h of reperfusion. Values are mean ± SE in n = 4–5 mice for each group. *, p < 0.001 vs WT mice treated with vehicle. H&E stains of the outer medulla are shown for WT C57BL/6 (b) and Rag-1 KO mice treated with vehicle (c) or with ATL146e (d). Magnification is ×200.

**FIGURE 2.** BM-derived IFN-γ contributes to IRI. Plasma creatinine level is shown for chimeric mice in which the BM cells of WT mice were ablated and reconstituted with IFN-γ KO BM (IFN-γ KO→WT) or WT BM (WT→WT) and subjected to 32-min ischemia and 24-h reperfusion. a, WT→WT chimera and IFN-γ KO→WT chimera were treated with vehicle or ATL146e (10 ng/kg/min) beginning 5 h before 32 min of ischemia and continuing for 24 h of reperfusion. Values are mean ± SE in n = 5 mice for each group. *, p < 0.001 vs WT→WT chimera. H&E stains of sections from the outer medulla are shown for WT→WT chimeras treated with vehicle (b), IFN-γ KO→WT treated with vehicle (c), or IFN-γ KO→WT treated with ATL146e (d) in n = 4–5 mice for each group. Magnification is ×200.
vein, and blood, kidney, and spleen were analyzed for CD4$^+$ cells. In comparison to WT mice (Fig. 3, a, d, and g), Rag-1 KO mice do not have appreciable amounts of CD4$^+$ cells in blood, spleen, and kidney (Fig. 3, b, e, and h, respectively); however CD4$^+$ cells were detected in blood, spleen, and kidney following adoptive transfer of CD4$^+$ cells into Rag-1 KO mice (Fig. 3, c, f, and i, respectively).

**Effect of CD4$^+$ A$_2$AR and IFN-γ on IRI**

To determine the target of A$_2$A agonist mediated tissue protection, we performed IRI on kidneys from WT CD4$^+$, A$_2$A KO, or IFN-γ KO CD4$^+$ cells were adoptively transferred into Rag-1 KO mice (2 x 10$^7$ cells injected via internal jugular vein) and subjected to IRI 7 days later. Mice were treated with vehicle or ATL146e (10 ng/kg/min) beginning 5 h before 32 min of ischemia and continuing for 24 h of reperfusion. Values are mean ± SE in n = 3–8 mice for each group.

**FIGURE 3.** Flow cytometric analysis of blood, spleen, and kidney from WT, Rag-1 KO, and CD4$^+$ adoptively transferred into Rag-1 KO mice. Freshly isolated leukocytes from blood (a–c), spleen (d–f), and kidney (g–i) from WT mice (a, d, and g), Rag-1 KO mice (b, e, and h), and Rag-1 KO mice after adoptive transfer of CD4$^+$ cells (WT CD4$^+$ → Rag-1 KO) (c, f, and i) were subjected to flow cytometric analysis. Density plots are shown for CD4$^+$ cells after gating for CD3 cells. CD4$^+$ cells were enriched by negative selection (see Materials and Methods).

**FIGURE 4.** ATL146e-induced tissue protection is mediated through CD4$^+$ cells. WT CD4$^+$, A$_2$A KO, or IFN-γ KO CD4$^+$ cells were adoptively transferred into Rag-1 KO mice (2 x 10$^7$ cells injected via internal jugular vein) and subjected to IRI 7 days later. Mice were treated with vehicle or ATL146e (10 ng/kg/min) beginning 5 h before 32 min of ischemia and continuing for 24 h of reperfusion. Values are mean ± SE in n = 3–8 mice for each group.

**FIGURE 5.** Kidney histology after ischemia-reperfusion. Representative H&E-stained sections of outer medulla from WT CD4$^+$ → Rag-1 KO mice (a and b), A$_2$A KO CD4$^+$ → Rag-1 KO mice (c and d), or IFN-γ KO CD4$^+$ → Rag-1 KO mice (e and f) whose kidneys were subjected to IRI and treated with either vehicle (a, c, and e) or with ATL146e (b, d, and f). Magnification is ×200.
0.13 mg/dl \((n = 5)\) and \(1.25 \pm 0.78\) mg/dl \((n = 6)\) for vehicle and ATL146e, respectively \((p = \text{NS})\) (Fig. 4). Histological analysis of the outer medulla showed that the injury score paralleled plasma creatinine and was \(2.90 \pm 0.43\) \((n = 5)\) and \(2.90 \pm 0.46\) \((n = 6)\) for A2A KO CD4+→Rag-1 KO treated with vehicle and ATL146e, respectively \((p = \text{NS})\) (Fig. 5, c and d). These results underscore the critical role of A2AR expressed on CD4+ cells. In the absence of A2AR on CD4+ cells, A2A agonists are ineffective in protecting kidneys from IRI.

To determine the role of CD4+ IFN-γ in IRI we adoptively transferred IFN-γ KO CD4+ cells into Rag-1 KO mice (IFN-γ KO CD4+→Rag-1 KO) and administered vehicle or ATL146e. Injury was not reconstituted in IFN-γ KO CD4+→Rag-1 KO mice subjected to renal ischemia-reperfusion and ATL146e had no effect on the extent of IRI. Plasma creatinine was \(0.52 \pm 0.02\) mg/dl \((n = 6)\) and \(0.64 \pm 0.01\) \((n = 3)\) mg/dl, for vehicle and ATL146e treated IFN-γ→Rag-1 KO, respectively \((p = \text{NS})\) (Fig. 4). Histological analysis of the outer medulla showed that the injury score paralleled plasma creatinine and was \(1.42 \pm 0.20\) \((n = 6)\) and \(1.33 \pm 0.17\) \((n = 3)\) for vehicle and ATL146e, respectively \((p = \text{NS})\) (Fig. 5, e and f).

Kidney sections were stained with a mAb to neutrophils to determine the extent of infiltration following IRI. Fig. 6 shows representative photographs of the outer medulla of kidney sections following IRI in the presence and absence of ATL146e. Neutrophil infiltration score was \(3.11 \pm 0.31\) \((n = 4)\) and \(0.56 \pm 0.46\) \((n = 4)\) for WT CD4+→Rag-1 KO treated with vehicle and ATL146e, respectively \((p < 0.001)\) (Fig. 6, a and b); \(2.60 \pm 0.1831\) \((n = 4)\) and \(2.70 \pm 0.17\) \((n = 4)\) for A2A KO CD4+→Rag-1 KO treated with vehicle and ATL146e, respectively \((p = \text{NS})\) (Fig. 6, c and d) and \(0.23 \pm 0.08\) \((n = 3)\) and \(0.32 \pm 0.04\) \((n = 3)\) for IFN-γ KO CD4+→Rag-1 KO treated with vehicle and ATL146e, respectively \((p = \text{NS})\) (Fig. 6, e and f).

**FIGURE 6.** Kidney neutrophil infiltration after ischemia-reperfusion. Kidney sections were stained with anti-neutrophil Ab. Representative sections of outer medulla from WT CD4+→Rag-1 KO mice (a and b), A2A KO CD4+→Rag-1 KO mice (c and d), or IFN-γ KO CD4+→Rag-1 KO mice (e and f) whose kidneys were subjected to IRI and treated with either vehicle (a, c, and e) or with ATL146e (b, d, and f). Magnification is \(\times 200\).
These results demonstrate that activation of A2AR on CD4+ T cells is a major target for A2AR-mediated renal tissue protection. It is interesting to note that others have reported that Rag-1 KO mice have similar severity of renal tissue damage as WT mice after exposure to renal ischemia-reperfusion (25, 26). It has been suggested that an increase in NK cell activity in Rag-1 KO serves as a compensatory mechanism for loss of T and B cells and is also responsible for the restoration of the reperfusion-induced tissue damage. To what extent NK cell activity has to be up-regulated in the Rag-1 KO mice to compensate for both T and B cell loss is not known. Differences do exist in the method of pedicle clamping, use of heparin and anesthetic agents, and degree of injury, which are variables that could potentially contribute to these differences (25, 26). Furthermore, the mice that were used in the current study were derived from B6.129S7-Rag1tm1Mom/J breeders (The Jackson Laboratory). These mice are isogenic and have been backcrossed 20 generations onto a C57BL/6 background. In other studies mice solely without B or T cells have been demonstrated to have less tissue damage upon ischemia-reperfusion challenge (15, 27). Other studies similar to ours show that the extent of IRI is lower in Rag-1 KO mice than in WT mice. Cardiac IRI (28) and liver IRI (Y. J. Day and J. Linden, unpublished observations) are reduced in Rag-1 KO mice. Rag-2 KO mice skeletal muscle is protected from hindlimb (29). Despite differences in published studies on Rag-1 KO, the observation that adoptive transfer of CD4+ cells reconstitutes injury clearly implicates the role of T lymphocytes in IRI and is consistent with previously published results (14).

Although a transient wave of serum IFN-γ level has been found in the early stage of murine liver IRI (10, 30, 31) the origin of this transient wave has not been defined in a renal IRI model. One of the plausible explanations for the appearance of an early transient wave of serum IFN-γ is that lymphocytes might participate in the initiation of reperfusion-induced inflammation. This possibility is supported by our findings that adoptive transfer of WT CD4+ cells but not IFN-γ KO CD4+ KO into Rag-1 KO mice reconstituted the injury phenotype following ischemia-reperfusion. Furthermore adoptive transfer of A2A KO CD4+ into Rag-1 KO mice reconstituted injury following IRI and ATL146e did not protect kidneys in the absence of A2A+R on CD4+ cells. These in vivo studies compliment in vitro studies by Lappas et al. (32). In these studies anti-CD3 mAb activation of CD4+ cells led to an increase in IFN-γ release from CD4+ cells, an effect that was blocked by 98% following incubation with an A2A agonist. Furthermore the ability of A2A agonists to inhibit IFN-γ release was blocked by 100% in CD4+ cells obtained from A2A KO mice. These data provide strong support that the mechanism by which A2A agonists mediate renal tissue protection from IRI is due in part to the ability of A2A agonists to suppress IFN-γ release.

Our studies also demonstrate that kidney neutrophil infiltration parallels injury. Pronounced neutrophil infiltration of the outer medulla was observed following IRI of WT CD4+→Rag-1 KO mice and A2A KO CD4+→Rag-1 KO mice. However the ability of A2A agonists to block neutrophil infiltration depended on the presence of A2A+R expressed on CD4+ cells. When reconstitution of Rag-1 KO mice was conducted with CD4+ cells from IFN-γ KO mice there was marked reduction of neutrophil infiltration. These results confirm the relationship between T cells and neutrophils (15) and suggest the participation of neutrophils in the early phase (<24 h) of ischemia-reperfusion.

Thus our data support other studies (14) that suggest early activation of T cells. The mechanism by which this occurs is unknown but could be the result of Ag-dependent or Ag-independent activation (33). H2O2 and RANTES are known to activate T lymphocytes through Ag-independent mechanisms. H2O2 is generated at the first few minutes of reperfusion (34, 35) and directly activates CD4+ T lymphocytes (36) through oxidation of cysteine residues that inactivate protein tyrosine phosphatases (37). Chemokines including RANTES mediate Ag-independent T lymphocyte activation (24). Alternatively, T lymphocytes can be activated through classical mechanisms that include TCR-agonist receptors presented by APCs followed by costimulation by CD80 (B7-1) and/or C86 (B7-2) (33). However Ag-dependent activation in response to IRI has yet to be demonstrated.

Interestingly, A2A have been identified as the predominantly expressed G protein-coupled receptors in T lymphocytes, especially on CD4+ T lymphocytes (38), and as the critical signaling pathway to suppress all TCR-triggered effector function of T lymphocytes (39, 40). Studies suggest that A2A could inhibit TCR-triggered CD25 up-regulation and block the proliferation of T lymphocytes by inducing apoptosis (41). These results suggest that the A2A signaling pathway acts as an intracellular negative regulatory mechanism in T cell proliferation and TCR-triggered responses (41). Furthermore, A2A may suppress intercellular interactions by interfering with cytokines released from Th cells during the inflammation process (42).

In conclusion, CD4+ T lymphocytes play an important role in A2A-mediated tissue protection. This role may involve complex interplay between CD4+ T lymphocytes and other lineages such as macrophage/monoocyte, platelet, and endothelium in recruitment of neutrophils at the first few hours of reperfusion. However, by using the adoptively transferred T cell- or B cell-deficient mouse model, our studies demonstrate that IFN-γ may be a critical mediator for this complex interplay. It will be interesting in future studies to determine how the CD4+ T lymphocyte interact with other BM cells during reperfusion injury and by which mechanisms A2A activation may provide protection from tissue injury.

Acknowledgments
We gratefully acknowledge Dr. Marc McDuffie (Department of Microbiology, University of Virginia) for providing Rag-1 KO mice, Melissa Marshall (Department of Medicine, University of Virginia) for expert technical assistance, Jiang-Fan Chen (Department of Neurology, Boston University School of Medicine, Boston, MA) for A2A KO mice, and Dr. Alaa Awad (University of Virginia) and Dr. Diane Rosin (Department of Pharmacology, University of Virginia) for careful reading of this manuscript.

Disclosures
J. Linden and M. D. Okusa own equity in Adenosine Therapeutics, which provided ATL313 and ATL146e for this study. All other authors have no financial conflict of interest.

References