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Article

Hematology

Activation of A<sub>2A</sub> adenosine receptors (A<sub>2A</sub>Rs) protects kidneys from ischemia-reperfusion injury (IRI). A<sub>2A</sub>Rs are expressed on bone marrow–derived (BM-derived) cells and renal smooth muscle, epithelial, and endothelial cells. To measure the contribution of A<sub>2A</sub>Rs on BM-derived cells in suppressing renal IRI, we examined the effects of a selective agonist of A<sub>2A</sub>Rs, ATL146e, in chimeric mice in which BM was ablated by lethal radiation and reconstituted with donor BM cells derived from GFP, A<sub>2A</sub>R-KO, or WT mice to produce GFP→WT, A<sub>2A</sub>-KO→WT, or WT→WT mouse chimera. We found little or no repopulation of renal vascular endothelial cells by donor BM with or without renal IRI. ATL146e had no effect on IRI in A<sub>2A</sub>-KO mice or A<sub>2A</sub>-KO→WT chimera, but reduced the rise in plasma creatinine from IRI by 75% in WT mice and by 60% in WT→WT chimera. ATL146e reduced the induction of IL-6, IL-1β, IL-1ra, and TGF-α mRNA in WT→WT mice but not in A<sub>2A</sub>-KO→WT mice. Plasma creatinine was significantly greater in A<sub>2A</sub>-KO than in WT mice after IRI, suggesting some renal protection by endogenous adenosine. We conclude that protection from renal IRI by A<sub>2A</sub>R agonists or endogenous adenosine requires activation of receptors expressed on BM-derived cells.

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# Renal protection from ischemia mediated by A<sub>2A</sub> adenosine receptors on bone marrow–derived cells

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Activation of A<sub>2A</sub> adenosine receptors (A<sub>2A</sub>Rs) protects kidneys from ischemia-reperfusion injury (IRI). A<sub>2A</sub>Rs are expressed on bone marrow–derived (BM-derived) cells and renal smooth muscle, epithelial, and endothelial cells. To measure the contribution of A<sub>2A</sub>Rs on BM-derived cells in suppressing renal IRI, we examined the effects of a selective agonist of A<sub>2A</sub>Rs, ATL146e, in chimeric mice in which BM was ablated by lethal radiation and reconstituted with donor BM cells derived from GFP, A<sub>2A</sub>R-KO, or WT mice to produce GFP→WT, A<sub>2A</sub>-KO→WT, or WT→WT mouse chimera. We found little or no repopulation of renal vascular endothelial cells by donor BM with or without renal IRI. ATL146e had no effect on IRI in A<sub>2A</sub>-KO mice or A<sub>2A</sub>-KO→WT chimera, but reduced the rise in plasma creatinine from IRI by 75% in WT mice and by 60% in WT→WT chimera. ATL146e reduced the induction of IL-6, IL-1β, IL-1ra, and TGF-α mRNA in WT→WT mice but not in A<sub>2A</sub>-KO→WT mice. Plasma creatinine was significantly greater in A<sub>2A</sub>-KO than in WT mice after IRI, suggesting some renal protection by endogenous adenosine. We conclude that protection from renal IRI by A<sub>2A</sub>R agonists or endogenous adenosine requires activation of receptors expressed on BM-derived cells.

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## Introduction

Bone marrow–derived (BM-derived) cells such as neutrophils (1, 2) and T lymphocytes (3–5) are thought to play prominent roles in the pathogenesis of ischemia-reperfusion injury (IRI). Activation of A<sub>2A</sub> adenosine receptors (A<sub>2A</sub>Rs) is known to reduce inflammation and has been shown to produce tissue protection from IRI (6, 7). We previously demonstrated that 4-[3-[6-Amino-

9-(5-ethylcarbamoyl-3,4-dihydroxy-tetrahydro-furan-2-yl)-9H-purin-2-yl]-prop-2-ynyl]-cyclohexanecarboxylic acid methyl ester (ATL146e), a specific A<sub>2A</sub>R agonist with 50 times higher affinity for A<sub>2A</sub>Rs than the commonly used A<sub>2A</sub> agonist CGS21680, attenuated plasma creatinine levels after renal injury by 70–80% when administered before or immediately after the onset of reperfusion and throughout the reperfusion period (8). The cellular target through which ATL146e mediates renal tissue protection is not known.

A<sub>2A</sub>R stimulation vasodilates the outer medullary descending vasa recta (9), an effect that could reduce ischemic damage. Inhibition of endothelial cell activation may contribute to renal protection by A<sub>2A</sub>R agonists. ATL146e reduces the expression of the endothelial adhesion molecules P-selectin and ICAM-1 (10). BM-derived cells such as neutrophils, macrophages, platelets, and T lymphocytes (T cells) may also be the target of protection by A<sub>2A</sub>R agonists. A<sub>2A</sub>Rs are expressed on most hematopoietic cells, and activation of A<sub>2A</sub>Rs on isolated cells has been shown to have direct anti-inflammatory effects (11).

In this study, we investigated the contribution of A<sub>2A</sub>Rs expressed on BM-derived cells to renal tissue protection from IRI. We created chimeric mice in which BM was ablated from WT mice and reconstituted with BM from congenic mice in which the A<sub>2A</sub>R gene had been deleted (A<sub>2A</sub>-KO). This method permits discrimination between A<sub>2A</sub> agonist effects on BM-derived cells and possible effects

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J. Stephen Fink is deceased.

**Conflict of interest:** Mark D. Okusa, Joel Linden, and Marcia J. McDuffie have equity in Adenosine Therapeutics, which supplied ATL146e.

**Nonstandard abbreviations used:** A<sub>2A</sub> adenosine receptors (A<sub>2A</sub>Rs); ischemia-reperfusion injury (IRI); bone marrow (BM); 4-[3-[6-Amino-9-(5-ethylcarbamoyl-3,4-dihydroxy-tetrahydro-furan-2-yl)-9H-purin-2-yl]-prop-2-ynyl]-cyclohexanecarboxylic acid methyl ester (ATL146e); periodate-lysine-paraformaldehyde (PLP); phosphodiesterase type IV (PDE 4); heart rate (HR); outer medulla (OM); macrophage inhibitory protein (MIP); IFN-γ-inducible protein 10 (IP-10); IL-1 receptor antagonist (IL-1ra); monocyte chemoattractant protein-1 (MCP-1).

on vascular or renal parenchymal cells. We also showed by using BM from GFP mice that few if any renal endothelial or smooth muscle cells are derived from donor BM, with or without renal IRI. Our findings demonstrate that activation of A<sub>2A</sub>Rs on BM-derived cells is primarily responsible for protection of the kidney from IRI. Furthermore, these studies provide evidence for a direct causal relation between activation of inflammatory cells and IRI.

## Methods

**Genotype and phenotype of congenic A<sub>2A</sub>-KO mice (C57BL/6).** Mice with functionally disrupted A<sub>2A</sub>Rs were initially generated on a mixed genetic background after several generations of inbreeding from (129/SvJae, A<sub>2A</sub>R<sup>-/-</sup> × C57BL/6) F1 founder mice (12). These mice were typed with a skeleton panel of 96 informative microsatellite loci covering every chromosome (except Y) at an average distance of 15 centimorgan. A marker-assisted breeding strategy was used to effectively move the mutant Adora2a allele onto the C57BL/6 background (99.3% homozygosity) by generation 3. The only detectable 129 contamination surrounds the Adora2a locus, which we mapped during gene transfer onto chromosome 10, in a region of conserved synteny with the human locus.

DNA was obtained from the tail of A<sub>2A</sub>-KO mice and subjected to a PCR genotyping strategy using the following oligonucleotides to amplify neomycin resistance inserted into the A<sub>2A</sub>-KO gene: NEO-F, 5'-AGACAATCG-GCTGCTCTGAT; NEO-R, 5'-CAAGCTCTTCAGCAATAT-CACG; and a portion of the WT gene deleted from the A<sub>2A</sub>-KO construct, WT-F, 5'-AGCCAGGGGTACATCTGTG, and WT-R, 5'-AGACAATCGGCTGCTCTGAT. PCR products were separated by 1% agarose gel electrophoresis and visualized by staining DNA with ethidium bromide. Tissue from WT C57BL/6 or A<sub>2A</sub>-KO mice was prepared for immunohistochemistry, and A<sub>2A</sub>R immunoreactivity was detected as described previously (13).

**Generation of chimeric mice.** C57BL/6 male mice (Hilltop Lab Animals, Scottsdale, Pennsylvania, USA), congenic A<sub>2A</sub>-KO mice, and GFP mice (Jackson Laboratory, Bar Harbor, Maine, USA) were used for BM transplantation. Donor mice (12 weeks of age, 25–28 g) were anesthetized with Nembutal (0.02 mg/g) and sacrificed by cervical dislocation. The marrow from the tibia and femur was harvested under sterile conditions. Bones were flushed with RPMI (Life Technologies, Grand Island, New York, USA) plus 10% FCS. The marrow was passed sequentially through a 22-gauge needle followed by three passages through a 25-gauge needle in order to obtain single-cell suspensions of BM cells. Cells were washed and resuspended, and viable cells were counted. The yield was approximately 50 × 10<sup>6</sup> nucleated BM cells per mouse. Recipient mice (8–10 weeks of age, 22–25 g) were lethally irradiated with two exposures to 6 Gy 4 hours apart. Immediately after irradiation, 3 × 10<sup>6</sup> BM cells were injected through the tail vein. The resulting chimeric mice were housed in microisolators for 6–8 weeks before experimentation and fed autoclaved food and water containing 5 mM sulfamethoxazole and 0.86 mM trimethoprim.

To evaluate the efficiency of reconstitution, a mutated mouse strain, B6.SJL-*Ptprca*<sup>a</sup> *Pep3*<sup>b</sup>/BoyJ, was used as the source of donor BM. The CD45.1 epitope, absent in cells of recipient mice, was detected by immunofluorescent cell sorting 6 weeks after BM transplantation. PBMCs from transplanted recipients were purified from 200 µl of tail vein blood using the Ficoll (density, 1.077) gradient method with centrifugation at 500 g for 10 minutes. Harvested PBMCs were placed in 1.5 mM NH<sub>4</sub>Cl to lyse red blood cells and then resuspended into 200 µl of RPMI with 5% fetal calf serum. After blocking nonspecific Fc binding with anti-mouse CD16/CD32 (BD Pharmingen, San Diego, California, USA), PBMCs were incubated with R-phycoerythrin conjugated anti-mouse CD45.1 monoclonal antibody (BD Pharmingen) and either allophycocyanin-conjugated rat anti-mouse CD11b (Mac-1 β chain) monoclonal antibody (BD Pharmingen), fluorescein isothiocyanate-conjugated rat anti-mouse CD8a monoclonal antibody (BD Pharmingen), or allophycocyanin-conjugated rat anti-mouse CD4 monoclonal antibody (BD Pharmingen) on ice for 30 minutes. CD11b-, CD8a-, and CD4-positive cells were sorted by flow cytometry, and the percentage of cells expressing CD45.1 was determined in each population of cells (FACScan, CellQuest, Becton Dickinson, San Jose, California, USA).

**GFP mouse chimera and anti-GFP immunohistochemistry.** GFP chimeras were created by transplanting BM from GFP mice into lethally irradiated C57BL/6 controls (GFP→WT) as described above. Immunohistochemistry was performed as described previously (14). Spleens or kidneys were harvested, fixed in 4% periodate-lysine-paraformaldehyde (PLP) and embedded in either paraffin (spleens) or epoxy resin (kidneys). Spleens and kidneys were cut to yield 4 and 0.5 µm sections, respectively. Epoxy resin was then removed (15). GFP was detected immunohistochemically, since direct detection of GFP fluorescence was found to be unreliable because of nonspecific green fluorescence in WT animals, particularly in tissues subjected to IRI. Sections were incubated with rabbit anti-GFP antibody (1:1000; Novus Biologicals Inc., Littleton, Colorado, USA) followed by goat anti-rabbit secondary antibody conjugated to FITC secondary (Vector Laboratories, Burlingame, California, USA). In some cases, kidney sections were subjected to dual labeling by incubating with rabbit anti-GFP antibody and mouse-anti Mac-2 (1:500; Accurate Chemicals, Westbury, New Jersey, USA) followed by goat anti-rabbit secondary antibody conjugated to FITC (1:1000) and goat anti-mouse secondary antibody conjugated to rhodamine (1:1000). Stained cells or tissue sections were cover slipped with an aqueous-based mounting solution consisting of *p*-phenylenediamine (1 mg/ml) and 70% glycerol. Sections were viewed under a Zeiss AxioSkop photofluorescence microscope. Photographs were taken with a SPOT RT Camera (software version 3.3, Diagnostic Instruments Inc., Sterling Heights, Michigan, USA) and imported into Adobe Photoshop (3.0) for brightness/contrast adjustment.

**Compound administration.** Mice were anesthetized with vaporized halothane (Halothan Vapor 19.1, North

American Drager, Telford, Pennsylvania, USA) before subcutaneous implantation of osmotic minipumps (model 1003D, ALZA Corp., Palo Alto, California, USA). The pumps released either vehicle (0.01% DMSO in PBS), ATL146e (10 ng/kg per minute) or rolipram (0.1 ng/kg per minute), a type IV phosphodiesterase (PDE 4) inhibitor. Doses of ATL146e and rolipram were chosen on the basis of previous dose-ranging studies to produce a 60–70% reduction of plasma creatinine levels 24 hours after renal IRI (8). Administration of vehicle or compounds began 5 hours before ischemia and continued throughout the 24-hour reperfusion period.

**Procedure for producing renal IRI.** Mice were anesthetized with ketamine (100 mg/kg intraperitoneally), xylazine (10 mg/kg intraperitoneally), and acepromazine (1 mg/kg intramuscularly) and subjected to bilateral flank incisions as previously described (6). Both renal pedicles were cross-clamped for 32 minutes. Surgical wounds were closed with metal staples, and mice were returned to cages for 24 hours. After 24 hours of reperfusion, animals were reanesthetized, blood was obtained by cardiac puncture, and kidneys were removed for various analyses.

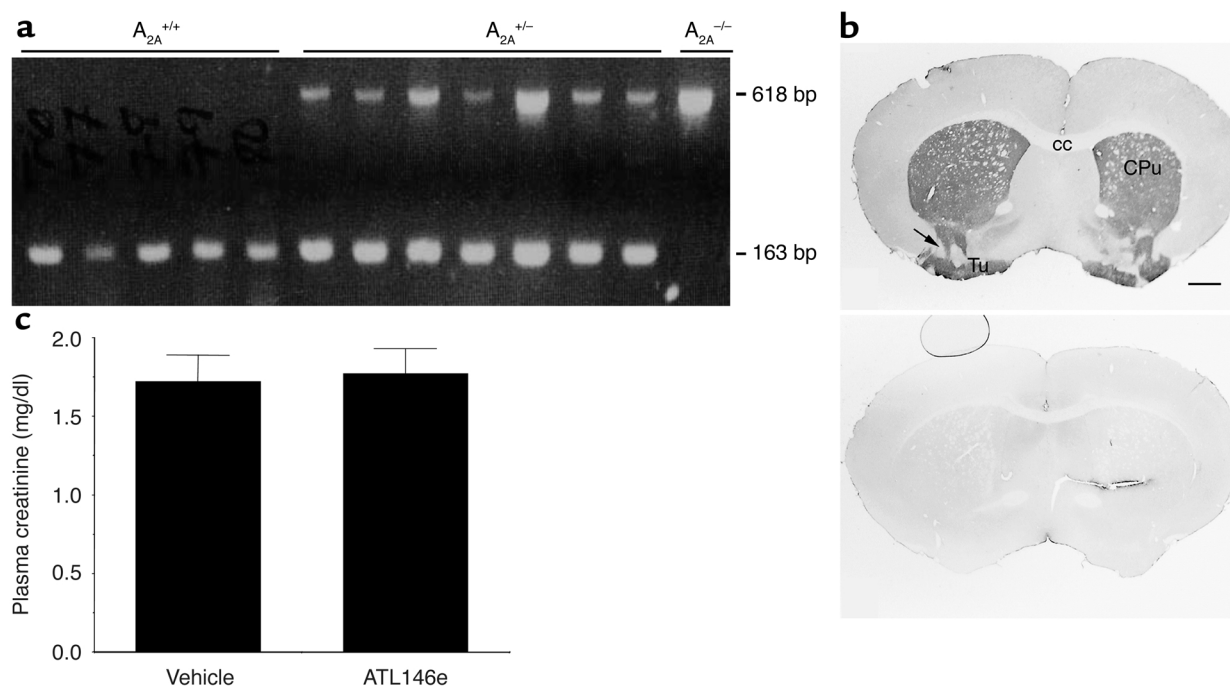
**Plasma and urine chemistry.** Plasma creatinine concentrations were determined using a colorimetric assay according to the manufacturer's protocol (Sigma-

Aldrich, St. Louis, Missouri, USA). Urine sodium and potassium concentrations were measured by an automated autoanalyzer (Massachusetts General Hospital Clinical Laboratory, Boston, Massachusetts, USA).

**Tail cuff blood pressure measurement.** Mean arterial pressure was measured by using a photoelectric sensor for pulse detection in mouse tail (IITC Model 179, IITC Inc./Life Science Instruments, Woodland Hills, California, USA). Mice were allowed to rest for 10 minutes in a chamber with the temperature controlled at 26°C. Blood pressures were measured twice and averaged.

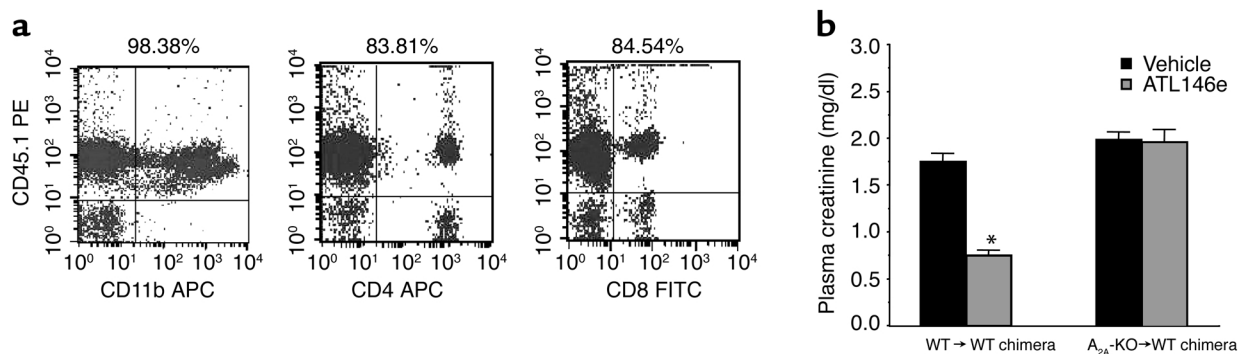
**Leukocyte isolation from spleen.** Spleens were removed from mice, gently dispersed, and filtered through gauze. Leukocytes were isolated by Ficoll density centrifugation, counted using a hemocytometer, and subjected to flow cytometry.

**Multiprobe RNase protection assays.** Total kidney RNA was extracted from homogenized tissue with RNazol B (Leedo Medical Laboratories, Houston, Texas, USA) and analyzed by 1.5% agarose gel electrophoresis to assess the integrity of RNA before solution hybridization. Cytokine mRNA expression was assessed by the RiboQuant multiprobe RNase protection system (BD Pharmingen) according to the manufacturer's protocol. In brief, mRNA-specific RNA probes were labeled with  $^{32}$ [P]UTP using multiprobe



**Figure 1**

Genotyping and phenotyping mice for the  $A_{2A}$ R gene. (a) Ethidium bromide-stained PCR amplification products of tail-clip DNA from WT ( $A_{2A}^{+/+}$ ) and  $A_{2A}$ -KO ( $A_{2A}^{-/-}$ ) mice. The  $A_{2A}$ -KO allele was identified by PCR of the inserted neomycin resistance cassette using oligonucleotide primers (see Methods) NEO-F and NEO-R and yielding a 618-bp product. The  $A_{2A}$  WT allele was identified by PCR of a portion of the WT  $A_{2A}$ R gene that was deleted from the KO construct using primers WT-F and WT-R and yielding a 163-bp band.  $+/+$ , WT;  $+/-$ , heterozygous;  $-/-$ , homozygous  $A_{2A}$ -KO. Each lane represents PCR products from individual mice. (b) Immunohistochemical localization of  $A_{2A}$ R in mouse forebrain sections. In the WT brain sections (top), dense  $A_{2A}$ R-like immunoreactivity is present in the striatum (caudate putamen) and extends through the cell bridges of the striatum (arrow) to the olfactory tubercles on the ventral surface of the brain. In  $A_{2A}$ -KO brain (bottom), specific immunoreactivity is completely absent. Scale bar, 1 mm. CPu, caudate putamen; Tu, tubercles; cc, corpus callosum. (c) Effect of ATL146e in  $A_{2A}$ -KO mice.  $A_{2A}$ -KO mice were subjected to IRI (see Methods). The rise in plasma creatinine after ATL146e was similar to that after vehicle administration ( $n = 6$ , not significant). Values are means  $\pm$  SE.



**Figure 2**

Effect of renal IRI and ATL146e on plasma creatinine in chimeric mice. (a) Reconstitution efficacy of granulocytes and lymphocytes at 6 weeks after BM transplantation. PBMCs from transplanted recipients were purified and incubated with anti-CD45.1 as a marker of donor cells and either anti-CD11b, anti-CD4, or anti-CD8a monoclonal antibodies (see Methods). Labeled cells were enumerated by flow cytometry. The number at the top of each panel indicates the percentage of cells that are positive for both CD45.1 and each of the other marker fluorescent antibodies. Data are representative of 12 independent experiments. (b) Effect of renal IRI and ATL146e on plasma creatinine in chimeric mice. Chimeric mice were prepared by transferring to lethally irradiated WT recipients BM from either WT (WT→WT) or A<sub>2A</sub>-KO (A<sub>2A</sub>-KO→WT) donor animals. Chimeric animals were treated with vehicle or ATL146e (10 ng/kg per minute) beginning 5 hours before 32 minutes of ischemia and continuing for 24 hours of reperfusion.  $n = 11$  for each group. Values are means  $\pm$  SE. \* $P < 0.0001$  versus vehicle.

template sets (mCK1, mCK2b, mCK3, and mCK5; BD Pharmingen) for cytokine/chemokine genes. Kidney total RNA was subjected to solution hybridization with each probe set. Hybridization was performed at 56°C before RNase treatment. After RNase treatment, protected fragments were separated by gel electrophoresis on 5% polyacrylamide gels and exposed to Kodak X-Omat AR film at -70°C with a single intensifying screen.

**Statistical analysis.** Unpaired Student's *t*-test was used for all comparisons. A *P* value of less than 0.05 was used to define statistical significance.

## Results

**Genotype and phenotype of A<sub>2A</sub>-KO mice.** The genotype of mice was determined by PCR using primers to amplify a 618-bp fragment of the neomycin cassette present in the KO allele and a 163-bp fragment present in WT sequence that was deleted from the KO allele (Figure 1a). Mouse phenotypes were assessed immunohistochemically using a selective A<sub>2A</sub>R monoclonal antibody (13). Consistent with our previous results in rats (13), immunohistochemical labeling of brain sections revealed the expected pattern of dense A<sub>2A</sub>R-like immunoreactivity in the striatum of WT mice (Figure 1b, top) and the absence of immunoreactivity in the striatum of A<sub>2A</sub>-KO mice (Figure 1b, bottom). Taken together, these results confirm disruption of the A<sub>2A</sub>R gene and the loss of expression of A<sub>2A</sub>R protein in A<sub>2A</sub>-KO mice.

No significant differences were detected between WT and A<sub>2A</sub>-KO mice in mean arterial pressure (MAP), heart rate (HR), pH, pCO<sub>2</sub>, pO<sub>2</sub>, or core body temperature (12), and the kidneys of A<sub>2A</sub>-KO mice were histologically indistinguishable from those of C57BL/6 mice (data not shown). Baseline plasma creatinine levels were  $0.128 \pm 0.013$  ( $n = 8$ ) and  $0.111 \pm 0.014$  mg/dl ( $n = 14$ , not significant) for WT and A<sub>2A</sub>-KO mice, respectively. Baseline 24-hour urine sodium excretion levels were

$0.096 \pm 0.020$  ( $n = 9$ ) and  $0.099 \pm 0.021$   $\mu$ mol per 24 hours ( $n = 8$ , not significant), and 24-hour urine potassium excretion levels were  $0.144 \pm 0.029$  ( $n = 9$ ) and  $0.138 \pm 0.019$   $\mu$ mol per 24 hours ( $n = 8$ , not significant) for WT and A<sub>2A</sub>-KO mice, respectively.

### Effect of ATL146e or rolipram on IRI injury in A<sub>2A</sub>-KO mice.

The primary goal of the current study was to determine the contribution of BM-derived cells to A<sub>2A</sub>R agonist-mediated renal protection from IRI. For this purpose, we generated chimeric mice in which BM of WT mice was replaced with BM-derived cells from A<sub>2A</sub>-KO mice. This strategy rests on the assumption that the protective effect of ATL146e is due to specific effects on A<sub>2A</sub>Rs. Preliminary experiments examined the effects of ATL146e on renal function in WT and A<sub>2A</sub>-KO mice following IRI. In WT mice, IRI produced a rise in plasma creatinine levels that was reduced 78% by administration of ATL146e to WT mice. Plasma creatinine levels in mice treated with vehicle or ATL146e were  $1.41 \pm 0.25$  and  $0.35 \pm 0.12$  mg/dl ( $n = 3$ ,  $P < 0.02$ ). This is similar to a 62% reduction ( $n = 9$ ,  $P < 0.001$ ) that we reported previously (8). In A<sub>2A</sub>-KO mice, the rise in plasma creatinine after IRI was not affected by ATL146e administration (Figure 1c). Plasma creatinine levels were  $1.72 \pm 0.17$  and  $1.77 \pm 0.16$  mg/dl for vehicle and ATL146e, respectively ( $n = 6$ , not significant). This lack of effect of ATL146e in reducing renal injury in A<sub>2A</sub>-KO mice is consistent with our previous demonstration that the selective competitive A<sub>2A</sub>R antagonist, ZM243185, inhibits ATL146e-mediated renal protection (6).

It is possible that deletion of the A<sub>2A</sub>R gene might cause mice to be nonspecifically insensitive to protection by ATL146e—for example, by enhancing the magnitude of IRI. To test this possibility, we examined the effects of the PDE 4 inhibitor, rolipram, which we have shown previously protects WT mice from renal IRI (8). Both A<sub>2A</sub>R activation and PDE inhibition are thought

to elicit tissue protection through a cAMP/PKA pathway (8). We reasoned that rolipram can increase cAMP in the absence of A<sub>2A</sub>Rs. Infusion of rolipram (0.1 ng/kg per minute) into A<sub>2A</sub>-KO mice significantly reduced plasma creatinine from  $2.36 \pm 0.16$  ( $n = 4$ ) to  $1.52 \pm 0.20$  mg/dl ( $n = 5$ ,  $P < 0.02$ ). These results suggest that failure of ATL146e to prevent renal IRI in A<sub>2A</sub>R KO mice is due specifically to deletion of receptors.

**BM reconstitution.** To determine the principal cellular targets of A<sub>2A</sub> action, we generated chimera in which BM was ablated from WT mice and reconstituted with BM cells lacking the A<sub>2A</sub>R gene (A<sub>2A</sub>-KO→WT) or with BM from WT mice (WT→WT). In preliminary studies, chimera prepared from different mouse strains were used to evaluate the extent of reconstitution of various BM-derived cells. Figure 2a shows representative examples demonstrating the percentage of cells expressing the donor marker, CD45.1, in PBMCs that are positive for CD11b ( $98.4 \pm 0.4$ ), CD4 ( $83.8 \pm 2.0$ ), and CD8 ( $84.5 \pm 2.3$ ) by flow cytometry. These results demonstrate efficient repopulation of BM-derived cells in chimeric animals.

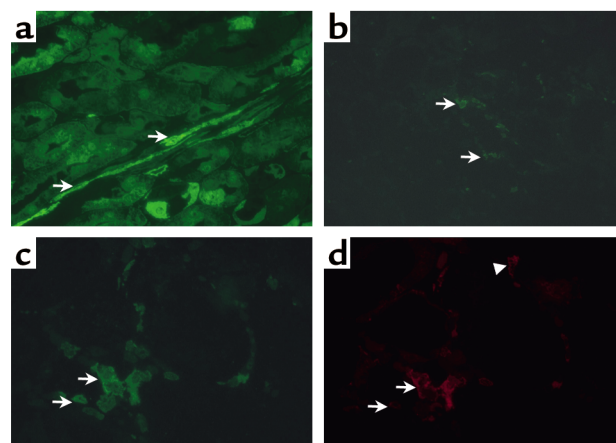
**Effect of ATL146e on renal IRI in chimeric mice.** We next sought to determine the contribution of BM-derived cells to the protective effects of A<sub>2A</sub>R activation. WT→WT and A<sub>2A</sub>-KO→WT chimeric mice were subjected to IRI, and plasma creatinine was used as a measure of renal injury (Figure 2b). In WT→WT chimera, the rise in the plasma creatinine level after IRI was reduced significantly in ATL146e-treated mice ( $n = 11$ ; vehicle,  $1.75 \pm 0.09$  mg/dl; ATL146e,  $0.75 \pm 0.06$  mg/dl;  $P < 0.0001$ ). The reduction in plasma creatinine by ATL146e in WT→WT chimeric mice (43%) was similar to that determined previously in WT mice (38% of control) (8). Plasma creatinine levels after IRI in vehicle-treated A<sub>2A</sub>-KO→WT chimeric mice ( $1.99 \pm 0.08$  mg/dl,  $n = 12$ ) were unaffected by ATL146e infusion ( $1.96 \pm 0.13$  mg/dl,  $n = 11$ , not significant).

There was no significant effect of IRI on MAP or HR in chimeric mice. During IRI with ATL146e, HR decreased in the WT→WT group ( $n = 6$ ,  $\Delta\text{HR} = -39.9 \pm 17$ ) but increased in A<sub>2A</sub>-KO→WT mice ( $n = 6$ ,  $\Delta\text{HR} = 42.5 \pm 17$ ). These results demonstrate that the protective effect of ATL146e in kidney IRI requires activation of A<sub>2A</sub>Rs on BM-derived cells.

**Evidence against transdifferentiation of transplanted BM cells into endothelial cells.** If renal endothelium is repopulated by BM-derived cells, then the deletion of A<sub>2A</sub>R on endothelial cell precursors could account for the absence of protection by ATL146e in A<sub>2A</sub>-KO→WT chimera. To determine the degree to which transdifferentiation of donor-derived endothelial progenitor cells to renal vascular endothelium occurs in chimera subjected to IRI, GFP→WT chimera mice were subjected to IRI. Cells expressing GFP were detected by anti-GFP antibody. Figure 3a shows diffuse GFP immunoreactivity in various cells of the outer medulla (OM) of kidney from a GFP mouse. In particular, endothelial cells are stained brightly and have a characteristic elongated morphology. Splens from GFP→WT chimera contained abundant GFP immunoreactivity (data not shown). After IRI in

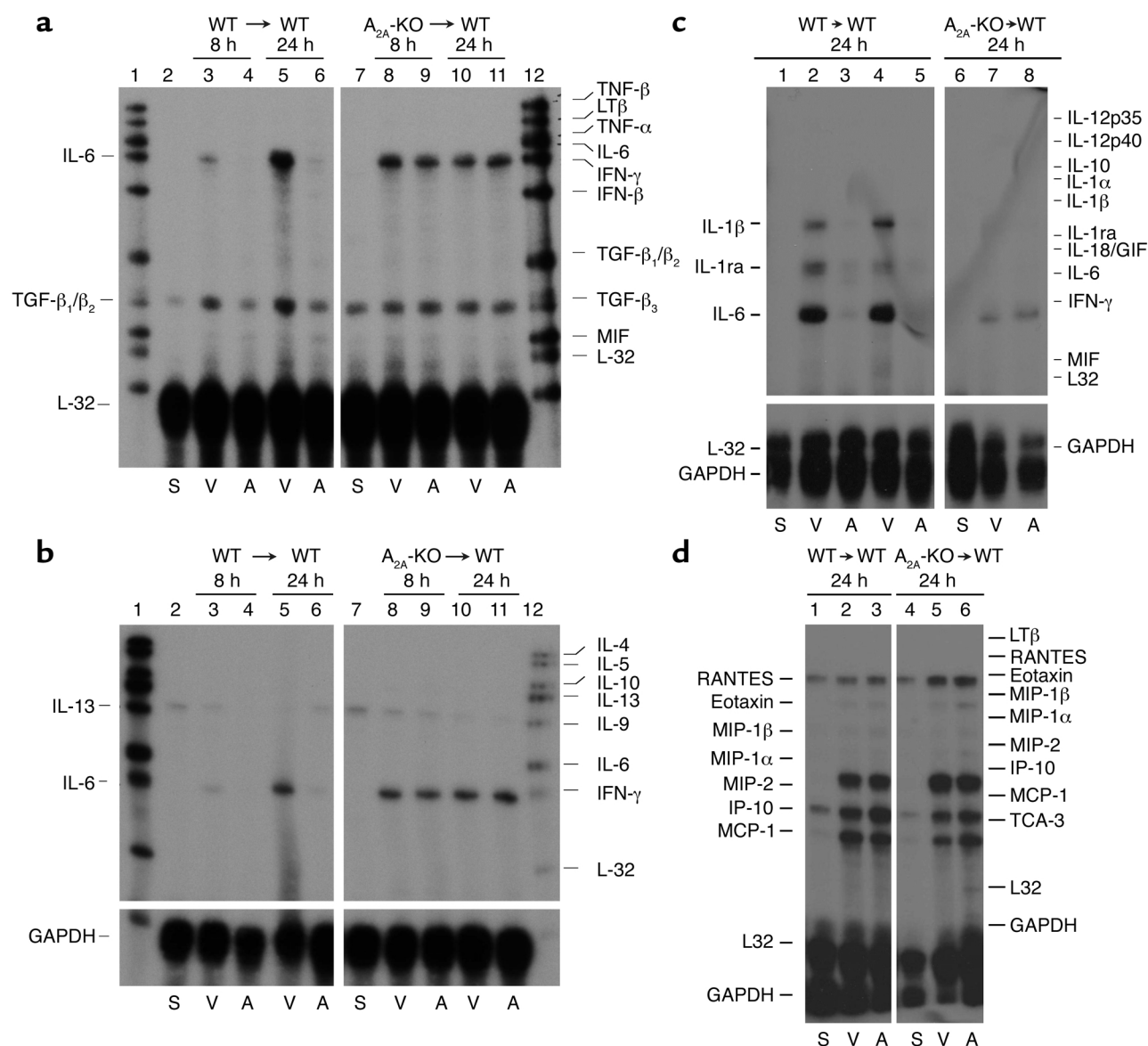
GFP→WT chimera, scattered GFP-immunoreactive cells that are morphologically distinct from endothelium are visible in kidney under low magnification (Figure 3b); these may be inflammatory cells that infiltrate the kidney after IRI. In dual-labeling studies, high-magnification images of the OM demonstrate GFP-positive cells in the peritubular capillaries (Figure 3c) that are identified as macrophages (Figure 3d). Occasional macrophages do not contain GFP immunoreactivity and may be of recipient mouse origin (arrowhead, Figure 3d). In renal sections from four GFP→WT chimeric mice, we could not detect any GFP-positive cells with an endothelial morphology. We conclude that 6 weeks after BM transplantation and 24 hours after IRI, there are few if any transdifferentiated endothelial cells in chimeric animals.

**Effect of ATL146e on cytokine and chemokine mRNA expression in kidneys of chimeric mice.** We used chimeric mice to determine the contribution of activation of A<sub>2A</sub>Rs on BM-derived cells to renal cytokine mRNA expression after IRI. In kidneys from WT→WT mice, 32 minutes of ischemia and 8 or 24 hours of reperfusion caused induction of multiple cytokine mRNAs that were not evident in sham mice. IL-6 and TGF- $\beta$  were induced at 8 and 24 hours after reperfusion (Figure 4a, lanes 3, 5, 8, and 10). ATL146e led to a pronounced reduction in the induction of IL-6 and TGF- $\beta$  in kidneys of WT→WT mice at 8 (lane 4) and 24 (lane 6) hours but had no effect on mRNA induction in A<sub>2A</sub>-KO→WT mice at 8 (lane 9) or 24 (lane 11) hours of reperfusion. A separate probe set was used to examine the effect of IRI on expression of various other cytokine transcripts after reperfusion. IL-6 mRNA was increased by IRI



**Figure 3**

Immunohistochemistry of GFP-positive cells in kidney of GFP mice and GFP→WT chimera. (a) Immunohistochemical localization of GFP in outer medulla of kidney from GFP mice. Magnification,  $\times 630$ . Endothelial cells are identified by arrows. (b) Outer medulla of kidney from GFP→WT chimeras after renal IRI. Donor BM-derived cells are identified by arrows. Magnification,  $\times 630$ . (c and d) Dual labeling of kidney from GFP→WT chimeras after renal IRI. Labeling with anti-GFP antibody (c) reveals cells of donor origin (arrows, c) that are identified as macrophages (arrows, d) by labeling with anti-macrophage antibody (d). Arrowhead indicates recipient macrophage. Magnification,  $\times 1000$  in (c) through (d).



**Figure 4**

Cytokine and chemokine gene expression from kidneys of chimeric mice subjected to IRI. Kidneys were subjected to 32 minutes of ischemia and either 8 or 24 hours of reperfusion in animals that were treated with either vehicle or ATL146e. V, vehicle; A, ATL146e. (a) RNase protection assay (RPA) analysis of selected cytokines from kidney mRNA using mCK3 probes. Sham-operated chimeric mice (lanes 2 and 7) and chimeric mice subjected to IRI (lanes 3–6 and 8–11). WT→WT mice at 8 (lanes 3 and 4) or 24 (lanes 5 and 6) hours and  $A_{2A}$ -KO→WT mice at 8 (lanes 8 and 9) or 24 (lanes 10 and 11) hours as indicated. Each lane represents solution hybridization with RNA derived from kidneys of separate mice. (b) RPA analysis using the mCK1 probes. (c) RPA analysis using mCK2b probes. WT→WT mice at 24 hours (lanes 1–5) and  $A_{2A}$ -KO→WT mice at 24 hours (lanes 6–8). Mice were subjected to sham operation (S) (lanes 1 and 6) or IRI (lanes 2–5, 7, and 8). Relative mobility of unprotected cytokine mRNA is illustrated on the right. S, sham operation. (d) RPA analysis using mCK5 probes. Kidney RNA was isolated from WT→WT mice at 24 hours (lanes 1–3) and from  $A_{2A}$ -KO→WT mice at 24 hours (lanes 4–6). Mice were subjected to sham operation (lanes 1 and 4) or IRI (lanes 2, 3, 5, and 6).

and reduced by ATL146e in WT→WT mice but not in  $A_{2A}$ -KO→WT mice (Figure 4b). IL-1β and IL1 receptor antagonist (IL-1ra) mRNA were also found to be increased after IRI, an effect blocked by ATL146e (Figure 4c). RANTES, eotaxin, macrophage inhibitory protein-1α (MIP-1α), MIP-1β, MIP-1β, IFN-γ-inducible protein 10 (IP-10), and monocyte chemoattractant protein-1 (MCP-1) mRNA were induced at 24 hours after IRI; however, ATL146e had no effect on their expression

(Figure 4d). At 24 hours after IRI, TNF-β, lymphotoxin β, INF-β, IL-4, IL-5, IL-10, IL-9, IFN-γ, IL-12p35, IL-12p40, IL-18/glycosylation-inhibiting factor, and macrophage migration-inhibiting factor (Figure 4, a–d) transcripts were not detected.

*Effect of endogenous adenosine on renal IRI in kidneys from C57BL/6,  $A_{2A}$ -KO, and chimeric mice.* Given the protective effect observed with  $A_{2A}$ R activation, we sought to determine whether  $A_{2A}$ -KO mice are more sensitive to the



effects of IRI than WT animals, as might be expected if endogenous adenosine exerts a protective action. We subjected kidneys of A<sub>2A</sub>-KO mice and of age- and sex-matched WT mice to IRI. IRI produced an increase in plasma creatinine levels in WT mice ( $1.36 \pm 0.18$  mg/dl) and a significantly greater increase in congenic A<sub>2A</sub>-KO mice ( $2.91 \pm 0.28$  mg/dl) ( $n = 5$ ,  $P < 0.002$ ). These data suggest that endogenous adenosine may protect kidneys. To determine the effect of endogenous adenosine on A<sub>2A</sub>Rs on BM-derived cells, we compared the effect of IRI on WT→WT and A<sub>2A</sub>-KO→WT chimera. Plasma creatinine levels after 32 minutes of ischemia and 24 hours of reperfusion were  $1.48 \pm 0.076$  ( $n = 7$ ) and  $1.78 \pm 0.78$  ( $n = 7$ ) in WT→WT and A<sub>2A</sub>-KO→WT mice, respectively ( $P < 0.02$ ). These data suggest that endogenous adenosine has a direct effect on BM-derived cells to reduce renal IRI injury.

## Discussion

The purpose of the current study was to determine the contribution of A<sub>2A</sub>Rs expressed on BM-derived cells to renal tissue protection from IRI. We found that when WT mice had their BM ablated and reconstituted with A<sub>2A</sub>-KO BM cells, the protective effect of ATL146e was completely abolished. This indicates that activation of A<sub>2A</sub>Rs expressed on BM-derived cells is necessary for ATL146e-mediated tissue protection. We also show that A<sub>2A</sub>R activation on BM-derived cells reduces IRI-induced elevation of IL-6, IL-1 $\beta$ , and IL-1ra and TGF- $\beta$  mRNAs in kidney. These cytokines may participate in renal damage in response to IRI. Lastly, endogenous adenosine, by acting on BM-derived A<sub>2A</sub>Rs, is probably a factor in limiting renal damage after IRI.

We have focused our efforts on defining the cellular target(s) through which A<sub>2A</sub>R activation affords protection from renal injury. In our previous studies in rats and C57BL/6 mice, we showed that infusion of ATL146e (a highly selective A<sub>2A</sub> agonist that was previously called DWH-146e) at doses below the threshold necessary to change blood pressure reduced elevated plasma creatinine seen after renal IRI (6, 8, 10). The mechanism by which A<sub>2A</sub> activation reduces IRI could involve effects on endothelial, epithelial, smooth muscle, or hematopoietic cells. Several groups of investigators have demonstrated that adenosine acts on activated neutrophils (16) *in vitro* to reduce production of oxygen radicals and neutrophil adherence to endothelial cells (17). These *in vitro* studies demonstrate that the renal protection observed *in vivo* may be due to direct effects of A<sub>2A</sub>R activation in suppressing adhesion and activation of neutrophils and release of toxic mediators from neutrophils. We demonstrated that neutrophil accumulation in the outer medulla of kidneys subjected to IRI is significantly reduced by ATL146e administration. The reduction of neutrophil accumulation may be related to a decrease in endothelial P-selectin and ICAM-1 expression (10) in mice given ATL146e, suggesting a direct or indirect effect on endothelial cells. Other groups have shown that the vasoconstrictive effects of angiotensin II in the outer medullary descending vasa recta are blocked by A<sub>2A</sub>R activation (9). Lastly, oxidant injury of immor-

talized human proximal tubule cells was reduced upon activation of A<sub>2A</sub>R with CGS21680 (18). These studies suggest multiple potential targets for adenosine A<sub>2A</sub> agonist action in ameliorating IRI.

To determine directly the contribution of A<sub>2A</sub>Rs on BM-derived cells, we generated A<sub>2A</sub>-KO→WT chimera. Our approach is based on the concept that repopulation of cells in recipient mice after BM ablation and transplantation with donor BM cells creates chimeras with reconstitution limited to BM-derived cells. Preliminary studies indicated that blood granulocytes were successfully reconstituted by approximately 98% and T cells by approximately 85%. These results are in keeping with previously published studies using this method (19).

The results provide direct evidence that the protective effect of ATL146e requires activation of A<sub>2A</sub>Rs expressed on BM-derived cells. Several important conclusions can be drawn from these results. Many studies of renal damage using IRI models in rats and mice (1), including our own (10), have suggested a pathogenic role from neutrophil infiltration of kidney tissue at various times after injury. Other studies either directly or indirectly implicate T cells or macrophages in IRI (4, 5, 20). In the current study, the finding that injury in A<sub>2A</sub>-KO→WT mice is unabated with an A<sub>2A</sub> agonist sheds additional light on the role of BM-derived cells in tissue injury. The possibility that leukocytes are important in IRI has been controversial (2). Our studies clearly suggest that BM-derived leukocytes are important in the pathogenesis of IRI and that suppressing circulating leukocyte function with ATL146e reduces injury.

Although our studies indicate that BM-derived cells are the target of ATL146e in mediating tissue protection, one possible explanation for the data is that donor-derived endothelial progenitor cells repopulate recipient endothelium. If most renal endothelial cells were replaced as a result of BM transplantation, the lack of effect of A<sub>2A</sub> agonists on renal IRI could be due to repopulation with endothelial cells bearing an A<sub>2A</sub>-KO phenotype. Reasoning that donor BM cells from GFP mice can be detected in recipient tissues, we made GFP→WT chimera to determine the extent to which endothelial progenitor cells repopulate recipient endothelium. Although the renal endothelium of GFP mice was brightly immunofluorescent, we observed only scattered cells of donor origin in GFP→WT chimeric mice subjected to IRI. The rare GFP-positive cells did not have endothelial morphology, and most were identified as macrophages. Although donor-derived circulating endothelial progenitors have been found in mice after bone marrow transplantation, the sites and magnitude of repopulation of these endothelial progenitors should be considered in the context of the present study. For example, Asahara et al. (21) have shown that tie2/lacZ transcript (donor origin) can be detected in various murine organs after BM transplantation, but this was detected by the extremely sensitive technique of RT-PCR and was not quantified. The most prominent evidence of donor endothelial cell repopulation has been found associated with vasculoge-



nesis or angiogenesis (21–23) after vascular injury (24). The outgrowth of neovessels is rare under normal physiological conditions. Significant neovascularization has been demonstrated in BM-transplanted mice only after various chronic injuries such as implantation of tumor cells (22), ocular neovascularization after surgery (23), murine ischemic hind limb (21, 23), and myocardial ischemia (25). Importantly, a detectable and very low level of donor BM cells in recipient endothelium is only reported in chronic neovascularization models (at least 1 week). Sata et al. performed BM transplantation in which irradiated WT mice received BM from ROSA26 mice, which express  $\beta$ -galactosidase (LacZ) (26). After BM transplantation, femoral artery of uninjured vessels did not express LacZ-positive cells. Wagers et al. demonstrated that BM transplantation using hematopoietic stem cells from GFP mice while reconstituting hematopoietic cells of recipient mice only rarely repopulated nonhematopoietic tissue. In the BM transplantation kidney ischemia-reperfusion model reported in this study, we clamped the renal artery for 32 minutes and sacrificed the mice after 24 hours of reperfusion. No angiogenesis occurs over this time frame. Our studies are consistent with the conclusion that endothelial repopulation by donor-derived progenitor cells occurs only to a very limited extent after IRI for 24 hours. Thus, it is likely that the absence of a protective effect of ATL146e in  $A_{2A}KO \rightarrow WT$  chimeras is due principally to the absence of  $A_{2A}$ -ARs on leukocytes.

Although we demonstrate that BM-derived cells are principal targets of action of ATL146e, the specific cell lineage is not certain. Because chimeric mice have hematopoietic cells from all cell lineages, tissue protection mediated by ATL146e may be due to effects on myeloid and/or lymphocytic lineages. Recent studies indicate that  $A_{2A}$ Rs are expressed on T cell subsets (27), and in light of the foregoing discussion, the effect of ATL146e could be mediated by action on T cells. Further support of the concept that T cell  $A_{2A}$ Rs could participate in tissue protection from IRI comes from *in vitro* studies.  $A_{2A}$  activation is also known to inhibit cytokines such as TNF- $\alpha$ , IL-6, IL-8, and IL-12 from mononuclear cells (28, 29). Identification of the specific cell lineage target of ATL146e will be the goal of future studies.

We observed that renal IRI in  $A_{2A}$ -KO mice was more severe than in control C57BL/6 mice as measured by plasma creatinine. A similar observation by Ohta and Sitkovsky showed that liver injury in response to toxins is more severe in mice that lack  $A_{2A}$ Rs (7). Furthermore, our data suggest that BM-derived cells are important for the effect of endogenous adenosine, because IRI in kidneys of  $A_{2A}$ -KO  $\rightarrow$  WT mice was more severe than in WT  $\rightarrow$  WT mice. These observations suggest that activation of  $A_{2A}$ Rs by endogenously produced adenosine may participate in a negative feedback mechanism to minimize tissue inflammation. The concept that endogenous adenosine contributes to tissue protection through activation of  $A_{2A}$ Rs is supported by our findings with the PDE 4 inhibitor rolipram. In the current studies, we observed that rolipram reduced renal IRI by 36%. The reduction of

injury is less than the 58% reduction observed when rolipram was administered to WT mice (8). This might occur because rolipram enhances the actions of endogenous adenosine in WT but not  $A_{2A}$ -KO mice.

The ability of endogenous adenosine to suppress tissue injury is consistent with *in vitro* and *in vivo* studies. It is well known that within 2 minutes of renal ischemia, interstitial adenosine concentration increases more than sixfold over the normal level of less than 1  $\mu$ M (30). Adenosine *per se* has suppressive effects on activated neutrophils or endothelial cells (17). It is likely that although activation of  $A_{2A}$ Rs by endogenous adenosine in WT mice mediates protection from injury, mechanisms that could contribute to the protective effects of endogenous adenosine and to the degree of injury associated with IRI may differ in WT as compared with  $A_{2A}$ -KO mice. The magnitude of the protective effect of endogenous adenosine is quite small as compared with the protection produced by ATL146e. Possible reasons for this are that (a) endogenous adenosine levels return to baseline within 10 minutes of reversal of ischemia due to washout (b) adenosine has a very short half-life in blood and is probably not accessible to receptors on circulating white blood cells (c) ATL146e has a higher affinity for  $A_{2A}$ Rs than adenosine, and (d) adenosine binds nonselectively to all four adenosine receptor subtypes, including  $A_1$ Rs and  $A_3$ Rs, which may promote inflammation. The net effect of increased adenosine levels will then depend on the balance of effects on multiple receptor subtypes and duration of action. In summary, adenosine can serve as a naturally occurring anti-inflammatory molecule and reduce tissue IRI, but this effect is probably limited primarily to the interstitial space of ischemic tissue.

We measured the effects of ATL146e on cytokine and chemokine mRNA expression in kidneys from chimeric mice. Our results demonstrate that expression of 11 of the 24 cytokines and chemokines measured in kidney is enhanced after IRI. The recruitment and activation of monocytes, macrophages, neutrophils, and T lymphocytes to the kidney that occurs after IRI (20) is probably due in part to the enhanced expression of IL-6, TGF- $\beta$ , MIP-2, MCP-1, IP-10, and RANTES. IL-6 is a major proinflammatory cytokine that interacts with TNF- $\alpha$  and IL-1 to stimulate dendritic cell migration. IL-6 is involved in Th1 lymphocyte differentiation and activation, production of IL-2 (31), a major clonal expansion cytokine, and induction of C-reactive protein and mannan-binding lectin from liver (32). C-reactive protein and mannan-binding lectin may bind to antigens released from damaged cells and then activate complement opsonization and phagocytosis. In this context, it is plausible that ATL146e exerts tissue protection partly by blocking IL-6 production at an early stage. TGF- $\beta$  is a dual immune modulator that may play a role in the inflammation process at an early stage and then act as an immune suppressor of activated T lymphocytes during the regeneration stage. TGF- $\beta$  produced by T lymphocytes is significant in immune suppression for its attenuation of the Th1 response. Recent findings also

indicate that TGF- $\beta$  may play a proinflammatory role by increasing chemotaxis of mononuclear cells, cytokine production (IL-1 $\alpha$ , IL-1 $\beta$ , TNF- $\alpha$ , PDGF-BB, bFGF), expression of integrin receptors (LFA-1, ICAM-1, VLA-3), and activity of phagocytes (33).

Our data show that activation of A<sub>2A</sub>Rs decreases the expression of TGF- $\beta$ , IL-1 $\beta$ , IL-1 $\alpha$ , and IL-6 transcripts in the kidney in WT $\rightarrow$ WT mice but not in A<sub>2A</sub>-KO $\rightarrow$ WT kidney. Because early effects of IL-1 $\beta$ , TGF- $\beta$ , and IL-6 may promote inflammation, their early appearance may contribute to damage observed in IRI.

Taken together, this study using genetically engineered and chimeric mice sheds light on the sequence of events that culminate in renal IRI and the mechanism by which A<sub>2A</sub>R activation can ameliorate this injury. First, the results suggest a causal link between BM-derived cells and the component of the injury response that occurs during the reperfusion period after renal ischemia. Second, tissue-protective effects of A<sub>2A</sub>R agonists require activation of receptors expressed on BM-derived cells. These observations are particularly interesting since A<sub>2A</sub>Rs are not limited in their distribution to BM-derived cells but are also found on several other tissues, notably on vascular endothelial cells. Although these data demonstrate that A<sub>2A</sub>Rs on BM-derived cells are necessary for A<sub>2A</sub> agonist-induced protection from renal injury, they do not exclude a minor contribution from nonhematopoietic cells expressing A<sub>2A</sub>Rs. For example, it is possible that A<sub>2A</sub>R activation on both BM-derived cells and on cells in the kidney is required to elicit significant renal protection. A third conclusion of this study is that endogenous adenosine modulates tissue injury by activating A<sub>2A</sub>Rs.

The participation of BM-derived cells in adenosine-mediated renal protection is consistent with in vitro studies demonstrating that A<sub>2A</sub> agonists such as ATL146e potently inhibit leukocyte adhesion, oxidative burst, and lymphocyte activation. It will be interesting in future studies to determine if the target of A<sub>2A</sub> agonists that protect the kidney is primarily on T cells or cells of myeloid lineage.

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