Renal ischemia-reperfusion injury and adenosine 2A receptor-mediated tissue protection: role of macrophages

Day, Yuan-Ji, Liping Huang, Hong Ye, Joel Linden, and Mark D. Okusa. Renal ischemia-reperfusion injury and adenosine 2A receptor-mediated tissue protection: role of macrophages. Am J Physiol Renal Physiol 288: F722–F731, 2005. First published November 23, 2004; doi:10.1152/ajprenal.00378.2004.—The role of monocytes/macrophages in the pathogenesis of ischemia-reperfusion injury (IRI) is unknown. We sought to determine whether activation of macrophage adenosine 2A (A2A) receptors (A2ARs) mediates tissue protection. We subjected C57Bl/6 mice infused with clodronate [dichloromethylene bisphosphonate (Cl2MBP)] to IRI (32 min of ischemia followed by 24 h of reperfusion) to deplete them of macrophages. IRI induced an elevation of plasma creatinine that was reduced with administration to animals subjected to renal IRI. Toward this end, we depleted mice of macrophages and examined the effect of A2AR agonist effect on IRI was blocked in macrophage-depleted A2A-knockout mice reconstituted with wild-type RAW 264.7 cells RNase protection assays 24 h after IRI demonstrated that macrophages are required for IL-6 and TGF-β mRNA induction. However, A2AR agonist-mediated tissue protection is independent of IL-6 and TGF-β mRNA. We conclude that the full extent of IRI requires macrophages and that A2AR agonist-mediated tissue protection is independent of activation of macrophage A2ARs.

THE CASCADE OF EVENTS that ensues after ischemia-reperfusion (IR) involves endothelial cell dysfunction (35) and activation of bone marrow-derived cells that contribute to the downstream effects of an active inflammatory process, leading to tissue injury (3, 38). This process consists of the coordinated release of cytokines/chemokines by specific hematopoietic cells that contribute to medullary vascular congestion or cytotoxic injury to epithelial cells. Previous studies indicate that neutrophils play an important role in IR injury (IRI) (16, 28, 33); however, not all studies support their involvement (25, 39). More recently, a number of different studies directly or indirectly support the role of T cells in IRI (15, 18, 20) or demonstrate a causal relation between T cells and IRI (4).

The role of macrophages in IRI is unknown. The classical paradigm suggests that macrophages produce a destructive proinflammatory response, whereas more recent studies suggest that macrophages are responsible for restoration of normal function (for review see Ref. 17). In IRI, correlative evidence supports the pathogenic role of macrophages (10). Kidney IRI is associated with increased infiltration of monocytes/macrophages (5, 37) and an increase in monocyte chemotactic protein (MCP-1) (11, 34). Furthermore, in mice lacking CCR2, the receptor for MCP-1 expressed on macrophages, the magnitude of IRI is reduced (11). Although these results suggest that macrophages contribute to the proinflammatory response in mediating kidney IRI, further studies are warranted to determine a direct causal relation between macrophages and the destructive process of early IRI.

Adenosine 2A (A2A) receptor (A2AR) agonists are potent inhibitors of inflammation and have been shown to decrease IRI by ~70% when administered before or after ischemic insult (22–24), primarily through the activation of A2ARs expressed on bone marrow-derived cells (9). Because A2ARs are expressed on macrophages (12), their activation could be responsible for the protection observed after A2AR agonist administration to animals subjected to renal IRI.

The purpose of the present study was twofold: 1) to determine the contribution of macrophages to the early phase of renal IRI and 2) to determine the protective role of A2ARs expressed on macrophages in renal IRI. Toward this end, we depleted mice of macrophages and examined the effect of A2AR agonist effect on IRI was blocked in macrophage-depleted A2A-knockout mice reconstituted with wild-type RAW 264.7 cells RNase protection assays 24 h after IRI demonstrated that macrophages are required for IL-6 and TGF-β mRNA induction. However, A2AR agonist-mediated tissue protection is independent of IL-6 and TGF-β mRNA. We conclude that the full extent of IRI requires macrophages and that A2AR agonist-mediated tissue protection is independent of activation of macrophage A2ARs.

METHODS

A2A agonists. Experiments were performed using A2A agonists, ATL-146e and ATL-303. ATL-146e has been previously characterized (30). To determine the relative activity of ATL-303, competitive binding assays were performed in HEK-293 cells transfected with A2ARs (31). Membranes were incubated with [125I]2-[2-(4-aminomethylphenyl)ethylamino]adenosine ([125I]APE, 0.3 nM), a selective A2A agonist radioligand, in the presence of competing concentrations (0.001 nM–100 μM) of ATL-303, ATL-146e, or CGS-21680 [2-(p-carboxyethyl)phenethylamino-5′-ethylcarboxamidoadenosine HCl; Research Biochemical, Natick, MA] for 2 h at 21°C. Four milliliters of ice-cold wash buffer (25 mM Tris-HCl, pH 7.2) were added to terminate the reaction, and the membranes were filtered through Whatman GF/C filters. Filters were washed twice with 4 ml of wash buffer. Bound radioactivity was determined by liquid gamma counting. Data were analyzed by nonlinear regression (GraphPAD Prism version 4.0, Graph PAD Programs, San Diego, CA). IC50 values were
Health procedures were performed in adherence to the National Institutes of Health and by the University of Virginia Animal Care and Use Committee. C57Bl/6 mice (7–8 wk of age, Charles River Laboratories, Wilmington, MA) and A2AKO mice (6–7 wk of age) (7, 9) were allowed free access to food and water until the day of surgery. Mice were anesthetized with ketamine (100 mg/kg ip), xylazine (10 mg/kg), and acepromazine (1 mg/kg im) and subjected to bilateral flank incisions as previously described (9). Both renal pedicles were cross-clamped for 32 min. Surgical wounds were closed with metal staples, and the mice were returned to cages for 24 h. After 24 h of reperfusion, animals were reanesthetized, blood was obtained by cardiac puncture, and kidneys were removed for various analyses.

Tissue culture. RAW 264.7 cells, a mouse monocyte/macrophage cell line established from ascites of a tumor induced in a female mouse by intraperitoneal injection of Absolon leukemia virus (A-MuLV; American Type Culture Collection, Manassas, VA), were cultured in high-glucose DMEM supplemented with sodium bicarbonate (0.075% wt/vol), l-glutamine, penicillin-streptomycin (1%), insulin (0.5 mg/ml), and 10% FBS in a humidified atmosphere of 5% CO₂ at 37°C. Cells were grown on tissue culture plates, and medium was replaced every 3–4 days.

Preparation of liposomes. Clostridium [dichloromethylene bisphosphonate (Cl₂MBP)] liposomes [lipo-Cl₂MBP] were prepared according to the method of van Rooijen et al. (40). Phosphatidylcholine [86 mg; egg lecithin (20 mg/ml) in chloroform] and cholesterol (8 mg; 8 mg/10 ml chloroform) were evaporated by rotation under reduced pressure (vacuum pump; Rotationsseptorator R-114, Buchi Labortechnik, Flavil, Switzerland). A suspension of 2.5 g of Cl₂MBP and 10 ml of PBS was stored under N₂ for 2 h, sonicated, and stored in PBS in a humidified atmosphere of 5% CO₂ at 37°C. Cells were grown on tissue culture plates, and medium was replaced every 3–4 days.

In vivo depletion of monocyte/macrophages and adoptive transfer of RAW 264.7 cells. C57Bl/6 mice were anesthetized with ketamine (50 mg/kg ip), xylazine (5 mg/kg), and acepromazine (0.5 mg/kg im) before administration of lipo-Cl₂MBP or RAW 264.7 cells. Cl₂MBP (100 µl/10 g mouse) was injected via a internal jugular vein, and a second dose (100 µl/10 g mouse) was injected 48 h later; the control group was subjected to the same protocol, except liposomes containing PBS (lipo-PBS) were injected. At 96 h after the first dose, PBS, murine RAW 264.7 macrophages (10⁶ cells), or siRNA-treated RAW 264.7 cells were injected intravenously, and the animals were subjected to IRI 5 h later. The experimental groups are summarized in Table 1.

Table 1. Experimental groups

<table>
<thead>
<tr>
<th>Adoptive Transfer</th>
<th>Recipient Mouse</th>
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<tr>
<td>MØ Depl WT</td>
<td>C57Bl/6</td>
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<tr>
<td>WT RAW → MØ</td>
<td>MØ depleted C57Bl/6</td>
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<tr>
<td>1) Control</td>
<td>C57Bl/6</td>
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<tr>
<td>2) MØ Depl WT</td>
<td>MØ depleted C57Bl/6</td>
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<td>3) WT RAW → MØ</td>
<td>WT RAW 264.7</td>
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<tr>
<td>Depl WT</td>
<td>MØ depleted C57Bl/6</td>
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<tr>
<td>4) A2AR KO RAW → MØ</td>
<td>A2AR siRNA RAW 264.7</td>
</tr>
<tr>
<td>Depl WT</td>
<td>MØ depleted C57Bl/6</td>
</tr>
<tr>
<td>5) WT RAW → MØ</td>
<td>WT RAW 264.7</td>
</tr>
<tr>
<td>A2AR KO</td>
<td>MØ depleted A2AR KO</td>
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MØ Depl WT, C57Bl/6 mice treated with dichloromethylene bisphosphonate (Cl₂MBP); WT RAW, RAW 264.7 cells; A2AR KO RAW, RAW 264.7 cells transfected with small interfering RNA (siRNA) adenosine 2A receptor (A2AR); control RAW, RAW 264.7 cells transfected with control plasmid siRNA; MØ Depl A2AR KO, A2AR-knockout (A2AR KO) mice treated with Cl₂MBP; Depl, depleted; MØ, macrophage; WT, wild-type.

Plasma creatinine and leukocyte counts. Plasma creatinine concentrations were determined using a colorimetric assay according to the manufacturer’s protocol (Sigma Chemical, St. Louis, MO). Anticoagulated blood was analyzed for leukocyte counts (HEMAMET 850, CDC Technologies, Oxford, CT).

siRNA. Recombinant pSilencer (Ambion, Austin, TX) expressing A2AR-siRNA was constructed as follows. A 21-nt targeting sequence beginning with an AA dinucleotide, AACACTTCTCGG-TATCTCT, was designed from A2AR mRNA by Oligo 6 and BLAST in G Tap. The hairpin siRNA template oligonucleotide was designed according to the manufacturer (Ambion): the sense strand sequence was 5'-GATC CCAC TCTT CGT GGT A TCT TTT CGA GAG AGT ACACA CGGA GAAG TTGG TTGG GAA A3'-3'. The sense and antisense strand sequences were annealed to form a hairpin siRNA template insert and ligated into pSilencer 2.1-U6 neo and ligated into pSilencer 2.1-U6 neo using T4 DNA ligase. The pSilencer was amplified by transformation into competent Escherichia coli DH5α cells and confirmed by agarose gel electrophoresis. The plasmid was digested with BamHI and HindIII. Three of the clones containing the 66-bp insert were sequenced to further confirm the recombinant pSilencer.

RAW 264.7 cells were plated into six-well plates (1 × 10⁵ cells/well) 24 h before transfection. For each well, 20 µl of siPORT Amine (Ambion) were diluted to 394 µl with OPTI-MEM I and incubated at room temperature for 30 min. siRNA (0.2 nmol) was added to diluted siPORT Amine transfection solution and incubated at room temperature for 20 min. The volume of the transfection solution was diluted to 2 ml with RPMI-10% FBS and added to the cells. Cells were cultured for 24 h with an additional 1 ml of RPMI-10% FBS to optimize cell growth. At 48 h after transfection, the cells were washed with PBS and incubated with RPMI 1640 containing G418 at 500 mg/ml for the first 24 h, 250 mg/ml for another 24 h, and 200 mg/ml for the rest of the week. The cells were replated to flasks and ready for the functional and reconstitution studies.

A2AR functional assay. RAW 264.7 cells were cultured in 96-well plates (5 × 10⁴/well) and the degree of A2AR modulation of TFN-α release from activated cells was measured by ELISA. RAW 264.7 cells were incubated overnight with IFN-γ (1 nM), and on the following day, H₂O₂ (5 mM) was added for 30 min with or without ATL-303 (10 nM), a selective A₂A agonist. To determine specificity, some cells were incubated with ATL-303 + ZM-243185 (50 nM), a selective A₂A antagonist (27). After 30 min of incubation, supernatants were subjected to ELISA for measurement of TFN-α concentration according to the manufacturer’s protocol (eBioscience, San Diego, CA). A similar protocol was followed for wild-type (WT) RAW 264.7 cells that were subjected to mock transfection and for WT RAW 264.7 cells that were transfected with recombinant pSilencer plasmid. Normalized data are presented to allow for comparisons of experiments on different days.

Histochemistry and immunohistochemistry. Kidneys and spleens were fixed in peridate-lysine-paraffin-embedded (4% paraformaldehyde) and embedded in paraffin, and 4-µm sections were cut. Sections were subjected to routine staining with hematoxylin and eosin and viewed by light microscopy (Zeiss AxioskOp).

For immunohistochemical studies, sections were subjected to antigen retrieval according to the manufacturer’s protocol (Vector Laboratories, Burlingame, CA); then sections were incubated with a well-characterized rat anti-mouse monoclonal antibody to murine macrophages (F4/80, Serotec, Raleigh, NC; 1:1,000 dilution) and then with a biotinylated goat anti-rat secondary antibody. The peroxidase reaction was performed according to the manufacturer’s protocol (Vectastain ABC Elite kit). Tissue sections were covered with an aqueous-based mounting solution consisting of p-phenylenediamine (1 mg/ml) and 70% glycerol, and coverslips were applied and affixed with nail polish. Sections were viewed under a Zeiss AxioskOp fluorescence microscope. Photographs were taken with a SPOT...
RT camera (software version 3.3, Diagnostic Instruments, Sterling Heights, MI). Images were imported into Adobe Photoshop (version 3.0) for brightness/contrast adjustment.

We quantified macrophages in the cortex and outer medulla 96 h after injection of liposomes containing Cl₂MBP or PBS. Kidney regions were viewed at ×200 magnification, and discrete immunoreactive cells were counted and averaged from 8–10 fields per mouse kidney.

**Multiprobe RNase protection assays.** Total kidney RNA was extracted from homogenized tissue with RNAzol B (Leedo Medical Laboratories, Houston, TX) 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. Briefly, mRNA-specific RNA probes were labeled with [32P]UTP using multiprobe template sets (mCK3b, BD PharMingen) for cytokine 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 −80°C with a single intensifying screen.

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

**RESULTS**

**Selective activation of A₂ₐRs by ATL-303 in transfected HEK-293 cells.** We examined the subtype selectivity and potency of a new A₂ₐ agonist, ATL-303, compared with our prototype A₂ₐ agonist, ATL-146e (30), and CGS-21680. Figure 1 shows the results of competitive binding experiments in membranes derived from HEK-293 cells transfected with human A₂ₐRs. Kᵢ values for ATL-303 and ATL-146e were similar (0.3 ± 0.07 and 0.6 ± 0.04 nM, respectively, n = 3) and significantly different from Kᵢ values for CGS-21680 (4.9 ± 0.30 nM, n = 3, P < 0.0001; Fig. 1). These results indicate that ATL-303 and ATL-146e bind with equal potency to A₂ₐRs in a dose-dependent manner, and ATL-303 has a 50-fold higher affinity for A₂ₐRs than CGS-21680. We used ATL-146e for in vivo experiments and ATL-303 for in vitro studies.

**Lipo-Cl₂MBP reduces tissue macrophage infiltration.** We administered liposomes containing PBS (lipo-PBS, group 1; Fig. 2, Aa and Ab) or Cl₂MBP (lipo-Cl₂MBP, group 2; Fig. 2, Ac and Ad) to mice and harvested the spleen and kidneys at 48 and 96 h to quantitatively examine the degree of macrophage depletion as determined by immunohistochemical localization of macrophages. At 96 h after lipo-PBS administration, macrophages are seen in the red pulp region of the spleen, a characteristic feature of the normal spleen (Fig. 2Aa), as well as in the kidney cortex (not shown) and outer medulla (Fig. 2Ab). In contrast, at 96 h after lipo-Cl₂MBP administration, marked macrophage depletion is observed in the spleen (Fig. 2Ac) and cortex (not shown) and in the outer medulla (Fig. 2Ad). At 48 h, there was an intermediate degree of macrophage infiltration with less well-defined cell margins that represented degenerating macrophages (data not shown). In the renal cortex and outer medulla, lipo-Cl₂MBP significantly reduced macrophage infiltration. Macrophage density (cell number/200 × field) was 16.9 ± 2.4 and 5.0 ± 1.4 in the cortex (n = 4, P < 0.01) and 211.8 ± 14.9 and 86.5 ± 9.6 in the outer medulla (n = 5, P < 0.0001) for lipo-PBS (group 1) and lipo-Cl₂MBP (group 2), respectively (Fig. 2B).

**Effect of macrophages and A₂ₐR activation on renal IRI.** We next sought to determine the contribution of macrophages to IRI and the contribution of activated A₂ₐRs expressed in macrophages to the protection observed with A₂ₐ agonists. Mice were injected with lipo-Cl₂MBP to deplete macrophages, and the kidneys were subjected to 32 min of ischemia followed by 24 h of reperfusion. First, we characterized the effect of lipo-Cl₂MBP on peripheral leukocyte counts, inasmuch as lymphocyte depletion and neutrophil depletion have been demonstrated to reduce renal tissue injury after IRI. Lipo-PBS and lipo-Cl₂MBP had no significant effect on total peripheral white blood cell or leukocyte subset counts in mice subjected to IRI (Table 2).

Control mice treated with lipo-PBS (group 1) and subjected to renal IR showed a rise in plasma creatinine (1.34 ± 0.07 mg/dl, n = 10) that was reduced with ATL-146e (0.32 ± 0.25 mg/dl, n = 3, P < 0.001; Fig. 3). Treatment with lipo-Cl₂MBP before IRI (group 2) reduced plasma creatinine to 27% of control lipo-PBS levels (0.36 ± 0.07 mg/dl, n = 11, P < 0.001). In mice treated with lipo-Cl₂MBP and ATL-146e, plasma creatinine was 29% of control (0.39 ± 0.06 mg/dl, n = 7, P < 0.001). These results suggest that macrophages contribute to IRI and that ATL-146e had no further protective effect after macrophage depletion. To exclude the possibility that the reduction of injury after macrophage depletion with lipo-Cl₂MBP was due to the nonspecific effects of lipo-Cl₂MBP, we adoptively transferred macrophages into mice previously depleted of macrophages. To infuse sufficient quantities of macrophages, we used a murine macrophage cell line, RAW 264.7 cells. Compared with mice depleted of macrophages (lipo-Cl₂MBP, group 2), adoptive transfer of WT RAW 264.7 cells into mice depleted of macrophages (lipo-Cl₂MBP + WT RAW 264.7, group 3) reconstituted injury as plasma creatinine rose to 1.10 ± 0.08 mg/dl (n = 8, P < 0.05), and ATL-146e led to a reduction of plasma creatinine (0.30 ± 0.03 mg/dl, n = 8, P < 0.001) in these mice.

To determine the effect of macrophages and A₂ₐ agonists on tissue histology in kidneys subjected to IRI, we stained kidney sections from groups 1–3. Renal IRI in control mice treated
with vehicle (lipo-PBS, group 1) led to marked tubule necrosis in the outer medulla with obstructing tubule lumen and loss of epithelial cells in the S3 segment (Fig. 4A); ATL-146e led to preservation of the tubule epithelium (Fig. 4B). These results are similar to those previously reported (24). Kidneys from mice depleted of macrophages (lipo-Cl\textsubscript{2}MBP, group 2) and subjected to IRI demonstrated relatively well-preserved tubule epithelium of the S3 segment in the absence (Fig. 4C) or presence of ATL-146e (Fig. 4D). Adapative transfer of WT RAW 264.7 cells into mice depleted of macrophages (WT RAW 264.7 → M\textsubscript{0} DEPL WT, group 3) reconstituted injury (Fig. 4E), and the effect was reversed with ATL-146e (Fig. 4F).

Protective effect of A\textsubscript{2A}R activation on renal IRI is independent of A\textsubscript{2A}Rs expressed on macrophages. Next, we sought to establish the contribution of A\textsubscript{2A}Rs expressed in macrophages to the protective effects of A\textsubscript{2A} agonists on IRI by silencing the A\textsubscript{2A}R gene in RAW 264.7 (A\textsubscript{2A}KO RAW 264.7) cells using siRNA and adaptively transferred the modified cells into mice previously depleted of macrophages (A\textsubscript{2A}KO RAW → M\textsubscript{0} DEPL WT, group 4). To verify that we successfully silenced the A\textsubscript{2A}R gene in macrophages, we used a functional assay for A\textsubscript{2A}Rs and measured the effect of an A\textsubscript{2A} agonist on TNF-α release from activated macrophages. Typically, A\textsubscript{2A} agonists block TNF-α release from activated macrophages (13), an effect reduced in A\textsubscript{2A} siRNA-transfected RAW 264.7 cells.
TNF-α release (expressed as the fraction of control) after A2A agonist treatment of A2A siRNA-transfected, control siRNA-transfected, and untransfected RAW cells was 0.72 ± 0.010 (n = 4), 0.11 ± 0.06 (n = 3, P < 0.01 vs. A2A KO RAW 264.7), and 0.21 ± 0.09 (n = 4, P < 0.01 vs. A2A KO RAW 264.7; Fig. 5), respectively. These results indicate effective A2A gene silencing in RAW 264.7 cells.

WT C57Bl/6 mice were depleted of macrophages (lipo-C3MBP) and reconstituted with RAW 264.7 cells transfected with siRNAs to silence the A2AR gene (group 4). Plasma creatinine in WT mice reconstituted with A2A siRNA-transfected, control siRNA-transfected, and untransfected RAW cells was 0.72 ± 1.23 mg/dl for vehicle and ATL-146e, respectively (Fig. 6A). Plasma creatinine for vehicle and ATL-146e was 1.46 ± 0.12 and 0.30 ± 0.014 mg/dl (n = 5, P < 0.0001), respectively. Corresponding histology demonstrated marked tissue necrosis of the outer medulla of kidneys from macrophage-depleted WT mice subjected to adoptive transfer of A2A KO RAW 264.7 cells (A2AKO RAW → MØ DEPL WT, group 4) and then subjected to renal IR (Fig. 6B), an effect that was markedly reduced with ATL-146e (Fig. 6C). These results indicate that the effect of A2A agonists in reducing IRI is independent of macrophage A2ARs.

To confirm this finding, we depleted macrophages from A2AKO mice and adoptively transferred WT RAW 264.7 cells (WT RAW → MØ DEPL A2AKO, group 5). In this manner, only the RAW 264.7 cells express A2ARs. In these mice, renal IR led to an increase in plasma creatinine after vehicle infusion, and ATL-146e had no protective effect (96% of control; Fig. 7A). Plasma creatinine was 1.40 ± 0.10 and 1.35 ± 0.07 mg/dl for vehicle and ATL-146e, respectively (P = not significant, n = 6). Corresponding histology demonstrated marked tissue injury of the outer medulla of vehicle-treated (Fig. 7B) and ATL-146e-treated (Fig. 7C) mice. These results further substantiate the previous findings and indicate that the protective effect of A2A agonists in IRI is due to action on bone marrow-derived cells other than macrophages.

**Effect of macrophages and macrophage A2AR on cytokine mRNA expression in IRI.** We next examined the contribution of macrophages and macrophage A2AR activation to IRI-induced cytokine mRNA expression. As we showed previously (9), cytokine (including IL-6 and TGF-β) mRNA expression increases after 32 min of ischemia followed by 24 h of reperfusion (Fig. 8). Macrophage depletion led to a marked reduction of IL-6 and TGF-β mRNA expression. The increase in expression of these cytokine mRNAs requires macrophages, as much as both transcripts are reduced after depletion and reconstituted after adoptive transfer of macrophages. Additionally, despite the tissue-protective effect of ATL-146e in A2AKO RAW 264.7 → MØ DEPL WT mice, IL-6 and TGF-β transcripts were not reduced. These results suggest that IL-6 and TGF-β mRNA expression requires macrophages but does not mediate the tissue-protective effect of A2A agonists.

**DISCUSSION**

The present study was performed to determine the role of macrophages in IRI and the role of macrophage A2AR activation in mediating renal tissue protection after IRI. Our results provide direct evidence that macrophages play an important role in IRI in the kidney. Furthermore, despite the essential role that macrophages play in IRI, they do not mediate the tissue-protective effect of A2A agonists. Given our previous findings that A2ARs on bone marrow-derived cells are a critical target for A2A agonists in mediating tissue protection, our present studies suggest that A2A agonists target bone marrow-derived cells other than macrophages. Recent studies implicate T cells in the pathogenesis of IRI and lead us to conclude that T cells...
may likely be the target of $A_{2A}$ agonist-mediated tissue protection.

Role of macrophages in IRI. Previous studies have implicated macrophages as participants in IRI; however, a causal relation between macrophages and IRI has yet to be reported (10). A number of studies have demonstrated that monocytes/macrophages (26, 37, 42), as well as macrophage-associated cytokines, such as IL-1, IL-6, and TGF-$\beta$ (37), and monocyte/macrophage chemoattractants, such as IFN-$\gamma$-inducible protein-10, MCP-1, and macrophage inflammatory protein-2 (9, 19, 36), appear in the kidney within 2–5 days of IRI. The late appearance suggests the possibility that macrophages participate in the repair process after IRI (10). Other animal models provide some insight into the potential role of macrophages. In experimental uveitis, liposomal dichloromethylene-diphosphonate depleted macrophages and prevented leukocyte-endothelial cell interaction, thus providing evidence for the central role of macrophages in this form of inflammation (2). In arterial injury, macrophage depletion using liposomal clodronate reduced inflammation involved in neointimal hyperplasia (8). Taken together, these studies provide strong evidence for the role of macrophages in inflammation and renal IRI.
In a similar manner, we used macrophage depletion to determine the role of macrophages in renal IRI. We chose the non-nitrogen-containing bisphosphonate (Cl2MBP) to deplete macrophages from mice. This method has been used previously and has been found to effectively reduce macrophage lineage without affecting peripheral blood leukocytes (1, 32). In our studies, we found that lipo-Cl2MBP produced a significant reduction in IRI, a marked reduction of macrophages in

![Fig. 6. A2A agonists reduce renal IRI in the absence of macrophage A2ARs. WT C57Bl/6 mice were injected with lipo-Cl2MBP to deplete mice of macrophages, reconstituted with RAW 264.7 cells with A2A gene “silenced” (A2A KO RAW 264.7 cells, group 4), and subjected to 32 min of ischemia followed by 24 h of reperfusion. A: plasma creatinine from mice treated with vehicle (open bar) or ATL-146e (solid bar). Values are means ± SE; n = 6. B and C: representative hematoxylin-eosin-stained sections of outer medulla from mice treated with vehicle and ATL-146e, respectively. Magnification, ×200.](image)

![Fig. 7. Nonmacrophage A2ARs mediate renal tissue protection from ischemia-reperfusion injury. A2A-R-knockout (A2A KO) mice were injected with lipo-Cl2MBP to deplete macrophages, reconstituted with WT RAW 264.7 cells (group 5), and subjected to 32 min of ischemia followed by 24 h of reperfusion. A: plasma creatinine from mice treated with vehicle (open bar) or ATL-146e (solid bar). Values are means ± SE; n = 6. B and C: representative hematoxylin-eosin-stained sections of outer medulla from mice treated with vehicle and ATL-146e, respectively. Magnification, ×200.](image)
spleen and kidney, and no change in peripheral blood leukocytes. In our studies, the peripheral monocyte count was slightly reduced with lipo-Cl2MBP (0.26 ± 0.04 counts/µl), but this difference did not reach statistical significance. The absence of a difference in monocyte count may reflect a difference in phagocytosis of lipo-Cl2MBP by monocytes and tissue resident macrophages. To exclude other nonspecific effects of our experimental protocol, adoptive transfer of macrophages into macrophage-depleted mice markedly reversed the protective effect of macrophage depletion. Additional information can be obtained from RNase protection assays, which demonstrated a reduction of cytotoxic cytokines from kidneys of mice subjected to macrophage depletion. Thus these studies provide evidence for a strong causal relation between macrophages and early proinflammatory-mediated renal tissue injury. Although macrophages may participate in the repair process, our studies directly implicate them in the genesis of renal tissue injury after IR.

Integrated action of macrophages and bone marrow-derived cells in mediating tissue injury. Several mechanisms may be involved in the protection from IRI produced by macrophage depletion. Our in vitro studies demonstrate that TNF-α is secreted by macrophages robustly on activation. Marked attenuation of macrophage infiltration will reduce TNF-α release locally and its direct cytotoxic effects on renal epithelial cells. In experimental uveitis, macrophage depletion with Cl2MBP reduced leukocyte rolling, arrest, and emigration, as well as plasma TNF-α levels (2). Activation of macrophages requires exposure to IFN-γ (17). Because CD4+ T cells are the primary hematopoietic cells that secrete IFN-γ, a reduction or attenuation of IFN-γ suggests that macrophages contribute to IRI through activation of CD4+ cells with subsequent release of IFN-γ.

T cells are believed to contribute to IRI (4). In studies using adoptive transfer of T cells into T cell-deficient mice, CD4+ cells were found to be key mediators of the neutrophil inflammatory response (4). In a warm IR model in which specific markers for inflammatory cells were used, ED1 cells (macrophages), CD4+ T cells, and CD8+ T cells have been identified in renal tissue (36). The T cell-derived cytokines, IFN-γ, IL-4, and IL-2, appear temporally with mononuclear cell infiltration (36). IR in CD4−/CD8− double-KO mice was associated with a reduction of neutrophil infiltration and injury (4). Additional evidence that suggests a role of T cells in renal IRI is derived from studies where blockade of the CD28-B7 costimulatory pathway reduces injury (36). Thus T cells may mediate tissue injury through coordinated actions with T cells, macrophages, and other bone marrow-derived cells. The coordinated action of T cells, neutrophils, and macrophages may be necessary to induce full expression of tissue injury after renal IRI.

Role of macrophage A2ARs in mediating A2A agonist-mediated tissue protection. A second goal of this study was to determine whether macrophages are the target of A2A agonist-mediated tissue protection. Our previous studies demonstrated that activation of A2ARs expressed on bone marrow-derived cells mediated renal tissue protection after IRI (9). In those studies, we lethally irradiated mice and repopulated the bone marrow of WT mice with marrow of A2AKO mice. In these chimeras, ATL-146e, an A2A agonist, lost its protective effect. In contrast, when marrow of WT mice was replaced with WT bone marrow, the protective effect was restored. These studies point to bone marrow-derived cells as the primary target of A2AR action in mediating IRI. The present study extended these findings by examining whether A2A agonists mediate renal tissue protection via macrophage A2ARs. In macrophage-depleted mice, administration of A2A agonists did not provide an additional protective effect. In macrophage-depleted mice, A2A agonists provided tissue protection after reconstitution with WT macrophages or macrophages in which A2AR expression was attenuated. Finally, the absence of an A2A agonist-mediated protective effect in A2AKO mice was not restored by reconstitution with WT macrophages.

IL-6 and TGF-β are dependent on the presence of activated macrophages; however, their role in renal IRI is uncertain. Our data demonstrate that the protective effect of A2A agonists is not dependent on these cytokines. Proinflammatory, anti-inflammatory, and reparative processes have been described for IL-6 (14, 29, 41). TGF-β has been implicated in inducing IRI (21) or reducing IRI in myocardial tissue (6). These results underscore the complex relation between these cytokines and injury.

Together, these studies provide direct insight into the pathogenic mechanisms of macrophages in IRI and the mechanism of tissue protection mediated by A2A agonists. Our studies demonstrate that macrophages play a critical role in mediating the full extent of IRI. Furthermore, these studies provide strong evidence that A2A agonist action in reducing IRI is independent of macrophages. Given previous studies that CD4+ cells con-
tribute importantly to IRI, it is likely that a coordinated action is necessary between bone marrow-derived cells to mediate IRI. Future studies will focus on the role of CD4+ cells in mediating the tissue-protective effect of A2A agonists in IRI. We believe that, in addition to the therapeutic benefits that A2A agonists may have in future clinical trials, A2A agonists offer an incisive pharmacological tool to investigate the mechanism of IRI.

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