Adenosine A_{2A} receptor activation on CD4⁺ T lymphocytes and neutrophils attenuates lung ischemia–reperfusion injury

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Objective: Adenosine A_{2A} receptor activation potently attenuates lung ischemia–reperfusion injury. This study tests the hypothesis that adenosine A_{2A} receptor activation attenuates ischemia–reperfusion injury by inhibiting CD4⁺ T cell activation and subsequent neutrophil infiltration.

Methods: An in vivo model of lung ischemia–reperfusion injury was used. C57BL/6 mice were assigned to either sham group (left thoracotomy) or 7 study groups that underwent ischemia–reperfusion (1 hour of left hilar occlusion plus 2 hours of reperfusion). ATL313, a selective adenosine A_{2A} receptor agonist, was administered 5 minutes before reperfusion with or without antibody depletion of neutrophils or CD4⁺ T cells. After reperfusion, the following was measured: pulmonary function using an isolated, buffer-perfused lung system, T cell infiltration by immunohistochemistry, myeloperoxidase and proinflammatory cytokine/chemokine levels in bronchoalveolar lavage fluid, lung wet/dry weight, and microvascular permeability.

Results: ATL313 significantly improved pulmonary function and reduced edema and microvascular permeability after ischemia–reperfusion compared with control. Immunohistochemistry and myeloperoxidase content demonstrated significantly reduced infiltration of neutrophils and CD4⁺ T cells after ischemia–reperfusion in ATL313-treated mice. Although CD4⁺ T cell–depleted and neutrophil-depleted mice displayed significantly reduced lung injury, no additional protection occurred when ATL313 was administered to these mice. Expression of tumor necrosis factor- α , interleukin 17, KC, monocyte chemoattractant protein-1, macrophage inflammatory protein-1, and RANTES were significantly reduced in neutrophil- and CD4⁺ T cell–depleted mice and reduced further by ATL313 only in neutrophil-depleted mice.

Conclusions: These results demonstrate that CD4⁺ T cells play a key role in mediating lung inflammation after ischemia–reperfusion. ATL313 likely exerts its protective effect largely through activation of adenosine A_{2A} receptors on CD4⁺ T cells and neutrophils. (*J Thorac Cardiovasc Surg* 2010;139:474–82)

Ischemia–reperfusion (IR) injury entails enhanced inflammatory responses during reperfusion and remains a major cause of respiratory failure and other complications after lung transplantation.¹ We^{2–4} have previously shown that pulmonary macrophages and neutrophils contribute importantly to lung IR injury, with macrophages serving as triggers and neutrophils as end effectors of tissue injury. Alveolar epithelial cells also interact with alveolar macrophages to augment the inflammatory response after IR.⁵ Although recent studies suggest that lymphocytes may play an important role in lung IR injury,^{6,7} the exact role

of specific subtypes of lymphocytes and their cross-talk with macrophages and neutrophils in the context of lung IR injury remain to be elucidated.

Multiple lines of evidence suggest that the adenosine A_{2A} receptor (A_{2A}AR) is critical for adenosine-mediated protection from IR injury. A_{2A}AR-mediated inhibition of IR injury has been documented in various organ systems including liver, lung, kidney, and heart^{8–11}; however, the precise mechanisms responsible for A_{2A}AR-mediated protection remain unknown. A_{2A}ARs are predominantly expressed on leukocyte populations and mediate a variety of physiologic responses. A_{2A}ARs couple to G proteins and activate adenylyl cyclase leading to increased cellular levels of cyclic adenosine monophosphate.^{12,13} The mechanisms of protection mediated by A_{2A}AR activation may include inhibition of leukocyte-mediated inflammatory responses,¹³ vasodilation,¹⁴ and direct effects on organ parenchymal cells.¹² We¹¹ have previously demonstrated a T lymphocyte–mediated mechanism for A_{2A}AR activation after myocardial IR in mice.

Our previous studies have shown that administration of a selective A_{2A}AR agonist during reperfusion significantly reduces lung and heart IR injury in both in vivo and ex vivo animal models.^{8,11,15} In a recent study, we¹⁶ have

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Abbreviations and Acronyms

A _{2A} AR	= adenosine A _{2A} receptor
BAL	= bronchoalveolar lavage
IgG	= immunoglobulin G
IL-17	= interleukin-17
IR	= ischemia–reperfusion
LV	= left ventricular
MCP	= monocyte chemotactic protein
MIP	= macrophage inflammatory protein
MPO	= myeloperoxidase
RANTES	= regulated on activation, normal T expressed and secreted
TNF- α	= tumor necrosis factor-alpha
WBC	= white blood cell

also demonstrated a key role of CD4⁺ T cells in the initiation and progression of lung IR injury and that activation of CD4⁺ T cells lies upstream of neutrophil activation and infiltration. Although *in vivo* experiments have demonstrated the protective effect of A_{2A}AR activation, these experiments have not identified the major cellular target(s) responsible for the salutary effects of A_{2A}AR activation in lung IR injury. In the present study, we used a potent and selective A_{2A}AR agonist, ATL313, in an *in vivo* mouse model of lung IR injury. By using specific antibodies to deplete CD4⁺ T cells or neutrophils, we demonstrate a primary role of A_{2A}AR activation on CD4⁺ T lymphocytes in protecting the lung against IR injury.

MATERIALS AND METHODS

Study Design

This study used 8- to 12-week-old, male C57BL/6 mice (Jackson Laboratory, Bar Harbor, Maine), which were assigned to a sham group or 1 of 7 experimental groups that underwent IR (1 hour of left lung ischemia followed by 2 hours of reperfusion). The duration of 1 hour of ischemia and 2 hours of reperfusion was chosen on the basis of our previous study, which determined that this time frame resulted in maximum pulmonary dysfunction after ischemia.¹⁶ A total of 8 groups were used in the current study ($n = 5$ – 9 /group), which are summarized as follows: (1) sham (sham thoracotomy with no ischemia); (2) IR (1 hour of ischemia followed by 2 hours of reperfusion); (3) IR+ATL313 (mice undergoing IR with treatment of ATL313 during reperfusion); (4) immunoglobulin G (IgG) (mice pretreated with control IgG antibodies that undergo IR); (5) neutrophil depletion (neutrophil-depleted mice that undergo IR); (6) neutrophil depletion+ATL313 (same as group 5 with treatment of ATL313 during reperfusion); (7) CD4⁺ T cell depletion (CD4⁺ T cell–depleted mice that undergo IR); and (8) CD4⁺ T cell depletion+ATL313 (same as group 7 with treatment of ATL313 during reperfusion). Note that all groups underwent left lung IR except the sham group. The A_{2A}AR agonist, ATL313 (gift of Adenosine Therapeutics, LLC, Charlottesville, Va), was administered via intravenous injection at a dose of 3 μ g/kg 5 minutes before reperfusion. This dose was based on previous studies that have shown that 3 μ g/kg ATL313 potentially attenuates IR injury without significant hemodynamic effects.⁸ This study was performed concurrently with a previously reported study,¹⁶ and thus much of the data from the antibody-treated, control groups of animals in the current study (IgG, neutrophil depleted, and CD4 depleted) were

shared with the previous study. This study conformed to the “Guide for the Care and Use of Laboratory Animals” published by the National Institutes of Health (NIH publication No. 85-23, revised 1985) and was conducted under protocols approved by the University of Virginia’s Institutional Animal Care and Use Committee.

Hemodynamic Study

The effect of ATL313 or vehicle (saline) on hemodynamics was evaluated in C57BL/6 mice. Mice were anesthetized with 1% (vol) isoflurane. The right common carotid artery was exposed and cannulated with a 1.4F Millar Mikro-Tip catheter (Millar Instruments, Inc, Houston, Tex). After peripheral arterial blood pressures were acquired, the catheter tip was advanced into the left ventricular (LV) chamber. After bolus injection of 3 μ g/kg ATL313 or saline (2 μ L/g) via the left external jugular vein, LV pressures (end-systolic and end-diastolic pressures), positive and negative developed pressures, as well as aortic arterial pressure were continuously recorded for 30 minutes.

Depletion of Neutrophils and T lymphocytes

Depletion of neutrophils and CD4⁺ T cells was achieved by using selective antibodies as described previously.¹⁶ Rat anti-Gr-1 monoclonal antibody was used to deplete neutrophils. In brief, 10 μ g anti-Gr-1 monoclonal antibody (eBioscience, San Diego, Calif) was injected via tail vein 24 hours before undergoing study. To deplete CD4⁺ T cells, anti-CD4 monoclonal antibody (GK1.5, eBioscience) was injected intraperitoneally on 2 consecutive days at a dose of 0.2 mg per mouse per day. Two days after the second injection, these animals were studied. We¹¹ have previously documented specific depletion of CD4⁺ T cells by anti-CD4 monoclonal antibodies in mice by flow cytometry of peripheral blood and spleen cells. Perioperatively, blood (30–40 μ L) was obtained by puncturing the left external jugular vein. Circulating blood leukocyte counts were performed with a HemaVet Hematology System (CDC Technologies, Oxford, Conn).

In Vivo Model of Lung IR

An *in vivo* hilar clamp model of IR was used as previously described.¹⁶ Mice undergoing IR were subjected to 1 hour of ischemia (left lung hilar occlusion) followed by 2 hours of reperfusion. In brief, mice were anesthetized with inhalation isoflurane, intubated with PE-60 tubing, and connected to a pressure-controlled ventilator (Harvard Apparatus Co, South Natick, Mass). Mechanical ventilation with room air was set at 150 strokes/min, 1.0 mL stroke volume, and peak inspiratory pressure less than 20 cm H₂O. Heparin (20 U/kg) was given via external jugular injection to minimize thrombosis in the pulmonary vasculature during ischemia. A left thoracotomy was performed by cutting the left fourth rib, and the left hilum was exposed. A 6-0 Prolene polypropylene suture (Ethicon, Inc, Somerville, NJ) was placed around the left hilum facilitated by a tip-curved (22-gauge) gavage needle. Both ends of the suture were threaded through a 5-mm long PE-50 tube. Hilar occlusion was achieved by pulling up on the suture and thus pressing the tube against the hilum to initiate ischemia. A small surgical clip was applied to the suture above the tube to maintain tension against the hilum. The thoracotomy was then suture-closed and the mouse was extubated and allowed to recover during the 1-hour hilar occlusion period. The average time on the ventilator for each animal was 10 minutes. Five minutes before reperfusion, the mouse was reanesthetized and reintubated. Reperfusion was achieved by removing the clip, tube, and suture. Again, the chest was suture-closed. The mouse was extubated and placed back in the cage during the 2-hour reperfusion period. Temperature was monitored during surgery by an anal probe and maintained between 36.5°C and 37.5°C. Sham animals received only thoracotomy without hilar occlusion. Cessation of blood flow during ischemia was confirmed in separate mice using Evans blue dye (Sigma–Aldrich, St Louis, Mo), which was injected intravenously immediately after hilar clamping, and the left lungs were harvested 1 hour later. We consistently found no detectable levels of Evans blue dye in these lungs, thereby confirming ischemia.

Measurement of Pulmonary Function

At the end of scheduled reperfusion, pulmonary function was evaluated with the use of an isolated, buffer-perfused mouse lung system (Hugo Sachs Elektronik, March-Huggstetten, Germany) as previously described.¹⁶ Once properly perfused and ventilated, the isolated lungs were maintained on the system for a 5-minute equilibration period before data were recorded for an additional 10 minutes. Hemodynamic and pulmonary parameters were recorded by the PULMODYN data acquisition system (Hugo Sachs Elektronik). The values during the 10-minute data acquisition period remained very constant in all animals, and the pulmonary function data reported in this study reflect the final values at the end of this period.

Bronchoalveolar Lavage (BAL)

After pulmonary function measurement, left lungs were lavaged with 0.4 mL of phosphate-buffered saline solution. The BAL fluid was centrifuged at 4°C (500g, 5 minutes), and the supernatant was collected and stored at -80°C until further analysis.

Lung Wet/Dry Weight Ratio

In separate groups of animals (n = 5/group), the left lung was harvested, weighed, and then placed in a vacuum oven (at 54°C) until a stable dry weight was achieved. The lung wet weight/dry weight ratio was then calculated.

Pulmonary Microvascular Permeability

In separate groups of animals (n = 5/group), lung microvascular permeability was estimated by the Evans blue dye extravasation technique,^{16,17} which is an index of change in protein permeability. Evans blue dye (20 mg/kg) was injected intravenously 30 minutes before the animals were humanely killed. The pulmonary vasculature was then perfused for 10 minutes with phosphate-buffered saline to remove intravascular dye. Lungs were then homogenized in phosphate-buffered saline to extract the Evans blue and centrifuged. The absorption of Evans blue was measured in the supernatant at 620 nm and corrected for the presence of heme pigments: $A_{620} \text{ (corrected)} = A_{620} - (1.426 \times A_{740} + 0.030)$. The concentration of Evans blue was determined according to a standard curve and expressed as microgram/gram wet lung weight.

Immunohistochemistry of Pulmonary CD4⁺ T Cells

Lung tissue was fixed with 1% paraformaldehyde at 4°C for 24 hours and then embedded in Tissue-Tek O.C.T compound (Sakura, Torrance, Calif) before storage at -80°C. Immunostaining was performed with rat anti-mouse CD4 antibody (GK1.5) (Santa Cruz Biotechnology, Santa Cruz, Calif) using a Vectastain ABC kit (Vector Laboratories, Burlingame, Calif) as described previously.¹⁶

Measurement of Myeloperoxidase (MPO)

MPO levels were measured in BAL fluid using a commercially available mouse MPO enzyme-linked immunosorbent assay kit (Cell Sciences, Canton, Mass).

Measurement of Cytokines/Chemokines

Cytokine and chemokine protein levels in BAL fluid were quantified by the Bioplex Bead Array technique with a multiplex cytokine panel assay (Bio-Rad Laboratories, Hercules, Calif) as described previously.^{5,16}

Statistical Analysis

Mean values for each group are plotted in the accompanying figures, along with bars indicating the standard error of mean. Tests of the statistical significance of the observed differences in mean values between groups were assessed by 1-way analysis of variance and the Satterthwaite *T* test, which provides an adjustment for unequal variance between groups.

RESULTS

Effect of ATL313 on Hemodynamics

Potential changes in hemodynamics were assessed after administration of ATL313 (3 µg/kg) or vehicle (saline) (n = 4/group). Heart rate and mean arterial pressure remained stable in vehicle-treated mice. There was a small but insignificant increase in heart rate of approximately 14% in ATL313-treated mice compared with vehicle treatment during the first 25 minutes (Figure 1, A). There was a small but insignificant decrease (<20%) in mean arterial blood pressure after ATL313 administration that returned to baseline level within 10 minutes (Figure 1, B). LV pressures and LV developed pressures had a similar pattern of changes as arterial pressures (data not shown).

Effect of ATL313 on Blood Cell Counts

Blood was sampled for complete counts of circulating leukocytes before surgery and 2 hours after sham thoracotomy (sham group) or at the end of IR (IR or ATL313-treated IR animals) (n = 5/group). The percent change in cell counts measured after reperfusion versus cell counts before ischemia (baseline) is shown (Figure 1, C). All preoperative and postreperfusion measurements were performed on the same mouse to eliminate animal-to-animal variability. The numbers of preoperative, circulating white blood cells (WBCs) (baseline = 4333 ± 799 cells/µL), neutrophils (baseline = 330 ± 83 cells/µL), lymphocytes (baseline = 3642 ± 675 cells/µL), and monocytes (baseline = 368 ± 116 cells/µL) did not significantly change after sham thoracotomy. In the IR group, IR had no effect on WBC counts but caused a significant increase in neutrophils by 3.5-fold and a significant decrease in lymphocytes by 57% when compared with corresponding preischemic baseline counts ($P < .05$). In the ATL313-treated IR group, there was a significant increase in neutrophils (2.8-fold) over preischemic counts with no effect on WBCs, lymphocytes, or monocytes (Figure 1, C). Intergroup analysis of the changes after 2 hours of reperfusion showed no significant difference in WBC or monocyte counts among the 3 groups. Circulating neutrophils were significantly elevated in the IR and IR+ATL313 groups versus sham, and lymphocytes were significantly lower in the IR group versus the sham and IR+ATL313 groups (Figure 1, C).

Pulmonary Function After IR Is Improved by ATL313

Pulmonary function, evaluated by airway resistance, lung compliance, and pulmonary arterial pressure, was significantly impaired after IR (Figure 2, A). Airway resistance, lung compliance, and pulmonary arterial pressure were significantly improved in ATL313-treated mice after IR. Moreover, airway resistance, lung compliance, and pulmonary arterial pressure after IR were partially but significantly

FIGURE 1. Effect of ATL313 on mouse hemodynamics and complete blood cell counts. A, Heart rate remained stable in vehicle (saline)-treated mice ($n = 4/\text{group}$). Heart rate was mildly elevated during the first 25 minutes after intravenous injection of $3 \mu\text{g/kg}$ ATL313 compared with vehicle-treated mice. B, Mean arterial pressure remained stable in vehicle-treated mice ($n = 4/\text{group}$). Small but insignificant changes in mean arterial pressure occurred after injection of ATL313 during the first 5 minutes. The time of injection of vehicle and ATL313 is indicated by an arrow. C, Complete blood cell counts after reperfusion are presented as the percentage of corresponding preoperative baseline counts. Intragroup comparison showed a 3.5-fold increase in circulating neutrophils in both IR and IR+ATL313 groups and a greater than 50% reduction in circulating lymphocytes after IR. Comparisons among the 3 groups revealed no significant differences in total white blood cell (WBC) counts. Circulating neutrophils were significantly elevated in the IR and IR+ATL313 groups. Circulating lymphocytes were significantly reduced in the IR group ($*P < .05$ vs sham; $\#P < .05$ vs other groups; $n = 5/\text{group}$).

improved in neutrophil-depleted and CD4⁺ T cell-depleted mice compared with IgG control (Figure 2, B). No additional improvement of lung function occurred after IR when

neutrophil- or CD4⁺ T cell-depleted mice were treated with ATL313 (Figure 2, B).

ATL313 Inhibits Infiltration of T Cells and Neutrophils

Immunostaining of peripheral lung tissue demonstrated significant infiltration of CD4⁺ T cells after IR compared with sham (Figure 3). ATL313 significantly reduced infiltration of CD4⁺ T cells after IR (Figure 3). MPO levels in BAL fluid, used as a biochemical marker of neutrophil infiltration into alveolar air space, were also significantly increased after IR and significantly reduced by ATL313 treatment (Figure 4, A).

Lung IR Injury Is Reduced by ATL313

Lung injury after IR was significantly reduced in ATL313-treated mice as determined by reduced microvascular permeability (via Evans blue content) and pulmonary edema (wet/dry weight) (Figure 4, A). Lung injury (measured by microvascular permeability and pulmonary edema) and neutrophil infiltration (measured by MPO level in BAL fluid) were significantly reduced after IR in CD4⁺ T cell-depleted and neutrophil-depleted mice compared with IgG control mice (Figure 4, B). There was no further improvement in lung injury when ATL313 was administered to neutrophil-depleted or CD4⁺ T cell-depleted mice (Figure 4, B).

Cytokine/Chemokine Expression in ATL313-Treated Mice

Expression of tumor necrosis factor- α (TNF- α), interleukin 17 (IL-17), monocyte chemoattractant protein (MCP-1; CCL2), macrophage inflammatory protein (MIP-1; CCL3), RANTES* (CCL5), and KC (CXCL1) were significantly increased in BAL fluid after IR, and ATL313 treatment significantly reduced the expression of each of these cytokines/chemokines (Figure 5, A). In neutrophil-depleted mice, expression of TNF- α , MCP-1, MIP-1, and KC remained elevated after IR; however, IL-17 and RANTES were significantly reduced compared with IgG control (Figure 5, B). ATL313 treatment significantly reduced expression of MIP-1, RANTES, and KC in neutrophil-depleted mice. A noticeable trend in reduction of TNF- α , IL-17, and MCP-1 production was also observed by ATL313 treatment in neutrophil-depleted mice (Figure 5, B). The expressions of IL-17, MCP-1, MIP-1, RANTES, and KC were all significantly reduced in CD4⁺ T cell-depleted mice versus IgG control but were not reduced further by ATL313 treatment (Figure 5, B). A noticeable trend in reduction of TNF- α production was observed in CD4⁺ T cell-depleted mice with or without ATL313 treatment.

*RANTES = regulated on activation, normal T expressed and secreted.

FIGURE 2. Pulmonary function. A, Airway resistance and pulmonary artery pressure were significantly increased whereas lung compliance was significantly reduced after ischemia–reperfusion (IR) compared with sham. Pulmonary function was significantly improved after IR by ATL313 treatment (IR+ATL313) (* $P < .05$ vs sham; # $P < .05$ vs IR; $n = 10$ /group). B, Pulmonary function in antibody-treated mice after IR. Compared with IgG-treated mice, airway resistance and pulmonary artery pressure were significantly decreased, and lung compliance was significantly increased in neutrophil-depleted (NE dep) and CD4+ T cell-depleted (CD4 dep) mice. No additional protection occurred after ATL313 treatment (ATL). (§ $P < .05$ vs immunoglobulin G; $n = 9$ /group.)

DISCUSSION

Using antibody depletion methods, we¹⁶ have previously demonstrated a key role of CD4+ T lymphocytes and neutrophils in lung IR injury wherein the role of neutrophils as end effectors of lung injury is secondary to CD4+ T cell activation. In the current study, we also used antibody-depletion methods to investigate the effects of A_{2A}AR activation on lung IR injury via its role on CD4+ T cells and neutrophils. We observed that depletion of CD4+ T cells significantly

attenuated lung injury, neutrophil infiltration, and induction of proinflammatory cytokines/chemokines after lung IR. Similarly, neutrophil-depleted mice also displayed significantly reduced lung IR injury. The current study showed that ATL313 exerts significant protection against lung IR injury in control mice but offers no additional protection in lung function or injury when administered to CD4+ T cell-depleted or neutrophil-depleted mice. Moreover, A_{2A}AR activation further decreased TNF- α , IL-17, KC, MCP-1,

FIGURE 3. Pulmonary infiltration of T cells. The average number of CD4+ T cells per high power field (HPF) analysis is shown in the graph, and examples of immunohistochemical staining are shown for each group above the corresponding bars. CD4+ T cell numbers were significantly increased after ischemia–reperfusion (IR) compared with sham and were significantly reduced after IR by ATL313 treatment (IR+ATL313). (* $P < .05$ vs sham; # $P < .05$ vs IR; $n = 5$ /group.)

MIP-1, and RANTES production in neutrophil-depleted mice after IR, but did not offer any additional protection in CD4+ T cell–depleted mice. The attenuation of neutrophil infiltration by ATL313 likely occurs by 2 means: (1) directly via activation of A_{2A} AR on neutrophils to prevent activation and (2) indirectly via activation of A_{2A} AR on CD4+ T cells to attenuate release of potent chemokines. The data from the current study indicate that the effect of A_{2A} AR activation on CD4+ T cells appears to be more prominent than on neutrophils. A_{2A} AR activation on CD4+ T cells provides protection from lung IR injury by attenuating neutrophil activation and infiltration as demonstrated by the decrease in MPO levels and significant decreases in IL-17, KC, and MIP-2 (potent neutrophil chemotactic cytokines). Taken together, the results demonstrate that the protective effect of A_{2A} AR activation against lung IR injury is due to its action on CD4+ T cells, as well as neutrophils, and suggest that protection may be largely due to A_{2A} AR activation on CD4+ T cells.

A_{2A} ARs are widely distributed largely in leukocyte cell populations. The A_{2A} AR agonist, ATL313, binds to recombinant mouse adenosine receptor subtypes with K_i values (nM) as follows: A_{2A} , $2.3 < A_3$, $43 < A_1$, $241 < A_{2B} > 1000$.¹⁸ We¹⁵ have previously used A_{2A} AR knockout mice to demonstrate that ATL313 reduces lung IR injury in mice via the specific activation of A_{2A} AR. The identity of the cell type(s) responsible for mediating the protective effects of A_{2A} AR activation against lung IR injury remains unknown and thus was a focus of the current study. A_{2A} AR

activation mediates immunosuppression by inhibiting the activation of T lymphocytes and neutrophils, predominantly by a cyclic adenosine monophosphate–dependent pathway. The immunosuppressive effects of A_{2A} AR activation have been confirmed by studies showing that genetic inactivation of A_{2A} ARs increases the intensity and prolongs the duration of T lymphocyte–dependent cytokine accumulation and tissue damage.^{19,20} Previous studies in lung injury and other IR models have demonstrated that A_{2A} ARs on bone marrow–derived cells are required for A_{2A} AR-mediated protection from reperfusion injury.^{17,21–23} In lung IR injury, it is postulated that A_{2A} AR activation on CD4+ T lymphocytes and neutrophils attenuates downstream intercellular signaling cascade events involving other cell types such as alveolar macrophages and epithelial and endothelial cells.

A_{2A} AR activation was found to inhibit the redistribution of circulating lymphocytes after IR as characterized by elevated circulating lymphocytes and reduced lung T cell infiltration (Figures 1 and 3). ATL313 was administered shortly before the onset of reperfusion, and its mild hemodynamic effect should not have induced any preconditioning effects against lung IR injury. Activation of A_{2A} AR resulted in reduced neutrophil infiltration as demonstrated by reduced MPO levels (Figure 4). The reduced inflammatory response as a result of A_{2A} AR activation was also documented by significantly reduced levels of CD4+ T cell–related cytokines/chemokines (Figure 5). Activated CD4+ T cells are known to amplify the inflammatory response by producing IL-17, RANTES, interferon- γ , granulocyte-macrophage colony-stimulating factor, as well as MCP-1, MIP-1, and TNF- α . The results showed that expression of IL-17, RANTES, KC, MCP-1, MIP-1, and TNF- β were significantly increased after IR. ATL313 significantly abrogated the induction of all measured cytokines and chemokines. These findings suggest that ATL313 may act on CD4+ T cells to inhibit proinflammatory responses after IR.

Our results suggest that both neutrophils and CD4+ T cells are involved in the same cellular signaling pathway that leads to lung IR injury. Neutrophils are end effectors that cause lung injury, and injury correlated with BAL MPO levels (neutrophil infiltration) in our study. CD4+ T cell depletion also significantly attenuated MPO levels, thereby implicating a role of the CD4+ T cell/neutrophil axis in lung IR injury. Both T cells and neutrophils express A_{2A} ARs, and activation of A_{2A} ARs on both of these cell types has been shown to inhibit inflammatory responses.^{13,19} It is plausible that lack of further protection by ATL313 in neutrophil-depleted mice is simply due to the deficiency of neutrophils; however, somewhat better protection by ATL313 occurred in these mice in terms of reduced pulmonary artery pressure and reduced expression of cytokines. However, in CD4+ T cell–depleted mice, there was no evidence that ATL313 provided any additional protection, which may be because CD4+ T cell depletion alone is enough to prevent neutrophil

FIGURE 4. Neutrophil infiltration and lung injury after reperfusion. Neutrophil infiltration was assessed by measuring myeloperoxidase (MPO) levels in bronchoalveolar lavage fluid (top). Lung injury was assessed by measuring pulmonary edema (wet/dry weight) (middle) and microvascular leak (Evans blue content) (bottom). A, Non-antibody-treated mice. B, Antibody-treated mice for IgG-treated control (IgG) and depletion of neutrophils (NE dep) or CD4⁺ T cells (CD4 dep). All antibody-treated mice underwent lung ischemia–reperfusion (IR). ATL, ATL313 treatment (* $P < .05$ vs sham; # $P < .05$ vs IR, § $P < .05$ vs IgG control; $n = 5$ /group.)

activation and infiltration after IR. The attenuation of neutrophil infiltration by CD4⁺ T cell depletion not only signifies the role of the CD4⁺ T cell/neutrophil axis as the major signaling pathway, but also implicates CD4⁺ T cells as a primary target for A_{2A}AR-dependent attenuation of lung IR injury. Furthermore, the spectrum of changes in cytokines/chemokines is comparable between ATL313-treated mice and CD4⁺ T cell-depleted mice, but not comparable with neutrophil-depleted mice (Figure 5, B). This suggests that activation of A_{2A}ARs on CD4⁺ T cells is sufficient to inhibit IR-induced inflammatory responses and reduce lung IR injury.

An interesting observation in this study was that IR-induced IL-17 production was attenuated by ATL313. IL-17, produced largely by CD4⁺ natural killer T cells and T helper 17 cells, is a key cytokine for the recruitment, activation, and migration of neutrophils.²⁴ Thus, reduced levels of KC (a potent neutrophil chemokine) and MPO (ie, neutrophil infiltration) in the ATL313-treated groups are likely secondary to the inactivation of CD4⁺ T cells. There was also a significant reduction of IL-17 in neutrophil-depleted mice that was not as robust as in CD4⁺ T cell-depleted mice (Figure 5, B). This supports prior studies suggesting that neutrophils may also be a source of IL-17.²⁵ However,

FIGURE 5. Production of cytokines/chemokines after ischemia–reperfusion (IR). Expression of tumor necrosis factor- α (*TNF- α*), interleukin (*IL*)-17, monocyte chemotactic protein (*MCP-1*), macrophage inflammatory protein (*MIP-1*), regulated on activation, normal T expressed and secreted (*RANTES*), and KC were measured in bronchoalveolar lavage fluid after reperfusion. Two separate statistical comparisons are shown: (A) non-antibody-treated mice and (B) antibody-treated mice for depletion of neutrophils (*NE dep*) or CD4+ T cells (*CD4 dep*). All antibody-treated animals underwent lung IR. ATL, ATL313 treatment (* $P < .05$ vs sham and IR+ATL; # $P < .05$ vs IgG control; § $P < .05$ vs *NE dep*; n = 5/group).

in the setting of lung IR, the release of IL-17 by neutrophils appears to be regulated by CD4⁺ T cells via abrogation of neutrophil chemotactic factors such as KC, IL-17, and MIP-2. These results further indicate that the anti-inflammatory effect of ATL313 may largely be due to its action on A_{2A}ARs on CD4⁺ T cells.

In summary, the current study aimed to define the cellular sources whereby A_{2A}AR activation exerts protection from lung IR injury. Although we found that CD4⁺ T cells mediate neutrophil activation during reperfusion, and the protective effect of ATL313 is largely due to its action on CD4⁺ T cells, a role for ATL313 in directly inhibiting neutrophil activation or infiltration could not be excluded. Activation of A_{2A}ARs by ATL313 protects the lung against IR injury. This protection is comparable with that found in CD4⁺ T cell-depleted mice. However, no additive effects occur when ATL313 is applied to CD4⁺ T cell-depleted mice. Collectively, these results suggest that CD4⁺ T cells mediate acute lung IR injury and that the protective effect of A_{2A}AR activation may be largely due to its actions on CD4⁺ T lymphocytes.

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