Pretreatment strategy with adenosine A_{2A} receptor agonist attenuates reperfusion injury in a preclinical porcine lung transplantation model

Damien J. LaPar, MD, MSc,^a Victor E. Laubach, PhD,^a Abbas Emaminia, MD,^a Ivan K. Crosby, MD,^a Vanessa A. Hajzus, BS,^a Ashish K. Sharma, MBBS,^a Heather M. Sumner, MD,^b David V. Webb, MD,^b Christine L. Lau, MD,^a and Irving L. Kron, MD^a

Objective: Adenosine A_{2A} receptor activation after lung transplantation attenuates ischemia–reperfusion injury by reducing inflammation. However, the effect of adenosine A_{2A} receptor activation in donor lungs before transplant remains ill defined. This study compares the efficacy of 3 different treatment strategies for adenosine A_{2A} receptor agonist in a clinically relevant porcine lung transplantation model.

Methods: Mature porcine lungs underwent 6 hours of cold ischemia before allotransplantation and 4 hours of reperfusion. Five groups (n = 6/group) were evaluated on the basis of treatment with ATL-1223, a selective adenosine A_{2A} receptor agonist: thoracotomy alone (sham), transplant alone (ischemia–reperfusion), donor pretreatment via ATL-1223 bolus (ATL-D), recipient treatment via ATL-1223 infusion (ATL-R), and a combination of both ATL-1223 treatments (ATL-D/R). Lung function and injury were compared.

Results: Blood oxygenation was significantly higher among ATL-D, ATL-R, and ATL-D/R groups versus ischemia–reperfusion (392.0 ± 52.5 , 428.9 ± 25.5 , and 509.4 ± 25.1 vs 77.2 ± 17.0 mm Hg, respectively, $P < .001$). ATL-1223–treated groups had lower pulmonary artery pressures (ATL-D = 30.5 ± 1.8 , ATL-R = 30.2 ± 3.3 , and ATL-D/R = 29.3 ± 4.5 vs IR = 45.2 ± 2.1 mm Hg, $P < .001$) and lower mean airway pressures versus ischemia–reperfusion (ATL-D = 9.1 ± 0.8 , ATL-R = 9.1 ± 2.6 , and ATL-D/R = 9.6 ± 1.3 vs IR = 21.1 mm Hg, $P < .001$). Likewise, ATL-1223–treated groups had significantly lower lung wet/dry weight, proinflammatory cytokine expression, and lung injury scores by histology compared with ischemia–reperfusion. All parameters of lung function and injury in ATL-1223–treated groups were similar to sham (all $P > .05$).

Conclusions: Pretreatment of donor lungs with ATL-1223 was as efficacious as other treatment strategies in protecting against ischemia–reperfusion injury. If necessary, supplemental treatment of recipients with ATL-1223 may provide additional protection. These results support the development of pharmacologic A_{2A}R agonists for use in human clinical trials for lung transplantation. (J Thorac Cardiovasc Surg 2011;142:887-94)

Despite recent advances in lung preservation and surgical care, outcomes after lung transplantation remain poor. Lung ischemia–reperfusion (IR) injury after transplantation continues to be a common and significant cause of morbidity and mortality in the early postoperative period, occurring in 20% to 35% of cases.¹⁻³ This condition, characterized by markedly elevated oxygen alveolar-arterial gradients, severe noncardiogenic pulmonary edema, increased

pulmonary vascular resistance, and decreased airway compliance,^{4,5} has been associated with approximately 30% of early deaths after lung transplantation in the current surgical era and often results in increased resource use with prolonged mechanical ventilation and longer hospital stays.^{1,3} Furthermore, the presence of IR injury has been shown to have long-term effects on the transplanted lung by increasing the risk of chronic graft rejection (bronchiolitis obliterans).⁶

Adenosine serves as a retaliatory metabolite in posttransplantation IR injury by exerting potent anti-inflammatory effects.⁷ This endogenous compound mediates its influence through interaction with 4 different G protein-coupled adenosine receptors: A₁R, A_{2A}R, A_{2B}R, and A₃R.⁸ Each of these receptor subtypes is expressed within the lung,⁹ and the effects of adenosine receptor signaling are classically propagated through the cAMP and phospholipase C second messenger pathways. Activation of the adenosine A_{2A} receptor (A_{2A}R) is well known to exert anti-inflammatory effects, and the A_{2A}R is predominantly expressed on inflammatory cells including neutrophils, mast cells, macrophages, monocytes, and T cells. Specifically, A_{2A}R activation prevents leukocyte adhesion to endothelial

From the Departments of Surgery^a and Pathology,^b University of Virginia Health System, Charlottesville, Va.

This study was supported by a research grant from the Roche Organ Transplant Research Foundation, Meggen, Switzerland (to V.E.L.) and by National Institutes of Health Grant T32HL007849 (to I.L.K.). The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Heart, Lung, and Blood Institute or the National Institutes of Health.

Disclosures: Authors have nothing to disclose with regard to commercial support.

Read at the 91st Annual Meeting of The American Association for Thoracic Surgery, Philadelphia, Pennsylvania, May 7-11, 2011.

Received for publication May 9, 2011; revisions received June 2, 2011; accepted for publication June 9, 2011; available ahead of print July 15, 2011.

Address for reprints: Irving L. Kron, MD, Professor of Surgery, Department of Surgery, University of Virginia Health System, PO Box 800679, Charlottesville, VA 22908 (E-mail: ilk@virginia.edu).

0022-5223/\$36.00

Copyright © 2011 by The American Association for Thoracic Surgery

doi:10.1016/j.jtcvs.2011.06.015

Abbreviations and Acronyms

A _{2A} R	= adenosine A _{2A} receptor
BAL	= bronchoalveolar lavage
IL	= interleukin
IR	= ischemia–reperfusion
PA	= pulmonary artery

cells and inhibits the release of toxic oxygen products during the respiratory burst.¹⁰ Our laboratory has previously demonstrated that pharmacologic activation of the A_{2A}R significantly attenuates lung IR injury in several different *in vivo* and *ex vivo* small and large animal models.^{11–15} However, the effect of different treatment strategies in a preclinical lung transplantation model remains unknown.

The purpose of this study was to compare the efficacy of 3 different strategies for administration of A_{2A}R agonist in a clinically relevant porcine lung transplantation model: pretreatment of donor lungs, treatment of the recipient after transplantation, and a combination of both treatments. Further defining the optimal treatment strategy for A_{2A}R agonist administration in a preclinical transplant model remains crucial to providing a solid foundation for the development of pharmacologic compounds, such as A_{2A}R agonists, for use in future human clinical trials to prevent IR injury after transplantation.

MATERIALS AND METHODS**Animal Care**

The University of Virginia Animal Care and Use Committee reviewed and approved all aspects of this experimental protocol before the commencement of this study. Humane animal care was observed in accordance with the “Guide for Care and Use of Laboratory Animals” (National Institutes of Health publication no. 85-23, revised 1985).

Study Groups

Mature domestic swine of both sexes (18–40 kg) were randomly distributed among 5 different study groups (*n* = 6/group) that were defined on the basis of treatment with or without ATL-1223 (Dogwood Pharmaceuticals, Charlottesville, Va), a selective A_{2A}R agonist. The sham group underwent a left thoracotomy alone with no ATL-1223 treatment and served as the negative control. The IR group underwent lung procurement and transplantation with no ATL-1223 treatment and served as the positive control. The ATL-D group received a single ATL-1223 bolus treatment to the donor animals (1.5 µg/kg) before transplantation. The ATL-R group received a continuous infusion of ATL-1223 (3.0 ng/kg/min) during the entire 4-hour reperfusion period after transplantation. The ATL-D/R group received a combination of both ATL-1223 treatment strategies. The dose of ATL-1223 during reperfusion was based on preliminary experiments with this drug and on previous studies from our laboratory.^{11,14} The ATL-1223 infusion was started 10 minutes before the initiation of reperfusion to ensure adequate circulation of the agonist to the transplanted lung on reperfusion. The ATL-1223 bolus was administered by intravenous injection 20 minutes before donor lung ischemia and procurement, a time chosen on the basis of an estimated 20-minute half-life for ATL-1223 in serum because of its similarity to other compounds with known pharmacokinetics in the same

chemical class (Dogwood Pharmaceuticals, data not shown). After transplantation or sham surgery, lungs of all animals were perfused *in vivo* for a continuous 4-hour period, after which parameters of lung function and injury were assessed as described below.

Porcine Left Lung Donor Procedure

Donor left-lung procurement was performed using a standard operative technique as previously described.^{14,16} Briefly, animals were anesthetized with ketamine (50 mg/kg) and xylazine (5 mg/kg) and ventilated with room air using a volume-control ventilator (Harvard Apparatus, Boston, Mass). After intubation, anesthesia was maintained with 2% isoflurane and 100% fraction of inspired oxygen, and the lungs were ventilated to a tidal volume of 8.0 mL/kg with a respiratory rate of 18 to 20 breaths/min and a positive end-expiratory pressure of 5.0 cm H₂O. Each animal was placed in the supine position and prepped and draped in a standard surgical fashion. Systemic anticoagulation was achieved using an intravenous bolus dose of heparin sodium (200 U/kg). Donor animals underwent a median sternotomy and pericardiotomy to expose the heart, great vessels, and both lungs. After dissection of the aortopulmonary window, the main pulmonary artery (PA) was cannulated with a cardioplegia cannula (Sarns, Ann Arbor, Mich) approximately 1.5 cm distal to the pulmonary valve. Prostaglandin E1 (10 mg/kg) was injected directly into the main PA and allowed to circulate for 10 minutes before crossclamping. The superior vena cava was then excluded, the left atrial appendage was incised, and the inferior vena cava was divided to ensure appropriate lung isolation and drainage. Antegrade flushing of both lungs was then performed with 1.5 L of 4°C Perfadex (Vitrolife Inc, Denver, Colo), a commercially available preservation solution widely used during human lung transplantation, through the PA cannula from a height of 30 cm while both lungs were cooled using 4°C saline slush. On completion of the flush, both lungs were inflated with 100% oxygen to tidal volume, and the trachea was clamped. The heart and both lungs were then explanted en bloc.

After explantation, the left and right lungs were separated and the heart was excised, ensuring the presence of a generous left atrial cuff. The donor lung was then retrograde perfused with an additional 500 mL 4°C Perfadex through each pulmonary vein to remove any remaining small clots from the pulmonary vasculature. The donor lung was then doubly wrapped in a sterile bag and stored in cold Perfadex at 4°C for 6 hours before transplantation. This cold ischemic time has been shown to result in significant and reproducible lung injury in the porcine model.¹⁶

Porcine Left Lung Recipient Transplantation

Size-matched recipient swine underwent identical preoperative preparation as donor animals, including induction and maintenance anesthesia, neck dissection with arterial and venous line placement, and PA catheter positioning. Each animal was placed in a right, lateral decubitus position and shaved, prepped, and draped in a normal sterile fashion. A left thoracotomy was then performed, and the left lung hilum was visualized. The hemiazygos vein was first suture ligated and divided, and the peribronchial lymph nodes were dissected free from all vascular and bronchial structures. The left hilum was then carefully exposed to obtain circumferential dissection of the main, left, and right pulmonary arteries, the right main bronchus, and the left superior and inferior pulmonary veins. A vessel loop was then passed around the right PA immediately distal to its bifurcation from the main PA for later occlusion. The left inferior pulmonary ligament was then divided, and the pleura was dissected to the level of the hilum. A left pneumonectomy was performed after clamping of the left PA just distal to the bifurcation with subsequent suture ligation and division of the left and right pulmonary veins and division of the left main stem bronchus. Care was taken to ensure adequate PA and bronchial cuffs before their division to allow for tension-free anastomoses during transplantation. The explanted lung was then discarded.

A left lung transplant of the preserved donor lung was performed after 6 hours of cold ischemia. Immediately after removing the donor lung from

ice, the donor left main stem bronchus was anastomosed to the recipient left main stem bronchial cuff using 4-0 Prolene suture (Ethicon, Inc, Somerville, NJ) in a continuous fashion. The bronchial anastomosis was then tested to ensure the absence of a leak before anastomosis of the left pulmonary arteries with continuous 5-0 Prolene suture. A generous portion of the left atrial appendage was then isolated with a side-biting vascular clamp and incised to provide an appropriate cuff for subsequent anastomosis to the donor pulmonary veins. Before completion of the left pulmonary vein anastomosis, the left PA was unclamped to establish reperfusion of the lung and to ensure adequate venting and drainage of the pulmonary vasculature. The pulmonary vein–left atrial anastomosis was then tied down securely, and adequate hemostasis was confirmed. The left thoracotomy was then closed using interrupted, figure-of-8, 0 Vicryl sutures (Ethicon, Inc), and the skin was reapproximated with staples.

Lung Physiology

All lungs underwent an initial 3.5 hours of reperfusion whereon the right main PA was occluded for an additional 30 minutes to assess pulmonary function of the isolated left lung at the 4-hour reperfusion time point. During the entire reperfusion period, hourly arterial blood gas measurements were obtained in addition to assessment of physiologic status through measurement of mean arterial pressure and heart rate. Ventilator settings were adjusted as needed to maintain normal pH, PCO_2 , and PO_2 during the initial 3.5 hours of reperfusion; however, no ventilation changes were made during the final 30-minute reperfusion period of the isolated graft. On isolation of the donor lung, baseline pulmonary function was assessed through measurement of mean airway pressure, tidal volume, peak inspiratory pressure, and minute volume using a pressure ventilator (Servo ventilator 900C, Siemens-Elma, Solna, Sweden), and the PA catheter was used to obtain measurements of PA pressure, wedge pressure, cardiac output, cardiac index, pulmonary vascular resistance, and systemic vascular resistance. On conclusion of the 4-hour reperfusion period, additional assessment of the recipient animal and the transplanted donor lung was performed to obtain final arterial blood gas, pulmonary function, and PA catheter measurements before explantation of the transplanted lung.

Bronchoalveolar Lavage and Cytokine Measurements

Bronchoalveolar lavage (BAL) of the upper lobe of the left lung in all groups was immediately performed after explantation using 40 mL of normal saline and repeated twice. BAL samples underwent centrifugation at 10,000 rpm at 4°C for 20 minutes to remove cells and debris, and the supernatant was collected and stored frozen at –80°C. Quantification of cytokine levels in BAL fluid was assessed using a commercially available porcine cytokine multiplex immunoassay kit (Aushon BioSystems, Inc, Billerica, Mass) according to the manufacturer's protocol. All BAL samples were tested in duplicate and averaged. Cytokine concentrations are expressed in picograms per milliliter.

Lung Wet/Dry Weight

Fresh lung tissue samples obtained from the lower lobe were blotted to remove excess blood and weighed before placement in a 52°C vacuum oven for 48 to 72 hours. The samples were weighed daily until the weight was stable. The wet-to-dry weight ratio was used to assess pulmonary edema, wherein high wet/dry weight is indicative of pulmonary edema.

Histopathology and Lung Injury Severity Score

The lower lobe was fixed in 4% paraformaldehyde, via tracheal inflation under constant 25 cm pressure, for 24 hours with subsequent paraffin embedding and hematoxylin–eosin staining. The portion of the lower lobe used to collect tissue for wet/dry weight was excluded from fixation via ligation of the airway supplying that portion of the lobe. Lung sections were blindly assessed by 2 separate lung pathologists and graded according to

the degree of interstitial infiltration, total neutrophil counts per high power field, and extent of alveolar edema. A score on a scale of 0 to 3 was assigned for each section using previously reported criteria: neutrophils per high-powered field (score: 0 = <5 cells, 1 = 6–10 cells, 2 = 11–20 cells, 3 = >20 cells), interstitial infiltration (score: 0 = none, 1 = minimal, 2 = moderate, 3 = severe), alveolar edema (score: 0 = <5%, 1 = 6%–25%, 2 = 26%–50%, 3 = >50%).¹⁴

Statistical Analysis

All experimental methodology was designed to test the null hypothesis that no significant differences in the degree of IR injury after lung transplantation would be observed despite $\text{A}_{2\text{A}}$ R agonist treatment strategy. Independent, pairwise group comparisons of mean differences in measured results were performed using analysis of variance. To provide a conservative estimate of these differences, all probability estimates were adjusted for the potential influence of multiple group comparisons using a post hoc Bonferroni correction. Experimental results are reported as mean $\pm$ standard error of the mean. All calculated test statistics were used to generate reported *P* values. All *P* values are 2-tailed.

RESULTS

Lung Function

Lung function after transplantation was significantly improved in all groups that received $\text{A}_{2\text{A}}$ R agonist treatment (Figure 1). Blood oxygenation was significantly higher among ATL-D, ATL-R, and ATL-D/R groups compared with IR control (392.0 ± 52.5 , 428.9 ± 25.5 , 509.4 ± 25.1 vs 77.2 ± 17.0 mm Hg, respectively, $P < .001$). Likewise, all ATL-1223-treated groups had significantly lower PA pressures (ATL-D = 30.5 ± 1.8 , ATL-R = 30.2 ± 3.3 , ATL-D/R = 29.3 ± 4.5 vs IR = 45.2 ± 2.1 mm Hg, $P < .001$) and lower mean airway pressures compared with IR control (ATL-D = 9.1 ± 0.8 , ATL-R = 9.1 ± 2.6 , ATL-D/R = 9.6 ± 1.3 vs IR = 21.1 mm Hg, $P < .001$). However, no significant differences were observed for these measurements either between the ATL-1223 treatment groups or between each treatment group and the sham group (all $P > .05$). No significant differences in median total anastomosis (graft implant warm ischemia) time existed between experimental groups (IR control = 37.5 minutes, ATL-D = 29.5 minutes, ATL-R: 32.0 minutes, ATL D/R = 26.5 minutes, all $P > .05$). In addition, no significant differences among all groups were observed for mean arterial pressure, cardiac output, cardiac index, systemic vascular resistance, or pulmonary capillary wedge pressure (data not shown).

Pulmonary Edema

The degree of pulmonary edema was determined by lung wet/dry weight ratios, which was significantly reduced in all ATL-1223 treatment groups (Figure 2). As expected, the IR group (7.7 ± 0.4) had significantly increased wet/dry weight compared with sham (5.0 ± 0.2) ($P < .05$). ATL-1223-treated lungs had significantly lower wet/dry weight compared with IR control animals (ATL-D = 4.8 ± 0.3 , ATL-R = 5.2 ± 0.3 , ATL-D/R = 4.9 ± 0.2 vs IR = $7.7 \pm$

FIGURE 1. Lung physiology after 4 hours of reperfusion. A, Mean blood oxygenation (mm Hg) as assessed by measured P_{O_2} on arterial blood gas analysis. B, Mean PA pressure (mm Hg). C, Mean airway pressure (mm Hg). Groups: thoracotomy alone (sham), left lung transplant (IR), ATL-1223 bolus pretreatment to donor lung (ATL-D), ATL-1223 infusion during reperfusion of donor lung (ATL-R), and a combination of ATL-1223 pretreatment and infusion during reperfusion (ATL-D/R). * $P < .05$ vs all. IR, Ischemia–reperfusion.

0.4, all $P < .05$). No significant differences in wet/dry weight existed between the ATL-1223 treatment groups or the sham group (all $P > .05$).

Proinflammatory Cytokine Expression

The effect of various ATL-1223 treatment strategies on the expression of proinflammatory cytokines in BAL fluid was assessed at the end of reperfusion (Table 1). As expected, the IR group had significantly increased expression of cytokines compared with sham. Pretreatment with ATL-

FIGURE 2. Mean lung wet/dry weight ratios after 4 hours of reperfusion. Groups: thoracotomy alone (sham), left lung transplant (IR), ATL-1223 bolus pretreatment to donor lung (ATL-D), ATL-1223 infusion during reperfusion of donor lung (ATL-R), and a combination of ATL-1223 pretreatment and infusion during reperfusion (ATL-D/R). * $P < .05$ vs all. IR, Ischemia–reperfusion.

1223 (ATL-D group), treatment of the recipient (ATL-R group), or combined treatment (ATL-D/R group) all resulted in significantly reduced expression of interleukin (IL)- 1β , IL-8, IL-10, interferon- γ , and tumor necrosis factor- α compared with IR control (Table 1). The expression of each of these cytokines in all ATL-1223–treated groups was similar to that for sham animals (all $P > .05$).

Gross and Histologic Evidence of Lung Injury

Overall, ATL-1223 treatment resulted in improved gross and histologic appearance (Figure 3) and decreased mean histologic injury scores (Table 2) compared with IR controls. Total lung injury scores for all 3 ATL-1223 treatment groups were significantly lower than IR control (ATL-D = 1.50 ± 0.22 , ATL-R = 1.52 ± 0.39 , ATL-D/R = 1.67 ± 0.21 vs IR control = 4.83 ± 0.31 , all $P < .05$). In addition, neutrophil infiltration, edema, and interstitial infiltrates were all elevated in the IR group versus sham and were significantly reduced in each of the ATL-1223–treated groups versus IR (Table 2). Lung injury scores for the ATL-1223 treatment groups were similar to those of sham animals (1.00 ± 0.45). On examination of each score category, the quantity of infiltrating neutrophils among IR controls proved to be the largest determinant of overall score differences when compared with ATL-1223 treatment and sham groups.

DISCUSSION

The present study used a preclinical porcine lung transplantation model to investigate the most efficacious and practical treatment strategy for the attenuation of acute lung IR injury through pharmacologic activation of $A_{2A}R$. The results demonstrate that a pretreatment strategy, using a single bolus dose of ATL-1223 administered to the donor before procurement, is efficacious and significantly

TABLE 1. Cytokine levels in bronchoalveolar lavage fluid

	Sham	IR	ATL-D	ATL-R	ATL-D/R
IL-1β (pg/mL)	28.6 ± 1.2*	265.1 ± 43.7	31.8 ± 7.0*	31.1 ± 2.7*	25.0 ± 2.1*
IL-8 (pg/mL)	51.3 ± 16.7*	222.4 ± 41.4	91.2 ± 26.9*	60.9 ± 24.3*	76.7 ± 33.9*
IL-10 (pg/mL)	2.7 ± 0.8*	63.1 ± 13.1	13.3 ± 9.9*	2.3 ± 0.2*	3.7 ± 1.1*
INF-γ (pg/mL)	5.1 ± 0.7*	248.2 ± 87.0	13.2 ± 6.2*	7.3 ± 2.4*	5.3 ± 2.1*
TNF-α (pg/mL)	2.5 ± 0.6*	25.9 ± 4.6	2.8 ± 0.6*	2.2 ± 0.4*	2.6 ± 0.6*

*P < .05 vs IR. Results reported as mean ± standard error.

attenuates posttransplantation IR injury. The preserved pulmonary condition afforded by a pretreatment strategy was associated with improved lung physiology, reduced pulmonary edema, decreased expression of proinflammatory cytokines, decreased neutrophil infiltration, and conserved lung histology. More important, pretreatment of donor lungs with ATL-1223 provided a similar level of protection from IR injury compared with either ATL-1223 treatment of the recipient alone or a combined ATL-1223 treatment strategy. In addition, the degree of protection after pretreatment of donor lungs resulted in parameters of lung function and injury that were not significantly different from the sham group. These results not only corroborate data from previous studies using small animal models of lung IR injury but, more important, also provide a solid foundation from which to advance the development of pharmacologic A_{2A}R agonists for use in human clinical trials.

The pharmacologic pretreatment strategy used in this study mimics the beneficial effects of adenosine modulation after ischemic preconditioning in other experimental reports.¹⁷⁻¹⁹ During ischemic preconditioning, brief periods of ischemia and hypoxia are associated with accumulating levels of extracellular adenosine. Elevated levels of adenosine have been demonstrated to confer benefits

before prolonged ischemia within several different organ systems, including the liver, lung, heart, and kidney.^{17,18,20} Although the precise mechanisms underlying the benefits of elevated adenosine during the preischemic state are not well understood, adenosine receptor activation appears crucial. With respect to lung IR injury, we have previously demonstrated the protective benefits of pharmacologic A_{2A}R activation as pretreatment in an ex vivo blood-perfused rabbit lung model,¹¹ results that were confirmed in the present study. In a related study by Yildiz and colleagues,¹⁹ similar benefits of adenosine pretreatment were demonstrated in an isolated, buffer perfused rat-lung model of IR injury. These 2 studies were limited, however, by the use of small animal, ex vivo models of lung inflammation and do not represent true transplant models. Thus, the use of an in vivo, clinically translatable animal model of lung transplantation was an important advantage in the present study.

The molecular and cellular mechanisms involved in lung IR injury and the role of adenosine modulation have been increasingly documented and are currently a source of continued investigation; however, the mechanisms involved in the abrogation of lung IR injury after an adenosine pretreatment strategy remain unknown. Accumulated data have

FIGURE 3. Representative gross (top) and histologic (bottom, hematoxylin-eosin sections, 40×) appearance of lungs after 4 hours of reperfusion. Groups: thoracotomy alone (sham), left lung transplant (IR), ATL-1223 bolus pretreatment to donor lung (ATL-D), ATL-1223 infusion during reperfusion of donor lung (ATL-R), and a combination of ATL-1223 pretreatment and infusion during reperfusion (ATL-D/R). IR, Ischemia-reperfusion.

TABLE 2. Lung injury severity scores by histology

	Neutrophils	Alveolar edema	Interstitial infiltrate	Total score
Sham	0.67 ± 0.33*	0.00 ± 0.00*	0.33 ± 0.21*	1.00 ± 0.45*
IR	2.17 ± 0.17	1.50 ± 0.22	1.17 ± 0.31	4.83 ± 0.31
ATL-D	0.67 ± 0.21*	0.50 ± 0.22*	0.36 ± 0.29*	1.50 ± 0.22*
ATL-R	1.17 ± 0.17*	0.00 ± 0.00*	0.67 ± 0.21*	1.52 ± 0.39*
ATL-D/R	1.00 ± 0.26*	0.00 ± 0.00*	0.67 ± 0.21*	1.67 ± 0.21*

See "Materials and Methods" for scoring criteria. * $P < .05$ versus IR. Results reported as mean ± standard error.

demonstrated that alveolar macrophages and infiltrating neutrophils and CD4⁺ T lymphocytes play an important role in acute IR injury.^{15,21-23} In addition, activation of A_{2A}R, which is predominantly expressed on inflammatory cells including neutrophils, mast cells, macrophages, monocytes, and T cells, has been shown to attenuate lung injury through a variety of mechanistic pathways involving decreased release of tumor necrosis factor- α and other proinflammatory cytokines, downregulation of adhesion molecules (P-selectin, intercellular adhesion molecule-1, and vascular cell adhesion molecule-1), and blocking of neutrophil activation and infiltration.^{10-12,14,15,21,24} In the current study, ATL-1223 treatment resulted in significantly decreased proinflammatory cytokine expression when administered either before ischemia or during reperfusion. In addition, neutrophil counts were similarly decreased after administration of ATL-1223 regardless of treatment strategy. Although the specific mechanisms underlying the improvement in lung function after pretreatment of donor lungs with ATL-1223 was not an objective of this study, indirect evidence of decreased neutrophil activation and reduced cytokine and chemokine expression suggests that pretreatment of the donor lung serves to "deactivate" immune cells in the donor lung to exert a protective effect on reperfusion. Deactivation of cells, such as alveolar macrophages and marginated neutrophils, before the ischemic insult may serve to inhibit the characteristic respiratory burst that typically occurs on reperfusion.

Several important considerations related to the experimental methodology of this study deserve further discussion. First, the total anastomosis time, which represents the amount of "warm" ischemic time that the donor lung experienced before reperfusion, was consistent and short (25–30 minutes) in our model. Prolonged warm ischemic time has a strong association with worse lung injury after transplantation.^{25,26} Thus the potential for this confounding influence was reduced between study groups. Second, all experimental groups had similar mean arterial pressures and cardiac function during the reperfusion period, which reduces the potential impact of variable hemodynamics between groups on lung injury outcomes. Third, a total of 38 transplants were performed in this study. Of the 8 transplants that were excluded, 3 were

excluded because of unexpected intraoperative death from technical complications, and 5 were excluded because of poor recipient lung condition from either active or prior pneumonia. The relatively short estimated half-life of ATL-1223 provided the rationale in our study for administering ATL-1223 to the pretreatment group 20 minutes before ischemia and to the recipient as a constant infusion during the 4-hour reperfusion period. However, in view of the efficacy of a single pretreatment bolus, we would speculate that a single bolus treatment of ATL-1223 on reperfusion might be as effective as a constant infusion. Finally, an established side effect of adenosine A_{2A}R agonists, such as ATL-1223, is vasodilation and systemic hypotension after administration of relatively high doses of these compounds. Thus, dosing strategies used in this study were designed to allow for the provision of the lowest, yet still efficacious dose of ATL-1223 to both donor and recipient animals without any significant systemic hypotension.

No clinical therapies are currently available that specifically target IR injury; however, the specific targeting of A_{2A}R may offer therapeutic strategies in this regard. Thus, the present results have significant clinical implications. The fact that a single bolus, pretreatment of ATL-1223 before donor lung procurement results in significant protection from acute lung IR injury, and that this level of protection is similar to that achieved after treatment during reperfusion, provides evidence for a simple and efficacious treatment strategy to prevent IR injury. Underscoring the significance of these results is the observation that the pretreatment strategy resulted in pulmonary function comparable to that of sham animals. Such a pretreatment strategy could be easily implemented into current lung transplantation procurement protocols similar to that currently used for the administration of pre-procurement prostaglandin. Although the combined treatment of ATL-1223 in our study did not significantly enhance protection above the single treatments, it is still possible that a combined treatment strategy, involving treatment of ATL-1223 before ischemia with supplemental treatment during reperfusion, could confer additional protection in some cases where unanticipated inflammation occurs after transplantation. Such an approach may prove especially beneficial for marginal donor lungs or for donor lungs that are subjected to prolonged ischemic times. In addition to benefiting the patient acutely, preventing lung IR injury through pharmacologic A_{2A}R activation would likely provide long-term benefits by reducing the incidence of bronchiolitis obliterans. A pretreatment strategy for A_{2A}R activation may also prove beneficial to reduce the incidence of IR injury after the transplantation of other solid organs, such as liver, kidney, small intestine, and pancreas. Thus, these results have significant and broad implications for the future of human organ transplantation.

The reported results yield potential areas for future investigation. The underlying mechanisms involved in protection

from IR injury after pretreatment with A_{2A}R agonists are warranted. In addition, further study to more clearly define the length of ischemic time that can be tolerated after pretreatment of donor lungs with ATL-1223 will be required to define the full potential of this treatment strategy. It is possible that treatment of donor lungs with ATL-1223 may allow for longer ischemic times than are currently acceptable after lung procurement, which ultimately may serve to help redefine transplantation protocols in the not-too-distant future.

CONCLUSIONS

Pretreatment of donor lungs with a bolus dose of A_{2A}R agonist proves efficacious and confers a similar level of protection from acute IR injury as other treatment strategies. This pretreatment strategy could be easily implemented into current, routine organ procurement protocols. A combined treatment strategy with supplemental A_{2A}R agonist administration to the recipient during reperfusion may provide additional attenuation of inflammation after transplantation. These results support the development of pharmacologic A_{2A}R agonists for use in human clinical trials to prevent acute graft failure after transplantation.

The authors thank Robert Figler, PhD, at Dogwood Pharmaceuticals, a subsidiary of Clinical Data, Inc, for providing us with the ATL-1223 compound used in this study, and Anthony Herring, Sheila Hammond, Cynthia Dodson, Shelby Catlett, and Richard Guyer for technical assistance during this study.

References

1. Ailawadi G, Lau CL, Smith PW, Swenson BR, Hennessy SA, Kuhn CJ, et al. Does reperfusion injury still cause significant mortality after lung transplantation? *J Thorac Cardiovasc Surg.* 2009;137:688-94.
2. Cottini SR, Lerch N, de Perrot M, Treggiari MM, Spiliopoulos A, Nicod L, et al. Risk factors for reperfusion injury after lung transplantation. *Intensive Care Med.* 2006;32:557-63.
3. Trulock EP, Christie JD, Edwards LB, Boucek MM, Aurora P, Taylor DO, et al. Registry of the International Society for Heart and Lung Transplantation: twenty-fourth official adult lung and heart-lung transplantation report-2007. *J Heart Lung Transplant.* 2007;26:782-95.
4. Aeba R, Griffith BP, Kormos RL, Armitage JM, Gasior TA, Fuhrman CR, et al. Effect of cardiopulmonary bypass on early graft dysfunction in clinical lung transplantation. *Ann Thorac Surg.* 1994;57:715-22.
5. Zenati M, Yousem SA, Dowling RD, Stein KL, Griffith BP. Primary graft failure following pulmonary transplantation. *Transplantation.* 1990;50:165-7.
6. Fiser SM, Tribble CG, Long SM, Kaza AK, Kern JA, Jones DR, et al. Ischemia-reperfusion injury after lung transplantation increases risk of late bronchiolitis obliterans syndrome. *Ann Thorac Surg.* 2002;73:1041-8.
7. Fredholm BB, Ap IJ, Jacobson KA, Klotz KN, Linden J. International Union of Pharmacology. XXV. Nomenclature and classification of adenosine receptors. *Pharmacol Rev.* 2001;53:527-52.
8. Olah ME, Stiles GL. Adenosine receptor subtypes: characterization and therapeutic regulation. *Annu Rev Pharmacol Toxicol.* 1995;35:581-606.
9. Chunn JL, Young HW, Banerjee SK, Colasurdo GN, Blackburn MR. Adenosine-dependent airway inflammation and hyperresponsiveness in partially adenosine deaminase-deficient mice. *J Immunol.* 2001;167:4676-85.
10. Linden J. Molecular approach to adenosine receptors: receptor-mediated mechanisms of tissue protection. *Annu Rev Pharmacol Toxicol.* 2001;41:775-87.
11. Gazoni LM, Laubach VE, Mulloy DP, Bellizzi A, Unger EB, Linden J, et al. Additive protection against lung ischemia-reperfusion injury by adenosine A_{2A} receptor activation before procurement and during reperfusion. *J Thorac Cardiovasc Surg.* 2008;135:156-65.
12. Gazoni LM, Walters DM, Unger EB, Linden J, Kron IL, Laubach VE. Activation of A₁, A_{2A}, or A₃ adenosine receptors attenuates lung ischemia-reperfusion injury. *J Thorac Cardiovasc Surg.* 2010;140:440-6.
13. Lisle TC, Gazoni LM, Fernandez LG, Sharma AK, Bellizzi AM, Schifflett GD, et al. Inflammatory lung injury after cardiopulmonary bypass is attenuated by adenosine A(2A) receptor activation. *J Thorac Cardiovasc Surg.* 2008;136:1280-7.
14. Reece TB, Ellman PI, Maxey TS, Crosby IK, Warren PS, Chong TW, et al. Adenosine A_{2A} receptor activation reduces inflammation and preserves pulmonary function in an in vivo model of lung transplantation. *J Thorac Cardiovasc Surg.* 2005;129:1137-43.
15. Sharma AK, Laubach VE, Ramos SI, Zhao Y, Stukenborg G, Linden J, et al. Adenosine A_{2A} receptor activation on CD4+ T lymphocytes and neutrophils attenuates lung ischemia-reperfusion injury. *J Thorac Cardiovasc Surg.* 2010;139:474-82.
16. Ross SD, Kron IL, Gangemi JJ, Shockey KS, Stoler M, Kern JA, et al. Attenuation of lung reperfusion injury after transplantation using an inhibitor of nuclear factor-kappaB. *Am J Physiol Lung Cell Mol Physiol.* 2000;279:L528-36.
17. Lee HT, Emala CW. Preconditioning and adenosine protect human proximal tubule cells in an in vitro model of ischemic injury. *J Am Soc Nephrol.* 2002;13:2753-61.
18. Lee WY, Lee SM. Ischemic preconditioning protects post-ischemic oxidative damage to mitochondria in rat liver. *Shock.* 2005;24:370-5.
19. Yildiz G, Demiryurek AT, Gumusel B, Lipton H. Ischemic preconditioning modulates ischemia-reperfusion injury in the rat lung: role of adenosine receptors. *Eur J Pharmacol.* 2007;556:144-50.
20. Toufektsian MC, Yang Z, Prasad KM, Overbergh L, Ramos SI, Mathieu C, et al. Stimulation of A_{2A}-adenosine receptors after myocardial infarction suppresses inflammatory activation and attenuates contractile dysfunction in the remote left ventricle. *Am J Physiol Heart Circ Physiol.* 2006;290:H1410-8.
21. Okusa MD, Linden J, Huang L, Rieger JM, Macdonald TL, Huynh LP. A(2A) adenosine receptor-mediated inhibition of renal injury and neutrophil adhesion. *Am J Physiol Renal Physiol.* 2000;279:F809-18.
22. Sharma AK, Lapar DJ, Zhao Y, Li L, Lau CL, Kron IL, et al. Natural killer T cell-derived IL-17 mediates lung ischemia-reperfusion injury. *Am J Respir Crit Care Med.* 2011;183:1539-49. Epub 2011 Feb 11.
23. Yang Z, Sharma AK, Linden J, Kron IL, Laubach VE. CD4+ T lymphocytes mediate acute pulmonary ischemia-reperfusion injury. *J Thorac Cardiovasc Surg.* 2009;137:695-702.
24. Glover DK, Riou LM, Ruiz M, Sullivan GW, Linden J, Rieger JM, et al. Reduction of infarct size and postischemic inflammation from ATL-146e, a highly selective adenosine A_{2A} receptor agonist, in reperfused canine myocardium. *Am J Physiol Heart Circ Physiol.* 2005;288:H1851-8.
25. Date H. [Experimental studies on reimplantation response after lung transplantation]. *Nippon Kyobu Geka Gakkai Zasshi.* 1989;37:510-21.
26. Le Gal YM. [Techniques involved in the protection of the lung and heart-lung for the purpose of transplantation]. *Rev Mal Respir.* 1990;7:535-9.

Discussion

Dr Patrick M. McCarthy (Chicago, Ill). That was a very clear presentation. Is there work ongoing in other organs? The concern would be about treating the lung donor if the effects on the other organs are unknown.

Dr LaPar. That is a very pertinent question. An extensive amount of work has been done with similar adenosine compounds in other organ systems, such as the kidney and the liver, and these investigations have demonstrated a significant benefit to using these agonists. To my knowledge, the use of the ATL-1223 compound as donor treatment in other organ systems has not been reported. However, on the basis of this study, we are now beginning experimental work with our other transplant colleagues to investigate the potential benefits of such treatment in other organ systems.

Dr J. William Gaynor (*Philadelphia, Pa*). Was any immunosuppression used in this model?

Dr LaPar. No.

Dr Gaynor. Are there any drug interactions of this agent with the commonly used immunosuppressive agents? If you give some other anti-inflammatory drug, such as a bolus of steroids, will you see the same marked effect?

Dr LaPar. To my knowledge, there are no documented interactions between immunosuppressive drugs such as steroids and some of the other A_{2A} agonists that we have used in the past, including ATL-146e or ATL-313. As for ATL-1223, the pharmacokinetics and drug interaction profiles are currently being investigated. However, on the basis of what we know of the other compounds, I would not anticipate any unforeseen negative interactions with concomitant immunosuppressive therapy.

Dr Michael E. Jessen (*Dallas, Tex*). This is a very interesting study. As you are moving toward clinical trials, I am concerned that your model does not quite model what happens in the clinical arena. Most donors have undergone some form of trauma or some

other event before organ procurement, which could incite the inflammatory response. Any time you are giving even a preoperative dose of the drug, there actually may already be an inflammatory response started. It sounded as though your protocol does not favor brain death, either, the process of which also incites a lot of inflammatory cytokine response. Would you be wiser to look at some of those scenarios before you take this to the clinical arena?

Dr LaPar. Thank you very much, Dr Jessen, for that question. You bring up an important point that a donor setting after multiple trauma or brain injury may be different from what we modeled in the laboratory in this study. In these settings, it is widely known that a profound inflammatory response is likely and that adenosine, in general, has profound anti-inflammatory effects. Thus I believe that even in these settings, provision of an agonist such as ATL-1223 to the donor is going to confer a greater level of protection than what can be achieved without it. I further believe that for certain patients supplemental treatment to the recipient could be beneficial to provide additional protection after establishing reperfusion.