Tissue-derived proinflammatory effect of adenosine A_{2B} receptor in lung ischemia–reperfusion injury

Farshad Anvari, MD,[a] Ashish K. Sharma, MBBS,[a] Lucas G. Fernandez, MD, DSc,[a] Tjasa Hranjec, MD, MS,[a] Katya Ravid, PhD,[b] Irving L. Kron, MD,[a] and Victor E. Laubach, PhD[a]

Objective: Ischemia–reperfusion injury after lung transplantation remains a major source of morbidity and mortality. Adenosine receptors have been implicated in both pro- and anti-inflammatory roles in ischemia–reperfusion injury. This study tests the hypothesis that the adenosine A_{2B} receptor exacerbates the proinflammatory response to lung ischemia–reperfusion injury.

Methods: An in vivo left lung hilar clamp model of ischemia–reperfusion was used in wild-type C57BL6 and adenosine A_{2B} receptor knockout mice, and in chimeras created by bone marrow transplantation between wild-type and adenosine A_{2B} receptor knockout mice. Mice underwent sham surgery or lung ischemia–reperfusion (1 hour ischemia and 2 hours reperfusion). At the end of reperfusion, lung function was assessed using an isolated buffer-perfused lung system. Lung inflammation was assessed by measuring proinflammatory cytokine levels in bronchoalveolar lavage fluid, and neutrophil infiltration was assessed via myeloperoxidase levels in lung tissue.

Results: Compared with wild-type mice, lungs of adenosine A_{2B} receptor knockout mice were significantly protected after ischemia–reperfusion, as evidenced by significantly reduced pulmonary artery pressure, increased lung compliance, decreased myeloperoxidase, and reduced proinflammatory cytokine levels (tumor necrosis factor-α; interleukin-6; keratinocyte chemoattractant; regulated on activation, normal T-cell expressed and secreted; and monocyte chemotactic protein-1). Adenosine A_{2B} receptor knockout→adenosine A_{2B} receptor knockout (donor→recipient) and wild-type→ adenosine A_{2B} receptor knockout, but not adenosine A_{2B} receptor knockout→wild-type, chimeras showed significantly improved lung function after ischemia–reperfusion.

Conclusions: These results suggest that the adenosine A_{2B} receptor plays an important role in mediating lung inflammation after ischemia–reperfusion by stimulating cytokine production and neutrophil chemotaxis. The proinflammatory effects of adenosine A_{2B} receptor seem to be derived by adenosine A_{2B} receptor activation primarily on resident pulmonary cells and not bone marrow-derived cells. Adenosine A_{2B} receptor may provide a therapeutic target for prevention of ischemia–reperfusion-related graft dysfunction in lung transplant recipients. (J Thorac Cardiovasc Surg 2010;140:871-7)

One of the most common causes of early morbidity and mortality after lung transplantation is primary graft dysfunction, which is a severe form of ischemia–reperfusion (IR) injury.[1] IR injury has been reported to account for up to 30% of early mortality after lung transplantation.[2] Although exact mechanisms of IR-induced injury remain unclear, numerous studies have established that acute inflammation is a key feature of IR injury.

Adenosine levels are known to increase in tissues as a result of inflammation, IR, hypoxia, and cellular stress. Adenosine is typically thought of as a retaliatory, anti-inflammatory response that exerts its effects via cell-surface G-coupled protein receptors, of which 4 subtypes have been identified: A_1 receptor (A_1R), A_{2A} receptor ($A_{2A}R$), A_{2B} receptor ($A_{2B}R$), and A_3 receptor (A_3R). The activation of adenosine receptors in different tissues has been shown to exert both pro- and anti-inflammatory responses.[3] The most recent adenosine receptor gene to be identified is $A_{2B}R$, which is expressed on a broad spectrum of cells in multiple organs, including the nervous system, intestines, lung, and heart. In the lung, $A_{2B}Rs$ are highly expressed on alveolar epithelial cells.[4] Among adenosine receptors, the $A_{2B}R$ has the lowest affinity for adenosine, and unlike other adenosine receptors it requires high levels of adenosine for activation that may not be reached under some physiologic conditions.[5] On the other hand, the expression of the receptor is induced under stresses, such as injury or oxidative stress, or by tumor necrosis factor (TNF)-α.[6] Some studies have suggested an anti-inflammatory role for $A_{2B}R$ in lung injury,[7] whereas others

From the Department of Surgery,[a] University of Virginia Health System, Charlottesville, Va; Departments of Biochemistry and Medicine,[b] Boston University School of Medicine, Boston, Mass.

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Address for reprints: Victor E. Laubach, PhD, Department of Surgery, University of Virginia Health System, P.O. Box 801359, Charlottesville, VA 22908 (E-mail: laubach@virginia.edu).

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Abbreviations and Acronyms

A_1R	= A_1 receptor
$A_{2A}R$	= A_{2A} receptor
$A_{2B}R$	= A_{2B} receptor
A_3R	= A_3 receptor
$A_{2B}R^{-/-}$	= $A_{2B}R$ knockout
BAL	= bronchoalveolar lavage
KC	= keratinocyte chemoattractant
IL	= interleukin
IR	= ischemia–reperfusion
MPO	= myeloperoxidase
RANTES	= regulated on activation, normal T-cell expressed and secreted
TNF	= tumor necrosis factor
WT	= wild-type

have shown a proinflammatory role.[8] These contradictory results underline the complexity in defining the role of $A_{2B}R$ in lung injury, because it could highly depend on the exact injury conditions applied and the relative contribution of bone marrow-derived versus tissue-derived $A_{2B}R$ in promoting inflammation. For instance, bone marrow cell-derived $A_{2B}Rs$ are clear protectors of inflammation,[9,10] whereas this might not apply to tissue-derived $A_{2B}R$.

We previously showed that activation of $A_{2A}R$ in the lung produces potent anti-inflammatory responses leading to improved lung function after IR.[11,12] The current study tested the ability of the $A_{2B}R$ to mediate lung injury and dysfunction after IR. $A_{2B}R$ knockout ($A_{2B}R^{-/-}$) mice were used to demonstrate the contribution of $A_{2B}R$ to lung IR injury as measured by pulmonary function and cytokine/chemokine production. Through the use of bone marrow chimeras, the role of $A_{2B}R$ on bone marrow versus non-bone marrow-derived cells in mediating IR injury was clarified.

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MATERIAL AND METHODS

Animals and Study Design

This study used 8- to 12-week-old male C57BL/6 wild-type (WT) mice (Jackson Laboratory, Bar Harbor, ME) and $A_{2B}R^{-/-}$ mice. The $A_{2B}R^{-/-}$ mice have been backcrossed onto the C57BL/6 background and display a normal phenotype. Their generation and characterization have been described.[9] Groups of mice (n = 5/group) underwent left lung IR or sham surgery (left thoracotomy). Preliminary analysis revealed that lung function in $A_{2B}R^{-/-}$ mice does not differ from WT mice, and thus a sham group of $A_{2B}R^{-/-}$ mice was not included in our comparisons. Separate groups of bone marrow chimeric mice were generated (n = 5/group) that underwent lung IR. This study conformed to the *Guide for the Care and Use of Laboratory Animals* published by the National Institutes of Health (publication No. 85-23, revised 1985) and was conducted under protocols approved by the University of Virginia's Institutional Animal Care and Use Committee.

In Vivo Model of Lung Ischemia–Reperfusion

An in vivo hilar clamp model of IR was used as previously described.[13] Mice were anesthetized using inhalational isoflurane, intubated, and ventilated at a rate of 120 strokes/min with room air (Harvard Apparatus Co, South Natick, Mass). Stroke volume was set at 1 mL, and peak inspiratory pressure was limited to less than 20 cmH_2O. Heparin (20 U/kg) was injected via the right external jugular vein to prevent thrombosis during ischemia. A left thoracotomy was performed in the third intercostal space. A 6-0 Prolene suture was passed around the hilum using a curved 22-G gavage needle. Both ends of the suture were then threaded through a 5-mm–long PE-50 tubing. Ischemia was initiated by pulling up on the suture and thus pressing the tube against the hilum and occluding it. A small surgical clip was applied to the suture at the end of the tube to maintain tension of the tube against the hilum. The thoracotomy was then closed, and the mouse was extubated and returned to its cage. Animals were kept warm by using a heat lamp. The average time for this stage of the procedure was 15 minutes per mouse. After a 1-hour period of ischemia, the mouse was reanesthetized and reintubated. Reperfusion was achieved by cutting the suture and removing the clip and the tubing. Again the thoracotomy was closed, and the mouse was extubated and returned to its cage. The average time for this stage of the procedure was 5 minutes. The animals were subsequently reperfused for 2 hours before analysis. To minimize pain and discomfort, an analgesic (buprenorphine, 0.2 mg/kg) was administered to all animals at the beginning of surgical intervention.

Generation of Chimeric Mice

Bone marrow chimeras were produced using standard techniques as previously described.[14] Briefly, donor mice (male, 24–26 g, age 8–10 weeks) were anesthetized with Nembutal (0.02 mg/g) and euthanized by cervical dislocation. Bone marrow from femurs was harvested under sterile conditions, yielding approximately 50 million nucleated bone marrow cells per mouse. The recipient mice (male, 22–25 g, age 6 weeks) were irradiated with 2 doses of 6 Gy each, 4 hours apart. Immediately after irradiation the mice were anesthetized using inhalational anesthesia and injected with 2 to 4×10^6 bone marrow cells via the tail vein. One control mouse did not receive an injection and subsequently died to confirm the efficacy of bone marrow depletion by irradiation. Transplanted mice were housed in micro-isolator cages for 6 weeks before experimentation. The following 4 different chimeras were produced (donor→recipient): WT→WT, $A_{2B}R^{-/-}$→$A_{2B}R^{-/-}$, WT→$A_{2B}R^{-/-}$, and $A_{2B}R^{-/-}$→WT (n = 5/group).

Measurement of Pulmonary Function

At the end of the 2-hour reperfusion period, pulmonary function was evaluated using an isolated, buffer-perfused mouse lung system (Hugo Sachs Elektronik, March-Huggstetten, Germany) as previously described by our laboratory.[15] Briefly, mice were anesthetized using a mixture of ketamine and xylazine. A tracheostomy was performed, and animals were ventilated with room air at 100 strokes/min and a tidal volume of 7 μL/g with a positive end-expiratory pressure of 2 cm H_2O. The animals were exsanguinated by transecting the inferior vena cava. The pulmonary artery was cannulated through the right ventricle, and the left ventricle was tube-vented through a small incision at the apex of the heart. The lungs were then perfused at a constant flow of 60 μL/g/min with Krebs–Henseleit buffer containing 2% albumin, 0.1% glucose, and 0.3% N-2-hydroxyethylpiperazine-N-2 ethanesulfonic acid (335–340 mOsm/kg H_2O). The perfusate buffer and isolated lungs were maintained at 37°C throughout the experiment using a circulating water bath. Once properly perfused and ventilated, the lungs were maintained on the system for a 5-minute equilibration period before data were recorded for an additional 5 minutes. Hemodynamic and pulmonary parameters were recorded using the PULMODYN data acquisition system (Hugo Sachs Elektronik).

Bronchoalveolar Lavage

After pulmonary function measurements, the right lung was occluded using a surgical clip. The left lung was lavaged with 0.4 mL of normal saline. The bronchoalveolar lavage (BAL) fluid was then immediately centrifuged at 4°C (500*g* for 5 minutes), and the supernatant was stored at –80°C until further analysis.

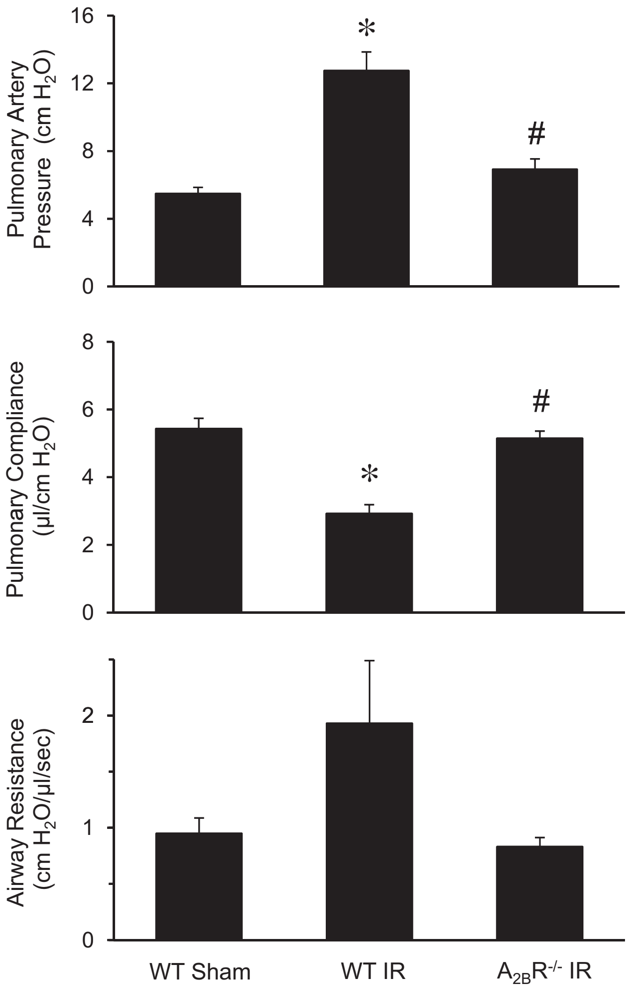

FIGURE 1. Pulmonary function after IR. Pulmonary artery pressure, pulmonary compliance, and airway resistance were improved after IR in $A_{2B}R^{-/-}$ mice compared with WT mice. *P < .05 versus WT sham, #P < .05 versus WT IR (n = 5/group). *$A_{2B}R^{-/-}$*, $A_{2B}R$ knockout; *IR,* ischemia–reperfusion; *WT,* wild-type.

Measurement of Myeloperoxidase

Myeloperoxidase (MPO) levels were measured in lung tissue using a commercially available mouse MPO enzyme-linked immunosorbent assay kit (Cell Sciences, Canton, Mass), and MPO levels were expressed as nanograms of MPO per micrograms of total lung protein. Lung tissue was homogenized in cell lysis buffer (Bio-Rad Laboratories, Hercules, Calif).

Measurement of Cytokines and Chemokines

Cytokines/chemokines in BAL fluid were quantified using a Bio-Plex Mouse Cytokine Multiplex Assay (Bio-Rad Laboratories) as previously performed.[16] The samples were analyzed as instructed with a Bioplex array reader, which is a fluorescent-based flow cytometer using a bead-based multiplex technology, each of which is conjugated with a reactant specific for a different target molecule.

Statistical Analysis

All data are presented as the mean ± standard error of the mean. Data were compared by 1-way analysis of variance, followed by Satterthwaite *t* test for unpaired data, which provides an adjustment for unequal variance between groups.

RESULTS

Pulmonary Function Is Improved After Ischemia–Reperfusion in $A_{2B}R^{-/-}$ Mice

As expected, pulmonary function was significantly impaired in lungs of WT mice after IR (WT IR) compared with WT sham mice (Figure 1). Pulmonary artery pressure was significantly increased, and lung compliance was significantly decreased in WT IR mice. Airway resistance was also higher in the WT IR mice but did not reach statistical significance. Compared with WT mice, pulmonary dysfunction was improved in strain-, age-, and sex-matched $A_{2B}R^{-/-}$ mice after IR, where the $A_{2B}R^{-/-}$ mice displayed significantly reduced pulmonary artery pressure, improved pulmonary compliance, and reduced airway resistance (Figure 1).

$A_{2B}R$ Deficiency Attenuates Cytokine/Chemokine Production After Ischemia–Reperfusion

The levels of TNF-α, interleukin (IL)-6, keratinocyte chemoattractant (KC) (CXCL1), and regulated on activation, normal T-cell expressed and secreted (RANTES) in BAL fluid were significantly increased in WT IR mice compared with sham (Figure 2). The $A_{2B}R^{-/-}$ mice showed significantly reduced levels of TNF-α, IL-6, KC, and RANTES after IR versus WT IR mice (Figure 2). MCP-1 level was also higher in WT IR lungs and was reduced in $A_{2B}R^{-/-}$ lungs, but this did not reach statistical significance (Figure 2).

Neutrophil Infiltration After Ischemia–Reperfusion Is Attenuated in the Absence of $A_{2B}R$

MPO is abundant in the azurophilic granules of polymorphonuclear neutrophils and was used as an indicator of neutrophil infiltration in lung tissue. As expected, MPO levels were significantly increased in lungs of WT mice after IR versus sham (Figure 3). MPO levels were significantly decreased in $A_{2B}R^{-/-}$ mice after IR compared with WT IR (Figure 3).

Proinflammatory Effects of $A_{2B}R$ Are Mediated by $A_{2B}R$ on Resident Pulmonary Cells

Pulmonary function was measured after IR in 4 groups of bone marrow chimeras. As expected, pulmonary artery pressure was significantly decreased and pulmonary compliance was significantly increased in $A_{2B}R^{-/-} \rightarrow A_{2B}R^{-/-}$ chimeras compared with WT→WT chimeras (Figure 4). Significant attenuation of lung dysfunction was also observed in the WT→$A_{2B}R^{-/-}$ chimeras, where these mice displayed reduced pulmonary artery pressure and increased pulmonary compliance compared with WT→WT (Figure 4). No protection was observed in the $A_{2B}R^{-/-}$→WT chimeras, where pulmonary artery pressure and compliance were similar to that of WT→WT controls. Airway resistance was not significantly different among the 4 groups of chimeras.

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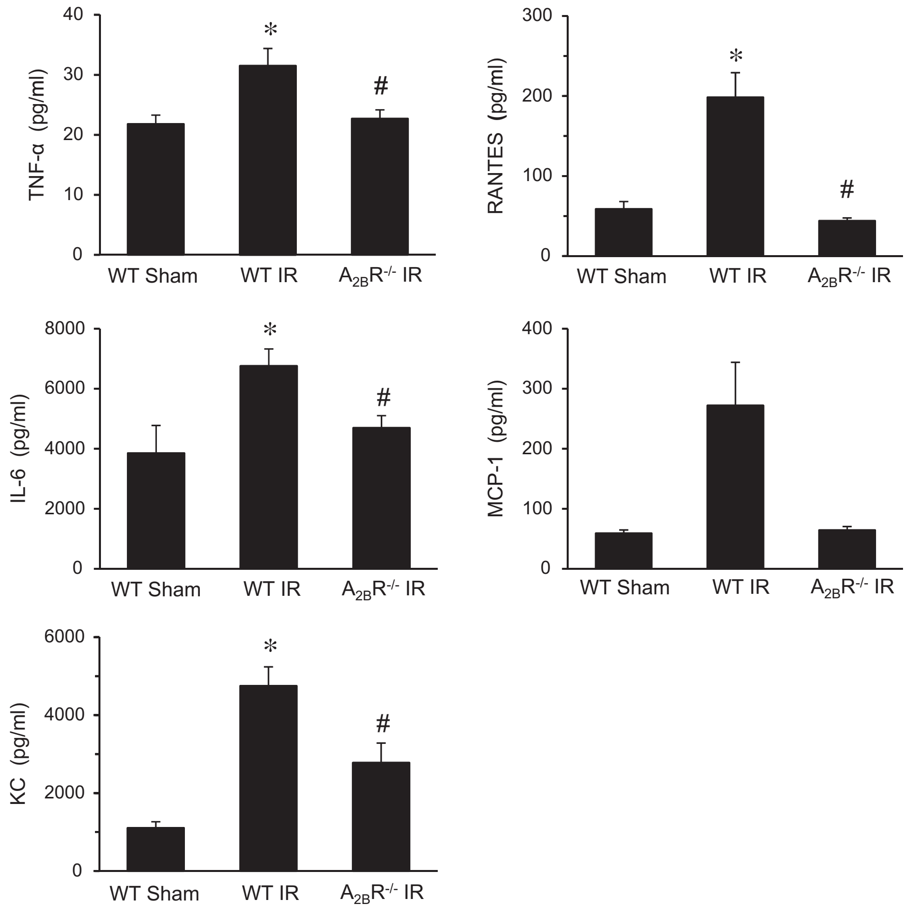

FIGURE 2. Expression of cytokines in BAL fluid after IR. Expression of TNF-α, IL-6, KC (CXCL1), RANTES, and MCP-1 were all significantly increased in WT mice after IR versus sham. Cytokine levels in $A_{2B}R^{-/-}$ mice after IR were significantly attenuated compared with WT IR. *$P < .05$ versus WT sham, #$P < .05$ versus WT IR (n = 5/group). *TNF,* Tumor necrosis factor; *IL,* interleukin; *KC*, keratinocyte chemoattractant; *IR,* ischemia–reperfusion; *WT,* wild-type; *MCP*, monocyte chemotactic protein; *RANTES,* regulated on activation, normal T-cell expressed and secreted; *$A_{2B}R^{-/-}$,* $A_{2B}R$ knockout.

DISCUSSION

This study used an in vivo mouse model to show that $A_{2B}R$ plays a proinflammatory role in lung IR injury. $A_{2B}R^{-/-}$ mice were used to demonstrate that $A_{2B}R$ gene deletion attenuates inflammatory responses in the lung after IR. Our data illustrate that pulmonary dysfunction is significantly attenuated in $A_{2B}R^{-/-}$ mice compared with WT mice, as evidenced by reduced pulmonary artery pressure and airway resistance and improved pulmonary compliance. In addition, lung inflammation after IR was significantly attenuated in $A_{2B}R^{-/-}$ mice, as evidenced by reduced production of proinflammatory cytokines/chemokines and MPO levels.

Bone marrow chimeric mice were used in an effort to identify key $A_{2B}R$-expressing cellular subsets that mediate proinflammatory responses after lung IR. In the WT$\rightarrow A_{2B}R^{-/-}$ chimeras, only bone marrow-derived cells (eg, leukocytes or macrophages) express $A_{2B}R$.[4] In the $A_{2B}R^{-/-}\rightarrow$WT chimeras, only non-bone marrow-derived cells (eg, smooth muscle cells, endothelial cells, and epithelial cells) express $A_{2B}R$.[4,9,17] Our results show that lungs of WT$\rightarrow A_{2B}R^{-/-}$ but not $A_{2B}R^{-/-}\rightarrow$WT chimeras were significantly protected after IR, suggesting that A_{2B}Rs on non-bone marrow-derived cells in the lung (ie, resident pulmonary cells such as epithelial cells) play a predominant role in mediating the inflammatory response after IR injury.

Compelling evidence has shown that lung IR injury is mediated by inflammatory responses.[13,16,18] Studies have also indicated that activated neutrophils are a primary mediator of this inflammatory response.[12,18,19] The results of the MPO assay in the current study (that IR-induced MPO levels are attenuated in the $A_{2B}R^{-/-}$ mice) are consistent with our pulmonary function data, suggesting neutrophil chemotaxis as a possible mechanism of the $A_{2B}R$'s role in lung IR.

Our data suggest that another mechanism for $A_{2B}R$-mediated proinflammatory responses in lung IR is through the modulation of cytokine/chemokine levels. The decrease in expression of KC, which is a potent neutrophil chemoattractant, in the $A_{2B}R^{-/-}$ mice suggests a proinflammatory role consistent with our MPO results. Furthermore, KC is known to be largely secreted by pulmonary epithelial cells, which

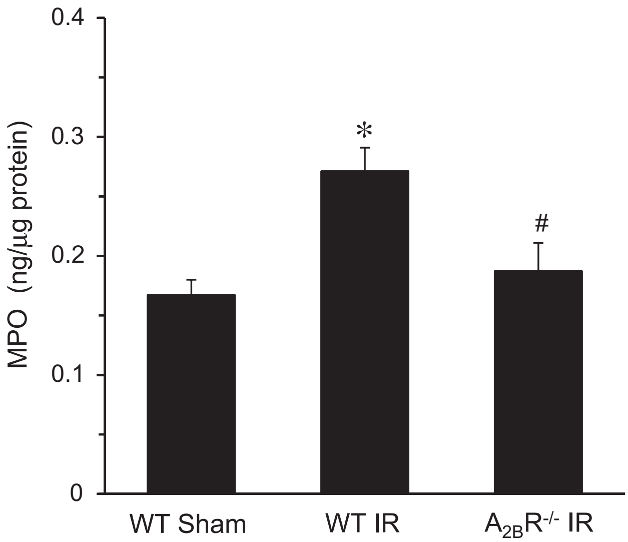

FIGURE 3. MPO levels in lung tissue were significantly elevated in WT mice after IR versus sham. MPO levels were significantly reduced in $A_{2B}R^{-/-}$ mice after IR compared with WT IR. *$P < .05$ versus WT sham, #$P < .05$ versus WT IR (n = 5/group). *$A_{2B}R^{-/-}$*, $A_{2B}R$ knockout; *IR,* ischemia–reperfusion; *WT,* wild-type.

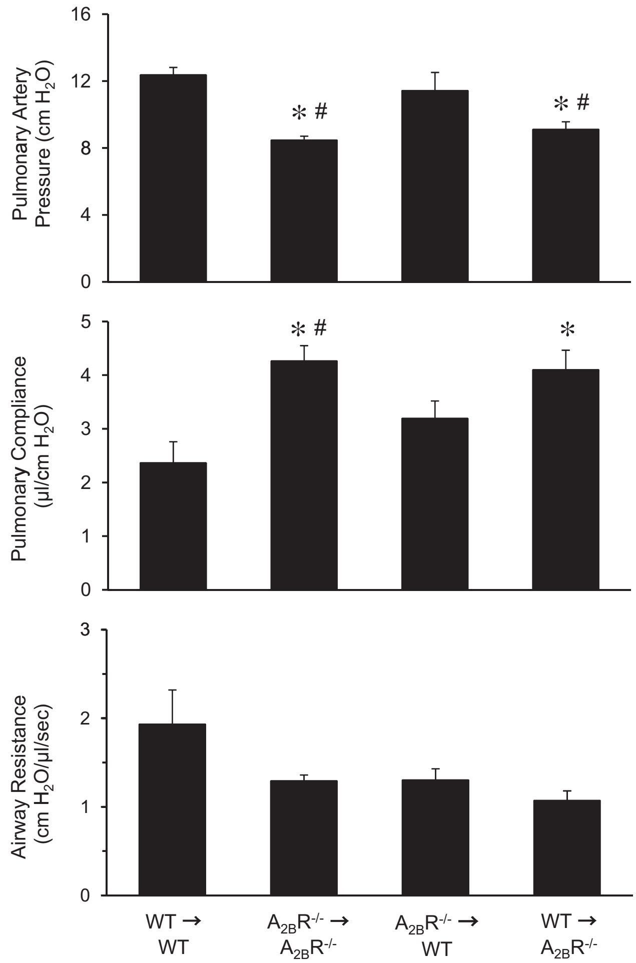

FIGURE 4. Pulmonary function after IR in bone marrow chimeras. All groups underwent lung IR. Pulmonary artery pressure and pulmonary compliance were significantly improved in $A_{2B}R^{-/-}\rightarrow A_{2B}R^{-/-}$ and WT$\rightarrow A_{2B}R^{-/-}$ chimeras compared with WT→WT chimeras (donor→recipient). *$P < .05$ versus WT→WT, #$P < .05$ versus $A_{2B}R^{-/-}\rightarrow$WT (n = 5/group). *$A_{2B}R^{-/-}$*, $A_{2B}R$ knockout; *IR,* ischemia–reperfusion; *WT,* wild-type.

supports the results of the bone marrow chimera experiment (that the $A_{2B}R$'s role in lung IR is mediated through resident pulmonary cells). This suggests that it is possible that $A_{2B}R$-mediated inflammation after IR is primarily directed by $A_{2B}R$ activation on lung epithelial cells. In addition, we observed that IL-6 levels were attenuated in the $A_{2B}R^{-/-}$ mice after IR. A prior study by Ryzhov and colleagues[20] demonstrated that $A_{2B}R$ exerts a proinflammatory response via secretion of IL-6, which is consistent with our observations.

The role of $A_{2B}R$ in modulating inflammatory responses to injury is complex and remains controversial. Studies have shown both pro- and anti-inflammatory responses for $A_{2B}R$ depending on the model and tissue used. A proinflammatory role of $A_{2B}R$ has been reflected in several studies.[21-24] For example, it has been shown that stimulation of $A_{2B}R$ exerts a proinflammatory role by stimulating IL-6 secretion and suppressing TNF-α production in mice.[20] In addition, Zhong and colleagues[24] showed that $A_{2B}R$ activation in human bronchial epithelial cells increases IL-19 secretion, which subsequently stimulates TNF-α release by monocytes. On the other hand, a number of studies have demonstrated an anti-inflammatory role for $A_{2B}R$ in different settings.[7,9,25,26] For example, basal levels of some proinflammatory cytokines, such as TNF-α, were found to be increased in tissues of $A_{2B}R^{-/-}$ mice,[22] and this has been interpreted as an anti-inflammatory role for $A_{2B}R$. In addition, a recent study by Schingnitz and colleagues[27] demonstrated a protective role of $A_{2B}R$ signaling in an endotoxin-driven lung injury model that was dependent on pulmonary $A_{2B}R$ signaling. These seemingly contradicting findings of $A_{2B}R$ may be due to its various signaling partners in different tissues and whether the signal in the system used originates from bone marrow cells or from tissues. Furthermore, some studies have shown that $A_{2B}R$ may exist in a multiprotein complex in the lung epithelia, and interactions with these partners may explain the different effects.[22,28] Endotoxin-induced inflammation is acute, involving a sharp elevation of inflammatory signals and cytokines, which typically involves bone marrow-derived cells. This has proven to be the case also with regard to the contribution of bone marrow cell $A_{2B}R$ to acute inflammation, as we have previously shown.[9] This is different from pulmonary tissue $A_{2B}R$ signaling, which evokes a milder and likely localized inflammatory profile. Indeed, in our lung IR injury model, tissue-derived rather than bone marrow-derived $A_{2B}R$ signals are involved. Our study leads us to suggest a paradigm according to which the response of $A_{2B}R$ to stress depends on whether it is an acute, systemic stress that involves bone

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marrow cells or rather localized stress that involves tissue signals. Continued investigations will be required to better understand the pro- and anti-inflammatory roles of $A_{2B}R$ under different settings.

Ventilator-induced injury could account for a significant part of the effects observed in various in vivo lung IR models. However, unlike many of these models in which mice are maintained on ventilation throughout reperfusion, we have reduced the total time of mechanical ventilation to less than 20 minutes to minimize injury caused by ventilation. This was done by extubating the animal during both the 1-hour ischemic and 2-hour reperfusion periods. The minimal lung injury and well-preserved function observed in our sham mice further support this point. Another limitation of our model is the assessment of lung function with the isolated, buffer-perfused lung system that includes both right and left lungs. As such, total lung function is measured and not just left lung function. However, this is a necessity of the system to prevent unacceptable injury to the left lung as a result of ventilation. Despite this, we have clearly demonstrated significantly impaired lung function after left hilar clamp versus sham and are confident that the dysfunction measured in our experiments is reflective of left lung injury.

An additional potential limitation of this study is that the expression of other adenosine receptors (A_1R, $A_{2A}R$ and A_3R) could have been abnormally affected in the $A_{2B}R^{-/-}$ mice after IR. This is unlikely, however, because we previously showed that $A_{2B}R$ gene deletion does not significantly affect the expression or activity of other adenosine receptor subtypes, as evidenced by reverse transcriptase-polymerase chain reaction.[9] Thus, the observed effects in the $A_{2B}R^{-/-}$ mice likely cannot be attributed to compensatory changes in expression of other adenosine receptors. We acknowledge that although the expression of any or all adenosine receptors could change after lung IR, this would apply to both WT and $A_{2B}R^{-/-}$ mice, which was not a focus of our study.

CONCLUSIONS

Pulmonary IR is a complex inflammatory response involving many components. The role of $A_{2B}R$ in this process remains the focus of ongoing investigations. The current study demonstrates a proinflammatory role for $A_{2B}R$ in the acute setting of lung IR injury, as evidenced by functional parameters, cytokine/chemokine expression, and MPO levels. Furthermore, the proinflammatory effects of $A_{2B}R$ can be attributed to $A_{2B}R$ activation primarily on resident pulmonary (non-bone marrow-derived) cells such as epithelia. These data suggest that $A_{2B}R$ may provide an additional therapeutic target for prevention or treatment of IR injury in lung transplant recipients. Although previous studies suggest that agonists for other adenosine receptors (A_1R, $A_{2A}R$, or A_3R) may be therapeutic in the setting of lung IR injury,[29,30] the present study highlights the importance of maintaining high specificity for these agonists to prevent the activation of $A_{2B}R$. Because a large amount of extracellular adenosine is known to be produced after IR, it may be optimal to use a combination therapy composed of an A_1R, $A_{2A}R$, or A_3R agonist along with an $A_{2B}R$ antagonist. Fortunately, because IR injury is rapidly initiated upon reperfusion and primary graft dysfunction usually occurs within 48 hours, the use of adenosine receptor agonists or antagonists to prevent IR injury would likely be required only during the initial 24 to 48 hours after transplant.

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Discussion

Dr Brendan Stiles *(New York, NY)*. Farshad, that was nicely presented. It is a good continuation of the work your group has done. I am a bit curious why you didn't use a sham experiment on the knockout mice. Or did you do that?

Dr Anvari. Our preliminary studies on these knockout mice showed that they are comparable to our WT mice in terms of pulmonary function, and we saw similar measurements as the WT mice. So we did not include 2 sham groups in these experiments.

Dr Stiles. So the baseline levels of cytokine expression, things like that, are no different between the knockout and the sham?

Dr Anvari. The baseline levels were similar for function. As far as cytokines, previous studies have shown that the baseline levels of some of the cytokines, including TNF-α and IL-6 are elevated in the knockout mice. So we did not repeat those experiments.

Dr Stiles. I am just concerned that you are knocking out a proinflammatory gene and then you are showing that there is a decrease in cytokine induction after IR, which you attribute to the treatment. Some of those things are downstream of the proinflammatory pathway that has been knocked out. I am concerned that what you are showing is more just a function of knocking that out rather than actual protection from IR. Do you think there is a way to tease that out?

Dr Anvari. This is an interesting question. The limitation with knockouts, as you mentioned, is that the observed effects may be the result of the creation of the knockout itself. We do know that the expression of other adenosine receptors are unchanged in these knockouts. I would look at this as a first step. We recently acquired agonists and antagonists for this receptor, and I think that will be the next step to validate these results. Our preliminary results look promising.

Dr Stiles. That is great. What is the delivery method of those, the agonists and antagonists?

Dr Anvari. Intravenous injections via the external jugular.

Dr Frank Sellke *(Providence, RI)*. I have a technical question. You used a *t* test for your statistical analysis, yet you had multiple groups. Shouldn't you use a multiple comparison test rather than a *t* test?

Dr Anvari. We performed an analysis of variance (ANOVA) followed by a *t* test for unpaired data.

Dr Sellke. Yes, but if you have multiple groups, you have to take the multiple comparisons into consideration, not just an ANOVA. You have to do a post hoc analysis with multiple comparisons like a Bonferroni correction or one of these tests into play.

Dr Anvari. Correct. We did an ANOVA in conjunction with a Tukey's test. We also performed additional analyses by looking at specific groups comparing 2 at a time using a *t* test.

Dr Sellke. So you are not really doing a Bonferroni correction if you are doing just a *t* test.

Dr Anvari. No. ANOVA in conjunction with Tukey's test. The *t* test was used for additional analysis.

Dr Michael Jessen *(Dallas, Tex)*. I enjoyed your study a lot. That is good work. The biggest area in cardiothoracic surgery I think where we see IR injury, as you pointed out, is in lung transplantation, but it is a tough thing to model. In some ways your model deviates from it. There is no denervation, no hypothermia, no preservation solution, and no immunosuppression on board. How confident are you that your findings will be maintained in a setting that is more clinically relevant to lung transplantation?

Dr Anvari. Those are all very relevant issues. We adopted this model as a first step in the process of better understanding the role of this receptor in pulmonary IR. We chose a mouse model because we had the benefit of the knockouts, and this provides a foundation. We need to continue this work by using drugs in this model, and if that confirms the results we are seeing, then we can take that to a bigger animal and use a more clinically relevant model. But I think this is an important first step in establishing that.

This in vivo model is, of course, as you mentioned, a warm ischemia model and doesn't take into account all the factors you mentioned, but in the mouse is the best model short of actual transplantation, which has recently been done. But, as you can imagine, it is technically challenging and not easily reproducible.

Dr Glen Van Arsdell *(Toronto, Ontario, Canada)*. Your group has been working with adenosine receptors for some time. The program also does lung transplantation. Have you taken human tissue to see whether there is a difference in expression and correlated that with IR that you see clinically?

Dr Anvari. We have not used any human tissue in our experiments so far.

Dr Van Arsdell. Is that coming down the line?

Dr Anvari. Hopefully, in the near future.