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The Role of Adenosine A_{2A} Receptor Signaling in Bronchiolitis Obliterans

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Abstract

Background—Binding of adenosine to the anti-inflammatory Gs-coupled adenosine 2A receptor (A_{2A}R) inhibits the activity of most inflammatory cells. Extensive preclinical evidence exists for the use of A_{2A}R agonists in the prevention of acute ischemia-reperfusion injury. Activation of A_{2A}Rs on lymphocytes and antigen-presenting cells also attenuates the alloimmune response. Because ischemia-reperfusion injury and alloimmunity are risk factors for the development of bronchiolitis obliterans (BO), the objective of this study was to determine the effect of A_{2A}R signaling on tracheal rejection in a mouse model of BO.

Methods—A non-revascularized heterotopic tracheal model across a total alloantigenic mismatch was used to study A_{2A}R signaling in a mouse model of BO. Tracheal transplants were performed using Balb/c donors into wild-type or A_{2A}R knockout C57BL/6 recipient mice. Another group of Balb/c transplants into C57BL/6 recipients were also treated with a selective A_{2A}R agonist. Tracheas were assessed at 3, 7, 12, 21, and 28 days after transplantation by hematoxylin and eosin staining, immunohistochemical staining, and collagen staining.

Results—Compared with allograft tracheas in wild-type recipients, allografts in A_{2A}R knockout recipients had increased inflammation and more severe BO development. Recipient wild-type mice treated with a selective A_{2A}R agonist were significantly protected from lymphocyte infiltration and luminal occlusion, but fibro-obliteration still developed by 28 days after transplantation.

Conclusions—Endogenous adenosine signals through the A_{2A}R to attenuate inflammatory and immune factors involved in BO development. Synthetic A_{2A}R agonists may provide a novel treatment strategy to prevent BO.

Keywords

bronchiolitis obliterans; Adenosine A_{2A} Receptor; lung injury; tracheal transplant

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INTRODUCTION

Lung transplantation currently is the preferred treatment option for a variety of end-stage pulmonary diseases. Remarkable progress in improving outcome has occurred through the refinement of technique and improved understanding of transplant rejection. Despite these improvements, bronchiolitis obliterans (BO), thought to be a manifestation of chronic allograft rejection [1], remains the major hurdle in preventing lung transplantation from reaching its full potential.

The mechanisms involved in the etiology of BO remain poorly understood. Alloimmune and nonimmune events are both believed to contribute. Persistent immunologic and inflammatory insults to the allograft airways are thought to eventually lead to chronic peribronchiolar leukocyte infiltration and subsequent fibro-obliteration of the airways. Because the lung is constantly exposed to the external environment through the airways, it may be particularly susceptible to injurious agents. Previous studies have identified several risk factors for the development of BO, including cytomegalovirus infection and pneumonia, mismatches at human leukocyte antigen (HLA) loci, development of antibodies to class I HLA, and gastroesophageal reflux with resultant aspiration [[2], [3], [4], [5] and [6]].

Adenosine is produced in response to ischemia or inflammation and protects tissues from injury [7]. Adenosine receptors are a critical part of the physiologic negative-feedback mechanism for limitation and termination of tissue-specific and systemic inflammatory responses [8]. Adenosine and the adenosine A_{2A} receptor (A_{2A}R) have been investigated in lung ischemia-reperfusion injury, and systemic administration of A_{2A}R agonists have been used successfully in preclinical models to reduce tissue injury in the setting of ischemia-reperfusion of the lung [9] as well as liver [10], kidney [11], heart [12], and skin [13]. The activity of most inflammatory cells, including macrophages, monocytes, T-lymphocytes, platelets, and polymorphonuclear leukocytes, is inhibited by activation of the anti-inflammatory Gs-coupled A_{2A}R, which results in reduced proinflammatory mediator production and decreased endothelial adhesion molecule expression [[10] and [14]].

Recent studies have shown the importance of A_{2A}R signaling in attenuating the adaptive immune system [[15], [16], [17], [18], [19] and [20]]. In murine skin transplants, allograft rejection was significantly attenuated with an A_{2A}R agonist, and this effect was blocked with an A_{2A}R antagonist [16]. In a mouse T cell-mediated model of colitis, A_{2A}R agonists prevented disease development [17]. In an in vivo mouse model of autoimmune-induced pneumonitis, A_{2A}R agonists prevented death and promoted tolerance [21].

In this article we present the results of a study of the role of A_{2A}R signaling in a mouse heterotopic tracheal model of BO. We show that endogenous adenosine imparts some protection against BO and that a selective A_{2A}R agonist produces even greater protection.

Material and Methods

Animals

For tracheal transplant experiments, Balb/c mice (Jackson Laboratory, Bar Harbor, ME) were used as donors. C57BL6 mice (Wild-Type; Jackson Laboratory) and A_{2A}R knock out (A_{2A}R KO/C57BL6 background) male mice (weight, 28 to 35 g; Linden's Laboratory) were used as recipients. The mice received humane care in accordance with *Principles of Laboratory Animal Care*, formulated by the National Society for Medical Research, and the *Guide for the Care and Use of Laboratory Animals*, prepared by the National Academy of Science and published by the National Institutes of Health. The study protocol was reviewed and approved by the Animal Care and Use Committee at the University of Virginia before experimentation.

Mouse Model of Heterotopic Tracheal Transplant

We used a heterotopic subcutaneous tracheal transplant model of BO as previously described [[22] and [23]]. A major histocompatibility complex (MHC) class I and class II mismatch was produced by transplanting Balb/c (H-2^d) tracheas into C57BL/6 or A_{2A} R KO (H-2^b) recipients. C57BL/6 into C57BL/6 transplants were used as isograft controls.

Experimental Group Design

Experimental mice were divided into five groups. Group 1: Balb/c tracheas were transplanted to C57BL/6 mice. Group 2: Balb/c tracheas were transplanted to A_{2A}R KO mice. Group 3: Balb/c tracheas were transplanted to C57BL/6 mice treated with the A_{2A}R agonist (ATL 313, Adenosine Therapeutics, Charlottesville, VA) delivered by Alzet Osmotic pumps (Models 1003D, 1007D, 1002D). In all cases the dose was 5 ng/kg/min, a dose that we have found is optimal for inhibiting reperfusion injury (Linden personal communication). Group 4: Balb/c tracheas were transplanted to C57BL/6 mice treated with Alzet pumps loaded with vehicle (0.1% dimethyl sulfoxide). The pumps were implanted in the frontal dorsal area immediately after the tracheal transplantation. For treatment at 21 and 28 days, the Model 1002 pump was replaced by implanting a new pump on day 14. Group 5: Transplants of C57BL/6 into C57BL/6 were performed for isograft control.

In groups 1 to 4, 4 donor tracheas were transplanted into 1 recipient, 3 recipients were used in each group and at day 3, 7, 12, 21, and 28. A total of 60 donors and 15 recipients were used in each group. In group 5, 3 donor tracheas were transplanted into 1 recipient, and 1 recipient was used at day 3, 7, 12, and 21, for a total of 12 donors and 4 recipients. The mice were euthanized, and the isograft and allografts were collected on days 3, 7, 12, 21, or 28 for histology and immunohistochemical staining.

Histology

Transplanted tracheal tissues were removed and immediately fixed in 4% formalin. After 24 hours they were embedded in paraffin, sectioned, and stained with hematoxylin and eosin or with anti-Mac-2, antineutrophil, and anti-CD3 antibodies. Collagen deposition was detected by trichrome staining (Gomori), followed by van Gieson stain.

Immunohistochemical Staining of Macrophages and Neutrophils

Macrophages and neutrophils were detected by immunohistochemical analysis. Rat antimouse neutrophil (AbD Serotec, Raleigh, NC) or rat antimouse macrophage (Mac-2, Accurate Chem, Westbury, NY) antibodies were detected with alkaline phosphatase-conjugated antirat immunoglobulin (Ig) G secondary antibody and Fast-Red (both Sigma, St Louis, MO). Purified normal rat IgG (eBioscience Inc, San Diego, CA) was used as a negative control. The sections were counterstained lightly with hematoxylin for viewing negatively stained cells. Macrophage and neutrophil infiltration were semi-quantified using Image-Pro Plus software (Media Cybernetics, Inc, Bethesda, MD). The densitometric value of positive staining area of each section was blindly selected and measured. The average value of each group (at least five different tracheal sections) was obtained for statistical analysis.

Immunohistochemical Staining of CD3+ T Cells

Transplanted tracheas were fixed with 4% paraformaldehyde at room temperature for 24 hours and then embedded, cut into serial sections (5 µm), dehydrated, and incubated with 1% hydrogen peroxide. After rinsing in phosphate-buffered saline, sections were boiled in unmasking solution (Vector Laboratories, Burlingame, CA) for 15 minutes and blocked with 10% serum. Immunostaining was performed with goat anti-mouse CD3ε antibody (SC-1127; Santa Cruz Biotechnology, Santa Cruz, CA) using Vectastain ABC kit (Vector). After

incubation with an avidin-biotin complex, immunoreactivity was visualized by incubating the sections with 3,3-diaminobenzidine tetrahydrochloride (DAKO Corp, Carpinteria, CA) to produce a brown precipitate. Sections were then counterstained with hematoxylin. The number of CD3+ T cells per high-power field was assessed by immunohistochemical staining of tracheal sections, and at least 5 fields were counted per trachea by blinded observers. The average cell number was used for statistical analysis.

Measurement of the Luminal Obliteration

Allografts were photographed at 4× magnification with a Nikon microscope (Nikon Corp, Tokyo, Japan) equipped with a charge-coupled device camera. The area of the obliterated lumen and the total area of lumen were measured using the Image-Pro Plus software. The percentage of the obliteration was calculated by the area of the fibrosis divided by the total area of lumen. Eight to 10 allografts were measured in each group. The data were used for statistical analysis.

Statistical Analysis

Data are presented as the mean ± standard error of the mean. The macrophage, neutrophil, and CD3+ T-cell counts were compared using one-way analysis of variance (ANOVA), followed by the *t* test for unpaired data with Bonferroni correction. Square roots of tissue cell counts were compared using one-way ANOVA. A value of $p < 0.05$ was considered significant.

Results

Tracheal Isografts

Isografts (C57BL/6 into C57BL/6) developed evidence of early ischemia-reperfusion injury with revascularization within 3 days and an associated loss of the epithelium from the basement membrane. There was reepithelialization of the basement membrane between 3 and 7 days. Beyond 7 days (even at 21 days), isografts appeared similar to nontransplanted C57BL/6 tracheas (data not shown); however, the allografts (Balb/c to C57BL/6) showed a very different pattern (Fig 1).

The histologic kinetics of allografts after transplantation from the different groups and different time points is shown in Figure 1 (vehicle group not shown, same results as allograft controls). In general, the airway obliteration, the wall thickness of the transplants, and the cellular infiltration increased with time from day 3 to day 28 in all of the allografts (data from day 28 are not shown in figures because all tracheas were completely obliterated.)

Protective Role of Endogenous Adenosine

Compared with controls, the intensity and timing of the rejection process was accelerated in A_{2A}R KO recipients (discussed subsequently) as evidenced by epithelial cell loss, cellular infiltration, luminal fibro-obliteration, collagen deposition, and luminal obliteration.

Epithelial loss—Control allografts (Balb/c into C57BL/6) exhibited loss of the airway epithelium within the first 3 days. From 3 to 7 days, early recovery was seen, followed by a later loss of epithelium. By day 12, 90% of the tracheal epithelium was lost in the controls. After 12 days, there was complete loss of epithelium (Fig 1).

Compared with controls, the intensity and timing of the epithelial loss was accelerated in A_{2A}R KO recipients. At 3 days, there was more extensive loss of epithelium; at 7 days, unlike in the controls, there was minimal reconstitution of the epithelium; and by 12 days, there was complete loss of epithelium (Fig 1, Table 1).

Leukocyte infiltration—In the control allografts, infiltration of macrophages peaked on day 7, whereas neutrophils peaked on day 3. Allografts in the A_{2A}R KO recipients had significantly increased macrophage and neutrophil infiltration, which peaked around day 12 but remained high throughout the time points. These findings show the allografts in the A_{2A}R KO mice have more inflammation than controls throughout the time points (Fig 2 and Fig 3, Table 1).

To determine if there is an adaptive immune role of A_{2A}R signaling in development of BO, immunohistochemistry was used to assess infiltration of CD3+ T cells in the different treatment groups. Few CD3+ T cells were detected in any allografts during the first 3 days after transplantation. Increasing infiltration of CD3+ T cells was apparent on day 7, peaked on day 12 (Fig 4, Table 1), and significantly decreased on day 21. The number of infiltrating CD3+ T cells was significantly increased in the A_{2A}R KO recipients compared with controls on days 7 ($p = 0.006$) and 12 ($p = 0.001$; Fig 4, Table 1). These results suggest that A_{2A}R activation by endogenous adenosine may protect transplants by inhibiting CD3+ T-cell infiltration.

Fibro-obliteration, collagen deposition, and luminal obliteration—Luminal fibro-obliteration was notable at 12 days and increased thereafter in all groups. The luminal obliteration of the allograft in A_{2A}R KO mice was significantly increased on days 7 ($p < 0.01$) and 12 ($p < 0.001$) compared with C57BL/6 controls (Fig 5A). On day 21, however, the difference between these two groups was not significant ($p = 0.126$).

Tracheas transplanted into A_{2A}R KO recipients had increased collagen deposition compared with the controls (Fig 5B). The collagen was located in the tracheal wall as well as intraluminally.

Role of A_{2A}R Agonist ATL 313

Epithelial loss—Significantly more cuboidal epithelium was preserved in recipient mice treated with ATL 313 compared with controls on day 3 (Fig 1, Table 1). At the other time points, no difference was seen in epithelial regeneration or loss between the control and the ATL 313 groups. These data suggest that ATL 313 protects against the early injury seen at day 3 in this model.

Leukocyte infiltration—Neutrophil and macrophage infiltration in the ATL 313-treated group was not different from controls at any time. Lymphocytes, however, were significantly decreased in the tracheal allografts of the ATL 313-treated recipients at 12 days ($p = 0.05$; Fig 4). At the other time points, the difference in lymphocytes between the two groups was not significant. These results suggest that inflammation is not improved by additional A_{2A}R stimulation (in excess of endogenous adenosine), but that adaptive immunity may be influenced by ATL 313 treatment.

Fibro-obliteration and collagen deposition—The tracheas transplanted into mice treated with ATL 313 did undergo eventual fibro-obliteration, but this process was delayed compared with the controls. At 12 days, fibro-obliteration was minimal in the allografts in the ATL 313-treated mice but was present in approximately 15% of the lumens in the controls. At 21 days, the lumens in the controls were obviously obliterated, but the allografts in the ATL 313-treated animals were less obliterated. The difference in these two groups was significant ($p < 0.01$; Fig 5A, Table 1). Fibro-obliteration was complete in all allografts by 28 days (data not shown).

Collagen deposition was stained with trichrome staining (Gomori), followed by van Gieson stain. Collagen was stained pink or deep red. The results demonstrated that collagen deposition was decreased at 12 days in the ATL 313-treated group compared with controls (Fig 5B).

Comment

The heterotopic tracheal model used in these studies is often referred to as obliterative airway disease [24] because it displays several features that are similar to human BO. Injury develops in heterotopic tracheas transplanted into HLA-mismatched recipients that can be divided into an acute phase characterized by inflammation resulting from ischemia-reperfusion injury and a later phase involving the adaptive immune system with lymphocyte infiltration and eventually fibro-obliteration. Our data show a beneficial effect of endogenous adenosine signaling by way of the A_{2A}R. The tracheas in the A_{2A}R KO animals showed increased epithelial loss, leukocyte infiltration, and accelerated luminal fibro-obliteration compared with control allografts. In addition, the use of a specific A_{2A}R agonist provided further protection from early epithelial cell loss and later BO development.

This early protection seen with both endogenous adenosine and use of ATL 313 is not surprising and goes along with previous studies on ischemia-reperfusion in other organs. Activation of the A_{2A}R on bone marrow-derived cells accounts almost entirely for the protective effects of A_{2A}R agonists in liver ischemia-reperfusion injury [7] and lipopolysaccharide-induced acute lung injury [8]. Cellular responses to A_{2A}R activation are widespread in leukocytes and are primarily mediated by cyclic adenosine monophosphate [8]. These effects include inhibition of oxidative burst in neutrophils [25] and reduced cytokine release from macrophages [[15] and [26]].

After lung transplantation, ischemia-reperfusion injury is associated with an increased risk that BO will develop, and this risk is directly related to the severity of acute primary graft dysfunction [[27] and [28]]. Some of the BO protection seen from endogenous adenosine and ATL 313 treatment could thus be a direct result of preventing the early ischemia-reperfusion injury, but in this study, we also show endogenous adenosine and an A_{2A}R agonist prevent lymphocyte infiltration into the trachea allografts. These findings suggest a protective role of adenosine by the A_{2A}R through the adaptive immune response in this model. Other models have shown that A_{2A}R signaling inhibits adaptive immunity by multiple mechanisms [[15], [16], [17], [18] and [19]].

Interestingly, an immunosuppressive loop involving adenosine was recently reported [20]. Expression of the ectoenzymes CD39 and CD73 was found to distinguish CD4+/CD25+/forkhead box P3+ T regulatory cells from other T cells [20]. Pericellular adenosine, generated from degradation of extracellular nucleotides by these ectoenzymes, interacts with A_{2A}R on T effector cells to down-regulate their effects and generate T regulatory cells. The importance of this cellular immunoregulation loop is seen by the failure of T regulatory cells from CD39-null mice to prevent allograft rejection in vivo [20].

Limitations of this study pertain to the heterotopic tracheal model, which is a well-described model, technically simple, and reproducible in its production of obliterative airway disease, but has significant shortcomings. It is not a good model to assess if A_{2A}R agonists benefit in BO is solely from their mitigation of ischemia-reperfusion because this model is not a vascularized or aerated model. Other models are used to study BO, including the orthotopic tracheal model; however this model is also not vascularized, is technically more challenging, and does not uniformly develop histopathologic BO. Of note, the orthotopic and the heterotopic tracheal transplant models are both large airway models for a small airway disease. It may be that other mouse models of rejection [29] or a recently reported novel murine model for BO [30] would be better to study the response of A_{2A}R agonists on the adaptive immune system. In the new murine model for BO, BO is caused by low-dose allogeneic T-cell infusion in a bone marrow transplant setting. Despite the lung being host-derived and the immune cells being donor-derived, it appears to have applicability for studying BO in a transplant setting [30].

In conclusion our results show a protective effect of endogenous adenosine and additional protection from ATL 313 by way of the A_{2A}R in BO development. Although more confirmation studies are needed, a synthetic A_{2A}R agonist (ATL 313) may provide a therapeutic strategy to prevent BO. BO was delayed in the animals treated with ATL 313 in this study compared with controls, but all allografts were completely fibro-obiterated by 28 days. These findings, however, are seen in the setting of no immunosuppression. We plan future experiments to see if using immunosuppression would allow a greater response with ATL 313.

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Fig 1.

Hematoxylin and eosin staining of the allograft from day (D) 3, D7, D12, and D21 after transplantation is shown in photomicrographs (original magnification $\times 4$). The high-power (original magnification $\times 40$) images of the selected area on D3 are shown on the up-right corner inset. Arrows indicates epithelial cells. (AT = ATL 313; BC = Balb/c; B6 = C57BL/6).

Fig 2.

Immunohistochemical staining of migratory macrophages into the allografts of Balb/c to adenosine 2A receptor ($A_{2A}R$) knockout mice, Balb/c to C57BL/6, and Balb/c to C57BL/6 mice treated with $A_{2A}R$ agonist ATL 313. (A) Migratory macrophages staining in allografts at 12 days after transplantation. Cells stained red indicate migratory macrophage infiltration. All sections were counterstained lightly with hematoxylin for viewing negatively stained cells. The slides were stained with anti-Mac-2 antibody. The magnification of all pictures is $\times 40$. (B) The bar graph shows the analysis of positive immunostaining of migratory macrophages in the allografts from day (D) 3 to D21. Data shown are the mean $\pm$ standard error values for each group.

Fig 3.

Immunohistochemical staining of neutrophils in the allografts of Balb/c to adenosine 2A receptor (A_{2A}R) knockout mice, Balb/c to C57BL/6, and Balb/c to C57BL/6 mice treated with the A_{2A}R agonist ATL 313. (A) Neutrophil staining in allografts at 12 days after transplantation is shown by cells stained red. All sections were counterstained lightly with hematoxylin for viewing negatively stained cells. The slides were stained with antineutrophil antibody. The magnification of all pictures is $\times 40$. (B) The bar graph shows the analysis of positive immunostaining of neutrophils in the allografts from day (D) 3 to D21. Data shown are the mean $\pm$ standard error values for each group.

Fig 4.

Immunohistochemical staining of CD3+ T cells in the allografts of Balb/c to adenosine 2A receptor (A_{2A}R) knockout mice, Balb/c to C57BL/6, and Balb/c to C57BL/6 mice treated with the A_{2A}R agonist ATL 313. (A) CD3+ T-cell staining in allografts at 12 days after transplantation is shown by cells stained brown. All sections were counterstained lightly with hematoxylin for viewing negatively stained cells. The slides were stained with anti-CD3 antibody. The magnification of all pictures is $\times 40$. (B) The bar graph shows the analysis of positive immunostaining of CD3+ T-cells in the allografts from day (D) 3 to D21. Data shown are the mean $\pm$ standard error values for each group.

Fig 5.

(A) Luminal obliteration of the allografts from day 3 to day 21. Data shown are the mean $\pm$ standard deviation. * $p < 0.05$; ** $p < 0.01$. (B) Collagen deposition in the allografts of Balb/c to adenosine 2A receptor ($A_{2A}R$) knockout mice, Balb/c to C57BL/6, and Balb/c to C57BL/6 mice treated with the $A_{2A}R$ agonist ATL 313 12 days after transplantation. The pink to red staining indicates collagen deposition. The magnification of all pictures is $\times 40$.

Table 1

Summary of the Key Effects of Adenosine 2A/Adenosine A_{2A} Receptor on Mouse Bronchiolitis Obliterans Development

Groups	Epithelial Lost or Regenerated	Infiltration			Luminal Obliteration	
		Macrophage	Neutrophil	CD3+ T cell		
BC to A _{2A} R KO	Lost on D3, no regeneration thereafter	+++	+++	+++		+++
BC to B6	Lost on D3, regenerated on D7, lost again on D12	++	++	++		++
BC to B6 and DMSO	Lost on D3, regenerated on D7, lost again on D12	++	++	++		++
BC to B6 and ATL	Lost partially on D3, regenerated on D7, 70% lost again on D12	++	++	+		+
B6 to B6	Lost on D3, regenerated on D7, remains thereafter	+	+	0		0

+ = minimal; ++ = moderate; +++ = extensive; ATL = ATL 313 (adenosine A_{2A} receptor agonist); BC = Balb/c; B6 = C57BL/6; D = day; DMSO = dimethyl sulfoxide; KO = knockout.