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Adenosine Signaling via the Adenosine 2B Receptor is Involved in Bronchiolitis Obliterans Development

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Abstract

Objectives—Adenosine is produced in response to ischemia or inflammation and protects tissues from injury. There are four adenosine receptors, which play a critical role in the physiological negative-feedback mechanism for limitation and termination of tissue-specific and systemic inflammatory responses. Accumulating evidence has focused on the anti-inflammatory and immunosuppressive role of the adenosine 2A receptor (A_{2A}R), and we have previously reported on its' role in the development of bronchiolitis obliterans (BO) following lung transplantation. However, few studies have reported on the role of the adenosine 2B receptor (A_{2B}R) in BO. Data suggests that the A_{2B}R has pro-inflammatory and profibrotic roles. We hypothesized that adenosine signaling through the A_{2B}R is involved in the development of BO.

Methods—A murine heterotopic tracheal model across a total alloantigenic mismatch was used to study A_{2B}R signaling in BO. Tracheal transplants consisted of Balb/c donor tracheas transplanted into wild-type or A_{2B}R knockout C57BL/6 recipients. Transplanted tracheas were removed 3, 7, 12, and 21 days after transplantation. The luminal obliteration was evaluated through hematoxylin and eosin staining and the cellular infiltration (macrophage, neutrophil, CD3+ and Foxp3+ regulatory T cell) was detected by immunohistochemical staining.

Results—In comparison to allografts in wild type recipients, tracheas transplanted into A_{2B}R knockout mice displayed less BO development on day 21. A_{2B}R knockout mice had an increase in CD3+ T cells and CD4+/CD25+/Foxp3+ regulatory T cells when compared to wild type on day 7. By day 12, more CD3+ T cells were present in the wild-type trachea compared to the A_{2B}R KO, but the percentage of CD4+/CD25+/Foxp3+ regulatory T cells remained higher in the tracheas of A_{2B}R KO mice.

Conclusions—A_{2B}R stimulation may promote the development of BO via inhibiting CD4+/CD25+/Foxp3+ regulatory T cell infiltration.

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Keywords

bronchiolitis obliterans; Adenosine_{2B} Receptor; lung injury

INTRODUCTION

Lung transplantation is currently recognized as an effective treatment for selected patients with end-stage pulmonary diseases. Despite notable advances in the last decade in operative techniques, immunosuppression and critical care, long term mortality for lung recipients remains the highest of all solid organ transplantations and is still hampered by the development of chronic rejection (1). The development of bronchiolitis obliterans (BO) following lung transplantation is a key factor contributing to patient mortality. BO is a form of chronic allograft rejection, characterized by histologic evidence of peribronchiolar leukocyte infiltration and subsequent fibro-obliteration of the small airways. Once BO develops, patients frequently experience a progressive decline in pulmonary function and most die of respiratory failure within 5 years of onset. Furthermore, BO responds poorly to immunosuppression. Thus, further understanding of the molecular mechanisms of BO development is critical to develop novel treatment strategies to prevent this devastating complication.

Extracellular adenosine elicits its' biological effects via activation of four distinct adenosine receptors (A₁, A_{2A}, A_{2B} and A₃)(2). Accumulated data indicates that endogenous adenosine released following cell injury confers protection against ischemia-reperfusion injury (IRI) in liver(3), kidney (4), heart(5) and lung(6). Commensurate with other studies, we have previously reported on the protective role of adenosine signaling via the A_{2A}R in IRI and BO (6, 7). Specifically, we have demonstrated that murine tracheal allografts in A_{2A}R knockout recipients displayed increased inflammation and more severe BO development compared to wild type C57BL/6 mice (7). Alternatively, since the A_{2B}R is the most highly expressed adenosine receptor within human donor bronchial epithelia (8) and human bronchial epithelial cell lines (9), it is plausible that it may also be involved in certain pulmonary diseases. Some reports suggest that adenosine signaling via the A_{2B}R exerts a proinflammatory effect within pulmonary tissue (9, 10), while other studies have demonstrated an anti-inflammatory effect in mouse acute lung injury models (11, 12). The role of A_{2B}R activation in the development of BO following lung transplantation remains unknown. Consequently, in this study, we investigated the potential role of adenosine signaling via the A_{2B} R in a murine heterotopic tracheal transplant model of BO development.

METHODS

Animals

Balb/c mice (Jackson Laboratory, Bar Harbor, ME) were used as donors. C57BL/6 mice (Wild-Type; WT, B6) (Jackson Laboratory, Bar Harbor, ME) and A_{2B}R knock out (A_{2B}R KO; on a C57BL/6 background) male mice (Linden's Laboratory) (25–35 grams) were used as recipients. The experimental protocol was reviewed and approved by the Animal Care and Use Committee at the University of Virginia.

Experimental group design

As previously described, we utilized a heterotopic subcutaneous tracheal transplant model of BO (7). Experimental mice were divided into two groups: (1) Balb/c tracheas transplanted to C57BL/6 mice; and (2) Balb/c tracheas transplanted to A_{2B}R KO mice. A total of 96 donors

and 24 recipients were used in each group: four donor tracheas were transplanted into one recipient, 3 recipients were used in each group and each time point (4 time points: day 3, 7, 12 and 21).

Histology

After sacrifice, all tracheal allografts were collected at each time point for histology and immunohistochemical staining. Transplanted tracheal tissues were processed according to our previous methods (7).

Immunohistochemical Staining of Migratory Macrophages and Neutrophils

Macrophages and neutrophils were detected by immunohistochemical staining as previously described (7, 13). We used either rat anti-mouse neutrophil (AbD Serotec, Raleigh, NC) or rat anti-mouse migratory macrophage (Mac-2, Accurate Chem, Westbury, NY) as primary antibodies. The secondary antibody was alkaline phosphatase - conjugated anti-rat IgG (Sigma, St Louis MO). Positively staining cells were detected with Fast-Red (Sigma, St Louis MO). Macrophage and neutrophil infiltration were semiquantified using Image-Pro Plus software (7, 13). The average value of each group (at least five different tracheal sections) was obtained for statistical analysis.

Immunohistochemical staining of CD3+ T cells, CD4+/CD25+/Foxp3+ regulatory T cells and CD31 molecule in the allografts

All CD3+ T cells, CD4+/CD25+/Foxp3+ regulatory T cells and CD31 were stained and quantified according to previous methods (7). After antigen unmasking and blocking, CD4+/CD25+/Foxp3+ regulatory T cells were detected using a polyclonal rabbit anti-mouse Foxp3 primary antibody (Abcam Inc, Cambridge, MA). The number of infiltrated CD3+ T cells and CD4+/CD25+/Foxp3+ regulatory T cells were counted and used for statistical analysis. In addition, goat anti-mouse CD31 antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA) was used as primary antibody to detect CD31 expression in all allografts. The expression of CD31 was semiquantified, and the average value of each group was obtained for statistical analysis.

Measurement of the luminal obliteration

Total luminal area as well as the area of the obliterated lumens of transplanted allografts were measured using the Image-Pro Plus software as previously described(7). The percent of obliteration was calculated by dividing the area of tracheal fibrosis by the total luminal area. Eight to ten allografts were measured in each group.

Statistical analysis

Data are presented as the mean $\pm$ SEM. For comparison between two groups, the Student's t test was used. For comparison of more than two groups, the macrophage, neutrophil and CD3+ T cell counts were processed using one-way ANOVA followed by Bonferroni correction. $P < 0.05$ was considered significant.

RESULTS

The following histopathological kinetics for untreated tracheal allografts post-transplantation have been characterized: 1) IRI within the first 3 days associated with loss of the airway epithelium, 2) AR from 3 to 14 days with early recovery then later loss of the epithelium; and 3) complete loss of epithelium and fibro-obliteration of the airway from 14 to 28 days post-transplantation(7).

Histopathological Comparison of Allografts between Two Groups at Various Time points

The histopathological staining patterns demonstrate the kinetics of allografts post-transplantation among different groups and different time points (Figure 1). Similar to our previous report (7), allografts within both A_{2B}R KO (Balb/c into A_{2B}R KO) and control mice (Balb/c into C57BL/6) exhibited a complete loss of the airway epithelium within the first 3 days (Figure 1A and 1B). From 3 to 7 days, lost epithelial cells were regenerated and formed a pseudoepithelial layer on the basement membrane of the allografts (Figure 1C and 1D). By day 12, the majority of the pseudoepithelial layer was absent again in both groups (Figure 1E and 1F). However, compared to controls, the intensity and timing of the epithelial loss was decreased in A_{2B}R KO recipients on day 12 (1E). Even on day 21, some of the epithelial cells were still observed on the basement membrane of the allografts in A_{2B}R KO recipients (Figure 1G), while the epithelial cells were completely lost and the lumen was obliterated in the C57BL/6 (Figure 1H). Within isograft control mice, epithelial cells loss similarly occurred at day 3 with regeneration at day 7; however, no further cell loss was observed up through day 21 (Figure 1I and 1J). Moreover, there was no histological evidence of BO development by day 21 among transplanted isografts (Figure 1I and 1J).

Macrophage and Neutrophil Infiltration into Allografts

Macrophage and neutrophil infiltration is a hallmark feature of IRI and the resultant inflammatory response. Consequently, we investigated migratory macrophage and neutrophil infiltration at four different time points following transplantation. Our results (Figure 2) demonstrate that macrophage infiltration peaked on days 3 and 7 and gradually decreased thereafter, while neutrophil infiltration (Figure 3) peaked on day 3 and day 12 and decreased on day 7 and day 21 within allografts of both groups. Moreover, macrophage infiltration in the allografts of A_{2B}R KO recipients was significantly elevated on day 7 compared to control recipients ($P=0.0465$), while neutrophil infiltration was decreased on days 3 ($P=0.0122$) and 21 ($P=0.0026$). These findings indicate that adenosine signaling via the A_{2B}R is involved in the early inflammatory response following tracheal transplantation.

CD3+ T-lymphocyte infiltration into allografts

In order to determine if there was an adaptive immune role of A_{2B}R signaling in the development of BO, infiltration of CD3+ T cells was assessed via immunohistochemistry in these two groups. As a result, few CD3+ T cells were detected within any allografts during the first 3 days after transplantation. The number of infiltrating CD3+ T cells was significantly increased in the A_{2B}R KO recipients compared to controls on days 7 ($P=0.0014$), but significantly decreased on day 12 ($P=0.029$). These results (Figure 4) indicate that adenosine signaling via the A_{2B}R alters CD3+ T cell infiltration, which may be critical for developing BO.

Identification of CD4+/CD25+/Foxp3+ regulatory T cells in the allografts

Among T cells, natural regulatory CD4+/CD25+/Foxp3+ T cells are known to play an important role in maintaining immunological tolerance to both self and to non-self antigens (14, 15). To further identify regulatory T cell populations in the allografts, immunohistochemical staining of Foxp3 was employed using allografts at various time points. The results (Figure 5) showed that depletion of A_{2B}R promoted CD3+/CD25+/Foxp3+ regulatory T cell infiltration into the allografts at days 3 ($P=0.0025$), 7 ($P=0.0014$), 12 ($P=0.0002$) and 21 ($P=0.0003$) compared to C57BL/6 recipients. These results suggest that endogenous adenosine signaling through the A_{2B}R facilitates chronic allograft rejection via suppression of Foxp3+ regulatory T cell infiltration following transplantation.

Expression of CD31 in the allografts

CD31 is generally used in the evaluation of angiogenesis (16). Accumulated evidence suggests that CD31 is a key participant in the adhesion cascade leading to extravasation of leukocytes during the inflammatory process (17–19). CD31 antibodies inhibit leukocyte migration across an endothelial cell monolayer in vitro (20) as well as in-vivo (21). CD31 may also be involved in activation of the T-cell receptor (18). Therefore, we examined allograft vascularization by comparing the density of CD31 staining among allografts of the two groups at different time points (Figure 6). CD31 immunostaining demonstrated that the density of CD31 expression was significantly increased in the allografts of A_{2B}R knockout recipients compared to control recipients on days 3 ($P=0.0069$), 7 ($P=0.0011$) and 12 ($P=0.0211$). However, no significant difference was observed on day 21 ($P=0.3589$). These results suggest that the increase of macrophage, CD3+ T cell and regulatory T cell infiltration maybe caused increased CD31 expression and density of vascularity in the allografts of A_{2B}R knockout recipients.

Luminal Obliteration

Minimum luminal fibro-obliteration was observed at 12-days but increased thereafter (Figures 1&7). There was no significant difference in luminal fibro-obliteration in the A_{2B}R KO recipients compared to controls by 12 days (Figure 1). However, the fibro-obliteration was decreased ($P=0.0426$) in the A_{2B}R KO recipients compared to controls by 21 days (Figure 7). These results suggest that adenosine signaling via the A_{2B}R contributes to the development of BO.

DISCUSSION

In this study, we have demonstrated that endogenous signaling via the A_{2B}R results in enhanced development of bronchiolitis obliterans following murine tracheal allotransplantation. The heterotopic tracheal transplantation model used in the current study displays several key features that are similar to human BO. In our experimental model, the allografts in A_{2B}R KO mice demonstrated delayed epithelial loss; alterations of CD3+ T cells, CD4+/CD25+/Foxp3+ regulatory T cells, and neutrophil infiltration; and delayed luminal fibro-obliteration compared to control allografts. As the A_{2B}R subtype has been proven to be the predominant adenosine receptor expressed in human lung fibroblasts (22), in donor specimens of human bronchial epithelial cell (8) and in human bronchial epithelial cell lines (9), adenosine antagonism at the A_{2B}R may be a potential therapeutic target for prevention or treatment of BO post lung transplantation.

Our experimental results implicate an important interaction between A_{2B}R activation and CD4+/CD25+/Foxp3+ regulatory T cell activity during BO development. Accumulated evidence suggests that the CD4+/CD25+/Foxp3+ regulatory T cell population is actively involved in the negative control of a variety of physiological and pathological immune responses, and that this cell population can be exploited for the induction of immunological tolerance to non-self antigens, including organ transplantation (14). Furthermore, regulatory T cells are defined by expression of the forkhead family transcription factor (Foxp3), which is found in CD4+/CD25+ peripheral T cells and CD4+/CD25+/CD8-thymocytes (23, 24) and is essential for the development and function of regulatory T cells (25). Our results reveal that Foxp3 expressing CD4+/CD25+/- T-regulatory cells were markedly increased in the allografts in the A_{2B}R KO recipients when compared with control mice at day 3, 7, 12, and 21. In addition, the depletion of A_{2B}R (A_{2B}R KO) significantly increased CD3+ T cell infiltration in the allografts on day 7; however, it prevented CD3+ lymphocyte infiltration into the trachea allografts at day 12 compared with the control group. As a result, it appears that endogenous adenosine promotes the rejection of transplanted trachea at least in part

through the binding and activation of the A_{2B}R which in turn inhibits CD4⁺/CD25⁺/Foxp3⁺ regulatory T cell infiltration in the allografts.

Another possible way that A_{2B}R depletion inhibits BO development is through an attenuated IRI as evidenced by decreased neutrophil infiltration on days 3 and 21. Inflammation in general may be decreased in these recipients as we have shown neutrophil infiltration to be decreased on day 21 as well. Reports have shown that lung IRI is associated with an increased risk of developing BO, and this risk is directly related to the severity of acute primary graft dysfunction after lung transplantation (26, 27). Additionally, our experimental results are consistent with previous studies that have shown that synthesized A_{2B}R-selective antagonist CVT-6694 attenuated Adenosine-5'-N-ethyluronamide (NECA) induced interleukin (IL)-6 and monocyte chemotactic protein-1 (MCP-1) in bronchoalveolar lavage smooth muscle cells (9). Collectively, these results suggest a novel mechanism whereby adenosine functions as a proinflammatory mediator in the bronchiole airways. Similarly, the A_{2B}R subtype is the predominant adenosine receptor expressed in human lung fibroblasts, which upon activation by NECA increases the release of IL-6 and induces the differentiation of fibroblast into myofibroblasts (22). Several other reports have corroborated the anti-inflammatory effects of A_{2B}R antagonism. In mice, A_{2B}R-selective antagonism with CVT-6883 significantly reduced AMP-induced airway reactivity (28) and bleomycin-induced pulmonary fibrosis and inflammation (10), and the antagonist MRE-2029-F20 has been shown to inhibit the levels of cAMP in neutrophils and lymphocytes (29).

Leukocyte infiltration and transmigration is a hallmark inflammatory event in the early and intermediate stages of BO development. This process is regulated at multiple levels by the expression and activation of different molecules, including platelet endothelial cell adhesion molecule-1 (PECAM-1 or CD31)(18, 19). In the current study, we have demonstrated that depletion of the A_{2B}R induced expression of CD31 may lead to increase macrophage, CD3⁺ T cell and CD4⁺/CD25⁺/Foxp3⁺ regulatory T cells infiltration in allografts on day 7 post-transplantation.

Despite our significant results, there are some limitations with the use of the heterotopic tracheal model. Although our experimental model is well described, technically simple, and reproducible in its production of obliterative airway disease, we have described some of its limitations in previous studies (7). A recently reported novel murine model for BO (30) may prove additionally valuable to investigate the role of A_{2B}R in BO development.

Conclusion

Based upon our experimental results, we conclude that depletion of A_{2B}R in mice confers delayed development of bronchiolitis obliterans compared to control mice and altered T-lymphocyte infiltration with increased T-regulatory cells migration in tracheal allografts. Manipulation of the A_{2B}R with a selective synthetic antagonist (31) may provide a therapeutic option for the prevention and treatment of bronchiolitis obliterans.

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Figure 1. Comparison of histopathological characters of the allografts in A₂B^R KO and C57BL/6 recipients on days 3, 7 12 and 21

A) Illustration of tracheal thickness measurement in the allografts. All the HE staining pictures were taken under microscope with 4X magnification. The donor tracheas were Balb/c mice. A, C, E and G are examples of allografts in A₂B^R KO recipients at days 3, 7, 12 and 21, respectively. B, D, F and H are examples of allografts in C57BL/6 recipients at days 3, 7, 12 and 21, respectively. The high magnification (40X) of the allografts are also displayed at the right-bottom corner of each representative picture to show the changes of epithelial cells post transplantation. I (4X magnification) and J (40X magnification) is a representative isograft of Balb/c mice at days 21.

Figure 2. Immunohistochemical staining of migratory macrophages in the allografts of Balb/c to A₂B₂R KO and Balb/c to C57BL/6

A) Representative migratory macrophages staining in allografts at days 7 and 12 post-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 rat anti-mouse Mac-2 antibody. All pictures are 40X magnification. **B) The bar graph shows the analysis of positive immunostaining of migratory macrophages in the allografts from days 3 to 21.**

Figure 3. Immunohistochemical staining of neutrophils in the allografts of Balb/c to A_{2B}R KO, Balb/c and C57BL/6

A) Representative pictures of neutrophil staining in allografts at days 7 and 12 post-transplantation. Cells stained red indicate neutrophil infiltration. All sections were counterstained lightly with hematoxylin for viewing negatively stained cells. The slides were stained with rat anti-mouse neutrophil antibody. All pictures are 40X magnification. **B) The bar graph shows the analysis of positive immunostaining of neutrophils in the allografts from day 3 to day 21.**

Figure 4. Immunohistochemical staining of CD3+ T-cells in the allografts of Balb/c to A₂B KO and Balb/c to C57BL/6

A) Representative pictures of CD3+ T-cell staining in allografts at days 7 and 12 post-transplantation. Cells stained brown indicate CD3+ T-cell infiltration. All sections were counterstained lightly with hematoxylin for viewing negatively stained cells. The slides were stained with goat anti-mouse CD3 antibody. All pictures are 40X magnification. **B) The bar graph shows the analysis of positive immunostaining of CD3+ T-cell in the allografts from day 3 to day 21.**

Figure 5. Immunohistochemical staining of Foxp3+/CD4+/CD25+ regulatory T-cells in the allografts of Balb/c to A₂B^R KO and Balb/c to C57BL/6

A) representative pictures of Foxp3+/CD4+/CD25+ regulatory T-cell staining in allografts at days 7 and 12 post-transplantation. Cells stained dark-brown indicate Foxp3+/CD4+/CD25+ regulatory T-cell infiltration. All sections were counterstained lightly with hematoxylin for viewing negatively stained cells. The slides were stained with polyclonal rabbit anti-mouse Foxp3 antibody. All pictures are 40X magnification. **B) The bar graph shows the analysis of positive immunostaining of Foxp3+/CD4+/CD25+ regulatory T-cells in the allografts from day 3 to day 21.**

Figure 6. Immunohistochemical staining of CD31 in the allografts of Balb/c to A₂B_R KO and Balb/c to C57BL/6

A) representative pictures of CD31 staining in allografts at days 7 and 12 post-transplantation. Brown (black arrows) indicates CD31 positive staining. All sections were counterstained lightly with hematoxylin for viewing negatively stained cells. The slides were stained with goat anti-mouse CD31 antibody. The magnifications of all pictures were 10X. **B) The bar graph shows the analysis of positive immunostaining of CD31 in the allografts from day 3 to day 21.**

Figure 7. Luminal obliteration in the transplanted tracheas from days 3 to 21
Data shown are the mean $\pm$ S.D.