Genetic removal of the $A_{2A}$ adenosine receptor enhances pulmonary inflammation, mucin production, and angiogenesis in adenosine deaminase-deficient mice

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Mohsenin A, Mi T, Xia Y, Kellems RE, Chen J-F, Blackburn MR. Genetic removal of the $A_{2A}$ adenosine receptor enhances pulmonary inflammation, mucin production, and angiogenesis in adenosine deaminase-deficient mice. Am J Physiol Lung Cell Mol Physiol 293: L753–L761, 2007. First published June 29, 2007; doi:10.1152/ajplung.00187.2007.—Adenosine is generated at sites of tissue injury where it serves to regulate inflammation and damage. Adenosine signaling has been implicated in the regulation of pulmonary inflammation and damage in diseases such as asthma and chronic obstructive pulmonary disease; however, the contribution of specific adenosine receptors to key immunoregulatory processes in these diseases is still unclear. Mice deficient in the purine catabolic enzyme adenosine deaminase (ADA) develop pulmonary inflammation and mucous metaplasia in association with adenosine elevations making them a useful model for assessing the contribution of specific adenosine receptors to key immunoregulatory processes in these diseases. The purpose of the current study was to examine the contribution of $A_{2A}$ receptor signaling to the pulmonary phenotype seen in ADA-deficient mice. This was accomplished by generating ADA/A2AR double knockout mice. Genetic removal of the $A_{2A}$ receptor from ADA-deficient mice resulted in enhanced inflammation comprised largely of macrophages and neutrophils, mucin production in the bronchial airways, and angiogenesis, relative to that seen in the lungs of ADA-deficient mice with the $A_{2A}$ receptor. In addition, levels of the chemokines monocyte chemoattractant protein-1 and CXCL1 were elevated, whereas levels of cytokines such as TNF-α and IL-6 were not. There were no compensatory changes in the other adenosine receptors in the lungs of ADA/A2AR double knockout mice. These findings suggest that the $A_{2A}$ receptor plays a protective role in the ADA-deficient model of pulmonary inflammation.

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Adenosine is generated at sites of tissue injury where it serves to regulate inflammation and damage by engaging cell surface adenosine receptors on various cell types. Four adenosine receptors have been identified: $A_1R$, $A_{2A}R$, $A_{2B}R$, and $A_3R$. These receptors are members of the G protein-coupled family of receptors and are expressed on a variety of cells including most immune cells, further implicating a role for adenosine in the modulation of immune cell activity. Substantial experimental data suggest that adenosine serves as an anti-inflammatory agent following injury; however, if inflammation progresses unresolved and becomes chronic in nature, excessive tissue damage and adenosine accumulation may activate exacerbation pathways (3). Efforts to understand how different adenosine receptor pathways are activated in specific disease situations will help guide the use of receptor-specific agonists and antagonists in the treatment of various inflammatory disorders.

There is evidence that adenosine signaling plays an important role in the regulation of pulmonary inflammation and damage. Asthmatics exhibit elevations in adenosine in bronchoalveolar lavage (BAL) fluid (14) and exhaled breath condensates (27), and lung adenosine levels are elevated in mouse models of Th helper type 2 (Th2) cytokine-induced lung disease where adenosine activates pathways that promote pulmonary inflammation and damage (4, 31). In addition, mice deficient in adenosine deaminase (ADA), which catalyzes the breakdown of adenosine, develop pronounced pulmonary inflammation and damage in association with elevations in lung adenosine concentrations (6). The pulmonary phenotype seen in ADA-deficient mice is characterized by an increase in alveolar macrophages, airway remodeling including increased mucin production, angiogenesis, and alveolar airway enlargement (6, 33). The ADA-deficient model of adenosine-mediated pulmonary inflammation and damage has been useful in investigating the contribution of individual adenosine receptors to aspects of pulmonary disease. Genetic removal of the $A_1R$ from ADA-deficient mice results in enhanced pulmonary inflammation and damage suggesting a protective role for this receptor in this model (42). In contrast, genetic removal of the $A_{2B}R$ (48), or pharmacological inhibition of the $A_{2A}R$ (43), revealed proinflammatory activities for these receptors in the adenosine-mediated lung disease seen in ADA−/− mice. These findings suggest that in this model different adenosine receptors regulate opposing pathways in the control of inflammation.

Many of the anti-inflammatory effects of adenosine observed during tissue injury have been attributed to $A_{2A}$ receptor engagement (30). This receptor is one of the earliest sensors of elevated adenosine concentrations and thus also functions as an early sensor of tissue damage (22). The $A_{2A}R$ is coupled to $G_{\alpha_\text{i}}$.
resulting in activation of adenylate cyclase and accumulation of cAMP (20, 28), a molecule with a long history of playing a prominent role in immune modulation and immunosuppression (8, 25). Studies using A2A-deficient mice have revealed the anti-inflammatory properties of this receptor. For example, A2A-R-deficient mice are more sensitive to challenges with infectious and noninfectious agents (34), and treatment of wild-type mice challenged with lipopolysaccharide (LPS) or live Escherichia coli with an A2A-R agonist results in increased survival, and this protection was ablated in A2A-R-deficient mice (40). Further evidence comes from in vitro studies using peritoneal macrophages stimulated with LPS that show an induction of the proinflammatory cytokine TNF-α (23). Treatment of these mice with an A2A-R agonist results in significantly reduced TNF-α production, and this protection is no longer present in A2A-R-deficient mice (23). These and many other studies provide evidence that the A2A-R functions to inhibit inflammation and promote tissue protection. However, the contribution of A2A-R signaling has not been examined in the ADA-deficient model of adenosine-mediated lung inflammation.

The purpose of the current study was to examine the contribution of A2A-R signaling to the pulmonary phenotype seen in ADA−/− mice. This was accomplished by generating ADA/A2AR double knockout mice. Genetic removal of the A2AR from ADA−/− mice resulted in an enhancement of inflammation, mucin production, angiogenesis, and chemokine expression in the lungs beyond that provided by ADA deficiency alone. These findings suggest that the A2AR plays a protective role in the A2A-R-deficient model of pulmonary inflammation.

MATERIALS AND METHODS

**Mice.** ADA−/− mice were generated and genotyped as previously described (5). ADA−/− mice were on a mixed background of 129/Sv, C57BL/6J, and FVB/N strains. Control mice, designated ADA+, were littermates that were either wild-type (+/+) or heterozygous (+/-) for the null Ada allele, as heterozygous mice do not display a phenotype (5). A2A-R-deficient (A2A-R−/−) mice were generated as previously described (9). A2A-R−/− mice were on a mixed background of 129-Steel and C57BL/6J. ADA/A2AR double knockout mice were generated by breeding ADA−/− mice with A2A-R−/− mice. All mice were maintained and experiments conducted in accordance with the Animal Care Committee at the University of Texas Health Science Center at Houston (protocol no. HSC-AWC-06-130). Mice were housed in ventilated cages equipped with microisorator lids and maintained under strict containment protocols. No evidence of bacterial, parasitic, or fungal infection was found, and serologies on cage water were negative. The studies were approved by the Animal Care Committee at the University of Texas Health Science Center at Houston (protocol no. HSC-AWC-06-130).

**Histology.** Aged-matched control and experimental animals were killed, and the lungs were infused with 4% paraformaldehyde in PBS at 25 cm of pressure and then fixed overnight at 4°C. Fixed lung samples were rinsed in PBS, dehydrated, and embedded in paraffin. Sections (5 μm) were collected on microscope slides and stained with hematoxylin and eosin (Shandon Lipshaw, Pittsburgh, PA) or periodic acid-Schiff (PAS; EM Science, Lawrence, KS) according to the manufacturer’s instructions.

**BAL fluid collection and analysis.** Mice were anesthetized with avertin, and lungs were lavaged four times with 0.3 ml of PBS; 0.95–1 ml of pooled lavage fluid was recovered. Total cell counts were determined using a hemacytometer, and aliquots were cytospun onto microscope slides and stained with Diff-Quick (Dade Behring, Newark, DE) for cellular differentials.

**Airway mucosal analysis and quantification.** The extent of mucin production in bronchial airways was determined by quantifying the amount of PAS-stained material in the bronchial airways using Image-Pro Plus analysis software as previously described (6). PAS-stained material was identified on digitized images, and the pixel intensities of each color channel (red, blue, and green) were averaged. This was repeated for each image, and the values were averaged and used to determine the area (A) and intensity (I) of PAS-stained material in bronchial airways. In addition, the area (A) of the total epithelium (including PAS-stained material) was determined. The mucus index was determined using the following equation: M = IA/A. Final indices were results of an average of eight images per lung encompassing large and small bronchial airways.

**CXCR2 immunohistochemistry.** CXCR2 immunohistochemistry was adopted from previously published protocols (36). Briefly, 5-μm sections from paraffin-embedded lungs were deparaffinized and rehydrated. Endogenous peroxidase activity was quenched, and sections were blocked for 1 h in 10% rabbit serum and 0.5% fish skin gelatin oil. Sections were incubated overnight at 4°C with a rat anti-mouse monoclonal antibody against CXCR2 (R&D Systems, Minneapolis, MN) at a concentration of 1 μg/ml. Signal was detected using a Vector Elite anti-rat ABC kit (Vector Laboratories, Burlingame, CA) and diaminobenzidine (Sigma-Aldrich, St. Louis, MO) as a chromogen.

**Muc5ac immunofluorescence.** Sections (5 μm) from paraffin-embedded lungs were deparaffinized and rehydrated. Sections were treated with a 1:1 ratio of acetic acid and methanol for 15 min at room temperature followed by 30-min incubation with 1% sodium borohydrate at room temperature (Sigma-Aldrich). Antibody retrieval was then conducted using Target Retrieval Solution according to manufacturer instructions (DAKO). Blocking was performed according to instructions provided with the Mouse-on-Mouse (M.O.M.) kit (Vector Laboratories). Sections were then incubated overnight at 4°C with a 1:200 dilution of anti-Muc5ac antibody (clone 45M1; Lab Vision, Fremont, CA) for 1 h at room temperature with goat anti-mouse Alexa Fluor 542 (Invitrogen, Carlsbad, CA) diluted 1:500 according to the Vector M.O.M. protocol. Sections were then mounted and visualized under ultraviolet light.

**Quantitative real-time RT-PCR.** Total RNA was isolated from whole lung tissue using TRIzol reagent (Invitrogen). Total RNA was treated using RNase-free DNase (Invitrogen). Transcripts were analyzed using TaqMan probes or the SYBR Green method on the SmartCycler (Cepheid, Sunnyvale, CA). Primer sequences for the transcripts of interest were designed using Primer3 (36). The expression of each target gene was normalized to the expression of the housekeeping gene GAPDH. The data were analyzed using the ΔΔCt method (36). Statistical analysis was performed using one-way ANOVA with Tukey’s post hoc test for multiple comparisons. Significant differences were determined at the level of p < 0.05.
manufacturer’s guidelines with an anti-rat IgG horseradish peroxidase detection kit (BD Pharmingen). CD31 localization was achieved by incubating the tissues overnight at room temperature with a 1:250 dilution of rat anti-mouse CD31 antibody (BD Pharmingen). Tracheas were dehydrated and mounted using Permount (Fisher Scientific, Houston, TX).

Morphometric measurements of blood vessels were made in whole mount tracheas stained with anti-CD31 antibody. Images were taken at ×40 using a digital camera. The number of vessels traversing a cartilage ring was used as a representative index of the total number of vessels in the trachea (45). This index was determined by placing a line of known length parallel to the long axis of the cartilage ring and counting the number of vessels intersecting the line. Results were expressed as the number of vessels per unit length of the line drawn. An average of 12 cartilage rings were counted per trachea, and those values were then used to obtain mean and SE values for tracheal vascularity.

Statistics. Values are expressed as means ± SE. As appropriate, groups were compared by one-way ANOVA with follow up comparisons between groups being conducted using Student’s t-test with a P value of ≤0.05 denoting significant differences.

RESULTS

Lifespan and pulmonary histology of ADA/A2AR double knockout mice. ADA−/− mice die between 18–21 days of postnatal life in association with severe pulmonary inflammation and histopathology that consists of enlarged alveolar air spaces and mucous metaplasia in the bronchial airways (6). To genetically examine the contribution of the A2AR to the pulmonary phenotype seen in these mice, A2AR−/− mice were crossed with ADA−/− mice. Examination of litters resulting from ADA/A2AR double heterozygous mating pairs revealed that ADA/A2AR double knockout mice died between postnatal day 14 and 16 (data not shown). Because of the precocious death of ADA/A2AR double knockout mice, pulmonary phenotypes were examined on postnatal day 14. At this stage, there was a substantial enlargement of alveolar air spaces in the lungs of ADA−/−A2AR++ mice relative to that seen in ADA++A2AR++ mice (Fig. 1, compare A and C). There was no evidence of alveolar airway enlargement in ADA−/− mice lacking the A2AR (Fig. 1B), and the alveolar air space enlargement was not enhanced in ADA/A2AR double knockout mice (Fig. 1, compare C and D).

Closer examination revealed differences in inflammation and hypertrophy of the bronchial airway epithelium among these mice. There were increases in alveolar macrophages within the airways of ADA−/−A2AR++ mice relative to ADA++A2AR++ mice together with thickening of the smooth muscle surrounding bronchial airways and hypertrophy of bronchial epithelium (data not shown). Comparison of lung sections from ADA−/−A2AR+ and ADA−/−A2AR− mice revealed increases in alveolar macrophages, thickening of the smooth muscle surrounding the bronchial airways, and bronchial epithelial hypertrophy (Fig. 1, compare E and F). In addition, there was increased accumulation of polymorphonuclear cells in the alveolar septae of ADA−/−A2AR−/− mice. These findings indicate that genetic removal of the A2AR from ADA−/− mice results in precocious death associated with enhanced pulmonary inflammation and hypertrophy of the bronchial epithelium.

Pulmonary inflammation in ADA/A2AR double knockout mice. To better understand the impact of the A2AR on pulmonary inflammation, total cell counts and cellular differentials were determined on postnatal day 14. ADA−/−A2AR++ mice displayed increased numbers of BAL cells compared with ADA++A2AR++ mice, and ADA−/−A2AR−/− mice exhibited enhanced total BAL cellularity (Fig. 2A). Differential staining of recovered airway cells demonstrated that the increased cellularity seen in ADA/A2AR double knockout mice was composed primarily of an increase in alveolar macrophages (Fig. 2B) with a trend toward an increase in the number of neutrophils (Fig. 2C). Interestingly, there were fewer total cells and alveolar macrophages recovered from the airways of ADA−/−A2AR−/− mice relative to ADA++A2AR++ mice.

To better visualize increases in lung tissue neutrophils, lung sections were reacted with antiserum to the chemokine receptor CXCR2 that is abundantly expressed on these cells. There were increased tissue neutrophils in the lung parenchyma of ADA−/−A2AR++ mice relative to ADA++A2AR++ mice (Fig. 3, A and C), and the number of CXCR2-positive neutrophils was increased in the interstitial spaces of lungs from ADA/A2AR double knockout mice (Fig. 3D). Taken together, these data demonstrate that there is enhanced pulmo-
with ADA−/−A2AR+/+ mice (Fig. 4, E and F). Lastly, morphometric analysis of PAS staining confirmed a significant increase in the mucus index seen in the bronchial airways of ADA/A2AR double knockout mice (Fig. 5). Together, these findings demonstrate enhanced mucin production in the bronchial airways of ADA/A2AR double knockout mice.

Inflammatory mediators in ADA/A2AR double knockout mice. Numerous inflammatory cytokines and chemokines are elevated in the lungs of ADA−/− mice (6, 42, 43). To better understand the mechanisms involved in the enhanced inflammation and mucin production seen in the lungs of ADA/A2AR double knockout mice, the levels of proinflammatory cytokines and chemokines were examined in whole lung RNA lysates using quantitative real-time RT-PCR. As expected, TNF-α and IL-6 were elevated in the lungs of ADA−/−A2AR+/+ mice; however, their levels were not significantly affected by the removal of the A2AR (Fig. 6, A and B). Other cytokines that were elevated in the lungs of ADA−/−A2AR+/+ mice but were not altered following the removal of the A2AR included IL-4, IL-5, IL-13, and IL-9 (data not shown). In contrast to the levels of cytokines measured, there were significant alterations in the levels of key inflammatory chemokines in the lungs of ADA/A2AR double knockout mice (Fig. 6, C–F). Transcripts for CXCL11 (eotaxin-1) and CCL17 (TARC) were found to be diminished in the lungs of ADA/A2AR double knockout mice, whereas levels of monocyte chemoattractant protein-1 (MCP-1) and CXCL1 (GRO-α) were found to be enhanced in the lungs of ADA/A2AR double knockout mice. MCP-3 was elevated in the lungs of ADA−/−A2AR+/+ mice but was not altered following the removal of the A2AR (data not shown). These data demonstrate that the A2AR differentially regulates cytokine and chemokine production in this setting of elevated adenosine levels.

Analysis of tracheal vascularity in ADA/A2AR double knockout mice. ADA−/− mice develop increased tracheal vascularity in an adenosine-dependent manner (33). To determine whether

Fig. 2. A–C: lavage cell counts and differentials. Bronchoalveolar lavage (BAL) was conducted on postnatal day 14, and total cell counts and cellular differentials were determined. A: total BAL cell counts of ADA−/− and ADA+/− mice with and without the A2AR. B: total BAL alveolar macrophage counts. C: total BAL lymphocyte, eosinophil, and neutrophil cell counts. Data are presented as the mean total cells × 10^6 ± SE; n = 8–10 mice per genotype. Significant changes were found among groups using one-way ANOVA. *P ≤ 0.05 using a Student’s t-test for the secondary comparisons indicated.
A2AR was involved in mediating increased tracheal vascularity, whole mount immunohistochemistry on tracheas from day 14 ADA/A2AR double knockout mice was conducted (Fig. 7). Results show that there is no difference in vessel numbers between ADA−/−A2AR+/+ and ADA+/A2AR+/+ mice indicating that the increases previously observed (33) must be occurring between day 14 and day 18. Interestingly, ADA/A2AR double knockout mice have increased tracheal vascularity compared with ADA−/−A2AR+/+ mice (Fig. 7). These results indicate that the A2AR is involved in regulating tracheal angiogenesis when adenosine concentrations are high.

Adenosine receptor transcript levels in the lungs of ADA/A2AR double knockout mice. To examine the potential for compensatory actions of adenosine receptors in the lungs of ADA/A2AR double knockout mice, receptor transcripts were measured on postnatal day 14 (Fig. 8). As previously noted (10), there were increases in transcript levels for all of the adenosine receptors except the A2AR in the lungs of ADA−/−A2AR+/+ mice. Transcripts for the A2AR were not found in the lungs of ADA−/−A2AR−/− or ADA+/−A2AR+/− mice, and transcript levels for the A1R, A2B, and A3R were not altered in the lungs of ADA−/−A2AR−/− mice relative to levels in the lungs of ADA−/−A2AR+/+ mice. These findings suggest that there are no compensatory changes in adenosine receptor transcripts in the lungs of ADA−/− mice following the removal of the A2AR.

**DISCUSSION**

Adenosine elevations have been linked to chronic lung disease (19). This is further supported by findings in the ADA−/− mouse model that demonstrate that elevations in adenosine are sufficient to induce features of chronic lung disease including chronic inflammation, mucin overproduction, and angiogenesis (6, 33). The purported mechanism by which adenosine exerts its effects is through the engagement of adenosine receptors located on the cell surface. This mode of action has been authenticated by receptor agonist/antagonist work both in vitro and in vivo and by animal models employing genetics to remove individual adenosine receptors. These approaches have revealed that different adenosine receptors have both pro- and anti-inflammatory functions in the lungs of ADA−/− mice (42, 43, 48). In the current study, ADA/A2AR double knockout mice were used to examine the contribution of the A2AR to the pulmonary phenotype seen in ADA−/− mice. Findings demonstrate that ADA/A2AR double knockout mice die on average 3 days earlier than ADA−/− mice competent in the A2AR. The earlier demise of these mice was associated with increased lung inflammation, mucin production, tracheal angiogenesis, and chemokine levels. These results demonstrate that the A2AR plays a tissue-protective role in the lung disease seen in ADA−/− mice.

A major aspect of the phenotype seen in the lungs of ADA/A2AR double knockout mice was the increased numbers of inflammatory cells. This inflammatory cell increase was composed primarily of alveolar macrophages and parenchymal neutrophils. This enhanced inflammation seen in the absence of the A2AR suggests this receptor may function to limit the recruitment of these cells into the lungs and airways of ADA−/− mice. This is consistent with numerous published findings demonstrating that A2AR−/− mice exhibit enhanced inflammatory responses following infectious and noninfectious challenges (22, 23, 34, 40). Moreover, studies have demonstrated that the A2AR is expressed on bone marrow-derived monocytes (30) and that the mechanism by which A2AR

![Fig. 4.](http://ajplung.physiology.org/)

**Fig. 4.** A–F: mucin production in the lungs of ADA/A2AR double knockout mice. Postnatal day 14 lung sections were reacted with periodic acid-Schiff (PAS; A–D) or anti-serum against Muc5ac (E and F) for the detection of mucin. A: lung section from an ADA−/−A2AR+/+ mouse. B: lung section from an ADA−/−A2AR−/− mouse. C: lung section from an ADA−/−A2AR−/− mouse. D: lung section from an ADA−/−A2AR+/+ mouse. E: lung section from an ADA−/−A2AR+/+ mouse immunostained for Muc5ac. F: lung section from an ADA−/−A2AR−/− mouse immunostained for Muc5ac. Findings are representative of 8–10 mice from each genotype. Scale bar = 100 μm.

![Fig. 5.](http://ajplung.physiology.org/)

**Fig. 5.** Airway mucus index for ADA/A2AR double knockout mice is shown. A mucus index was determined on PAS-stained lung sections as described in MATERIALS AND METHODS. Data are presented as mean mucus index ± SE; n = 8–10 mice per genotype. Significant changes were found among groups using one-way ANOVA. Asterisk (*) denotes significant differences between ADA−/−A2AR−/− and ADA+/−A2AR−/−, whereas ** denotes significance between ADA−/−A2AR−/− and ADA−/−A2AR+/+. Significance of secondary comparisons was determined using a Student’s t-test, P = 0.05.
agonists mediate protection from ischemia-reperfusion injury requires direct activation of A2ARs on bone marrow-derived cells (35). A2AR involvement in macrophage recruitment has also been demonstrated in other disease models. Tubulointerstitial disease of the kidneys induced by unilateral ureteral obstruction (UUO) results in ischemic injury and the recruitment of macrophages (29). Activation of the A2AR with the selective agonist ATL-1466 after UUO results in a transient decrease in renal macrophage infiltration suggesting a role for A2AR in the regulation of macrophage recruitment (29). In addition, studies have demonstrated that A2AR signaling confers anti-inflammatory actions on neutrophils (11, 12, 32, 41), which is consistent with the enhanced neutrophilia seen in ADA/ADA double knockout mice. These observations suggest that the A2AR is involved in limiting the degree of neutrophil and macrophage recruitment to sites of tissue injury.

Neutrophil and macrophage accumulation in the lung occurs as a result of proinflammatory stimuli that mediate the recruitment of these cells from the circulatory system into tissue. Thus the greater number of neutrophils and macrophages observed in the lungs of ADA/ADA double knockout mice likely parallels a rise in mediators capable of recruiting these cells. Indeed, analysis of inflammatory mediators in the lungs of ADA/ADA double knockout mice (42) were affected by the removal of the A2AR. Levels of MCP-3, another important macrophage chemoattractant molecule (44), did not change with loss of the A2AR in ADA−/− mice, whereas levels of CXCL11 and CCL17 were decreased following the removal of the A2AR. These observations suggest that A2AR signaling may selectively downregulate certain inflammatory pathways while concomitantly increasing others. Interestingly, similar decreases in CXCL11 and CCL17 were seen following blockade of the A2BR in this model (43), suggesting that the A2AR and A2BR may share common pathways in downregulating these chemokines. Chemokines such as CXCL11 and CCL17 are commonly associated with Th2 inflammatory environments such as those seen in asthmatics (15), whereas chemokines such as CXCL1 and MCP-1 are often, but not exclusively, associated with Th1 inflammatory environments (36, 44) where neutrophils are prominent. There is substantial evidence that adenosine may play a proinflammatory role in Th2-associated diseases such as asthma (19), although it is clear that adenosine can serve as an anti-inflammatory signal in Th1-associated diseases (22). Results form the current study suggests that at least part of the differential effects of adenosine in these various disease states may be through the selective A2AR-mediated downregulation or upregulation of key regulatory chemokines.

Adenosine signaling through the A2AR can downregulate the production of proinflammatory cytokines such as TNFα and
ADA signaling in the lungs of ADA-deficient mice

In addition to its role as a neutrophil chemoattractant (36), CXCL1 is a potent stimulator of angiogenesis, particularly in pathological angiogenesis as such that seen in tumors (46) and in chronic lung disease (1). ADA-/- mice have increased tracheal angiogenesis in association with increased CXCL1 levels (33). Similarly, the current study demonstrates that elevations in CXCL1 seen in the lungs of ADA/A2AR double knockout mice are associated with enhanced tracheal vascularity. These findings suggest that CXCL1 production in the lung may be an important regulator of pathological angiogenesis and an important target for adenosine regulation.

Overall, elevations in adenosine promote inflammation and damage in the lungs of ADA-/- mice (6). Results in the current study suggest an anti-inflammatory role for the A2AR in the lung disease seen in ADA-/- mice. In addition to the A2AR, the A1R has also been shown to be tissue-protective as ADA/A1R double knockout mice display enhanced pulmonary inflammation, mucus metaplasia, and alveolar destruction (42). Thus other adenosine receptors must be involved in mediating the tissue remodeling effects of adenosine in the lungs of ADA-/- mice. The A2BR has the lowest affinity for adenosine and has been implicated in both anti- and proinflammatory pathways (2, 16, 26, 47). Evidence for the A2BR’s tissue destructive properties comes from studies showing that engagement of the A2BR on pulmonary fibroblasts promotes their differentiation into myofibroblasts, which are responsible for depositing collagen and promoting fibrosis (50). In addition, the A2BR induces the production of proinflammatory mediators important in the progression of lung disease including IL-4, IL-6, IL-8, and IL-13 (17, 39, 49). Further support for a tissue-destructive role for the A2BR comes from studies using an A2BR antagonist in the ADA-/- mouse that show that blockade of the A2BR results in the prevention of pulmonary inflammation, air space enlargement, and airway fibrosis (43). This indicates that many of the pathological lung changes that occur in the ADA-/- mouse are mediated by the A2BR. These findings differ somewhat from recent observations in A2BR knockout mice that demonstrate that the A2BR has anti-inflammatory functions in macrophages (47) and mast cells (26) in vivo. The discrepancies between these models and the

IL-6 that are stimulated following endotoxin exposure or ischemic injury (13, 24). Surprisingly, the current study demonstrated that cytokines that have been shown to be diminished by activation of the A2AR, such as TNF-α and IL-6, were not elevated following the removal of the A2AR from the lungs of ADA-/- mice. These findings suggest that other pathways, such as A2BR signaling (47), may serve to limit production of proinflammatory cytokines in this model. However, another explanation is that adenosine itself may mediate the production of these cytokines in this model through other adenosine receptors. Engagement of the A2BR has been shown to increase IL-6 in a number of cellular systems (18, 37), and treatment of ADA-/- mice with a selective A2BR antagonist can reduce expression of both TNF-α and IL-6 in the lungs of these animals (43). Thus the ability of A2AR signaling to limit the production of key inflammatory chemokines, but not cytokines, in the ADA-/- model of adenosine-mediated lung injury.

Fig. 7. A and B: tracheal angiogenesis in ADA/A2AR double knockout mice. A: tracheas were removed from postnatal day 14 mice and analyzed by whole mount CD31 immunostaining for the visualization of vessels. Results are representative of 4 mice from each genotype. B: tracheal vascularity was quantified in the samples shown in A by counting the number of vessels intersecting a line down the length of the cartilage ring. At least 12 cartilage rings were analyzed per sample. Data are represented as mean vessels (in millimeters) ± SE; n = 4. Significant changes were found among groups using one-way ANOVA. Asterisk (*) denotes significant differences between ADA+/A2AR+/+, ADA+/A2AR-/-, ADA-/A2AR+/+, and ADA-/A2AR-/- mice. Significance was determined using a Student’s t-test, P ≤ 0.05.

Fig. 8. Adenosine receptor transcript levels in the lungs of ADA/A2AR double knockout mice are shown. Total cellular RNA was isolated from the lungs of postnatal day 14 mice, and quantitative real-time RT-PCR was used to quantify the levels of various adenosine receptors. Data are presented as mean transcripts ± SE; n = 8–10 mice per genotype. Significant changes were found among groups using one-way ANOVA. Asterisk (*) denotes significant differences compared with ADA+/A2AR+/+. Significance was determined using a Student’s t-test, P ≤ 0.05. ND, not detected.
findings in ADA−/− mice treated with an A2B antagonist may be related to the levels and duration of adenosine elevations, the nature of the inflammation seen, or the fact that the A2B antagonist was given after established disease in the ADA−/− model. The latter may be particularly important in that mice genetically deficient in the A2B may possess exaggerated inflammatory responses because of the loss of tropic anti-inflammatory activities that make assessment of proinflammatory and tissue-destructive properties of the A2B such as fibrosis difficult to assess. Additional studies investigating the effect of genetically removing the A2B from the ADA−/− model are needed to address this issue. The A2R also plays a tissue destructive role in the ADA−/−, as it contributes to airway inflammation and mucus production, but the effect is not as pronounced as that of A2B antagonism (48). Taken together, we can now paint a broad picture for the role of each adenosine receptor in the ADA-deficient model of adenosine-mediated lung injury: the A1R and A2AR appear to play tissue-protective roles, whereas the A2R and A3R may serve a proinflammatory role.

Understanding the contribution of adenosine signaling is convoluted given the pleiotropic effects of this nucleoside in various disease models and cell types. The ADA−/− mouse demonstrates that elevations in adenosine result in lung disease with many features seen in patients suffering from asthma and COPD (3). The results presented in this manuscript demonstrate that the A2AR plays a tissue-protective and anti-inflammatory role in the pulmonary phenotype of ADA−/− mice, a finding consistent with the vast majority of studies found in the literature. How elevations in lung adenosine and subsequent differential engagement of receptors are ultimately regulated to impact disease is far from understood; however, examination of specific receptors in the ADA-deficient model of lung disease may be helpful in unmasking specific pathways that are regulated by individual receptors. For example, in the context of chronic lung diseases where adenosine levels are elevated, A2AR agonists may have utility in limiting the production of key regulatory chemokines that impact pulmonary inflammation and angiogenesis.

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