Genetic removal of the A2A adenosine receptor enhances pulmonary inflammation, mucin production, and angiogenesis in adenosine deaminase-deficient mice

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Mohsenin A, Mi T, Xia Y, Kellemes RE, Chen J-F, Blackburn MR. Genetic removal of the A2A 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 adenosine-mediated pulmonary disease. Studies suggest that the A2A adenosine receptor (A2AR) functions to limit inflammation and promote tissue protection; however, the contribution of A2AR 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 A2AR signaling to the pulmonary phenotype seen in ADA-deficient mice. This was accomplished by generating ADA/A2AR double knockout mice. Genetic removal of the A2AR from ADA-deficient mice resulted in enhanced pulmonary inflammation and mucin production, lung adenosine levels are elevated in mouse models of T helper type 2 (Th2) cytokine-induced lung disease (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 (ADA−/−) 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 A1R from ADA−/− 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 A3R (48), or pharmacological inhibition of the A2BR (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 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 T helper type 2 (Th2) cytokine-induced lung disease (4, 31). In addition, mice deficient in adenosine deaminase (ADA), which catalyzes the breakdown of adenosine, develop pronounced pulmonary inflammation and damage (4, 31). In contrast, genetic removal of the A3R (48), or pharmacological inhibition of the A2BR (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 A2A R 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 A2AR is coupled to Gαi1040-0605/07 $8.00 Copyright © 2007 the American Physiological Society

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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 A2AR-deficient mice have revealed the anti-inflammatory properties of this receptor. For example, A2AR-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 A2AR agonist results in increased survival, and this protection was ablated in A2AR-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 A2AR agonist results in significantly reduced TNF-α production, and this protection is no longer present in A2AR-deficient mice (23). These and many other studies provide evidence that the A2AR functions to inhibit inflammation and promote tissue protection. However, the contribution of A2AR 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 A2AR 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 ADA-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). A2AR-deficient (A2AR⁻/⁻) mice were generated as previously described (9). A2AR⁻/⁻ mice were on a mixed background of 129-Steel and C57BL/6J. ADA/A2AR double knockout mice were generated by breeding ADA⁻/⁻ mice with A2AR⁻/⁻ 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 microisolator lids and maintained under strict containment protocols. No evidence of bacterial, parasitic, or fungal infection was found, and serologies on cage littermates were negative for 12 of the most common murine viruses.

ADA enzyme therapy and zymogram analysis of ADA enzyme activity. Polyethylene glycol-modified ADA (PEG-ADA) was generated by the covalent modification of purified bovine ADA with activated PEG as described previously (48). Levels of ADA enzyme activity in tissues were measured using zymogram analysis according to established procedures (5).

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 mucins 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 ImagePro 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 (M) 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 × I/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 acetone and methanol for 15 min at room temperature followed by 30-min incubation with 1% sodium borohydride at room temperature (Sigma-Aldrich). Antigen 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). On the following day, sections were incubated 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 Smart Cycler (Cepheid, Sunnyvale, CA). Primer sequences for the transcripts examined were the same as used previously (10, 33, 42). Specific transcript levels were determined using Smart Cycler analysis software through comparison to a standard curve generated from the PCR amplification of template dilutions.

Whole mount immunohistochemistry for CD31 on tracheas. Procedures for tracheal whole mount immunohistochemistry were as previously described (33). Briefly, mice were anesthetized, and the trachea was removed and placed into 1× PBS. Surrounding tissue was teasing away, and the trachea was then flattened down using insect pins onto a silicone polymer. Tracheas were washed in 1× PBS for 10 min and then fixed using zinc fixative (BD Pharmingen, San Jose, CA) for 24 h. Following fixation, tracheas were washed three times in 1× PBS for 5 min per wash. Tracheas were then permeabilized using PBS containing 1% Triton X-100 for 30 min at room temperature. Endogenous peroxidase activity was blocked by incubating tissues in prechilled 0.6% hydrogen peroxide for 30 min at room temperature. Immunohistochemistry for CD31 was performed according to the
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RESULTS

Lifespan and pulmonary histology of ADA/A2AR double knockout mice. ADA2−/− mice die between 18–21 days of postnatal life in association with severe pulmonary inflammation and hypertrophy of the bronchial epithelium. Enhanced pulmonary inflammation and hypertrophy of the bronchial epithelium (Fig. 1, compare A and C). There was no evidence of alveolar airspace enlargement in ADA2−/− mice lacking the A2AR (Fig. 1B), and the alveolar space enlargement was not enhanced in ADA/A2AR double knockout mice (Fig. 1, compare A and C).

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 ADA2−/−A2AR+/+ mice relative to ADA2+/+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 ADA2−/−A2AR+ and ADA2−/−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 ADA2−/−A2AR−/− mice. These findings indicate that genetic removal of the A2AR from ADA2−/− 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. ADA2−/−A2AR+/+ mice displayed increased numbers of BAL cells compared with ADA2+/+A2AR+/+ mice, and ADA2−/−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 ADA2−/−A2AR−/− mice relative to ADA2+/+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 ADA2−/−A2AR+/+ mice relative to ADA2+/+A2AR+/+ mice (Fig. 3A, C and D), 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-
Presented as the mean total cells.

C: total BAL lymphocyte, eosinophil, and neutrophil cell counts. Data are ADA
PAS-positive bronchial epithelial cells demonstrated that contribution of A2AR signaling to mucin production in this
model, mucin production was quantified in the airways of ADA/A2AR double knockout mice. Gross examination of
mice with and without the A2AR. nary inflammation in the lungs of ADA
A2AR double knockout mice. 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 CXCL1 (eotaxin-1) and CCL17 (TARC) were found to be diminished in the lungs of ADA/A2AR double knockout mice, whereas levels of monocyte chemotactic 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

Mucin production in ADA/A2AR double knockout mice. ADA/*−/− mice display mucin over production in association with elevated adenosine concentrations (6). To examine the contribution of A2AR signaling to mucin production in this model, mucin production was quantified in the airways of ADA/A2AR double knockout mice. Gross examination of PAS-positive bronchial epithelial cells demonstrated that ADA/*−/− A2AR/*+/+ mice have increased mucous compared with ADA*+ mice without regard to A2AR status (Fig. 4, A–C). However, comparing PAS staining in the airways of ADA/*−/− A2AR/*−/− and ADA/*−/− A2AR/*+/+ mice demonstrated that the lack of the A2AR results in enhanced levels of PAS staining in the bronchial airways of ADA/*−/− mice (Fig. 4, C and D). Furthermore, examination of the major mucin gene, Muc5ac, demonstrated enhanced Muc5ac immunoreactivity in the bronchial airways of ADA/*−/− A2AR/*−/− mice compared 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 CXCL1 (eotaxin-1) and CCL17 (TARC) were found to be diminished in the lungs of ADA/A2AR double knockout mice, whereas levels of monocyte chemotactic 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
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, A2BR, 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
agonists mediate protection from ischemia-reperfusion injury requires direct activation of A2R 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-146e 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/A2AR 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/A2AR double knockout mice likely parallels a rise in mediators capable of recruiting these cells. Indeed, analysis of inflammatory mediators in the lungs of ADA/A2AR double knockout mice revealed enhanced production of key chemokines known to regulate the recruitment of these cells. Namely, expression of CXCL1 and MCP-1 was increased in the lungs of ADA/A2AR double knockout mice. CXCL1 plays a central role in the recruitment and trafficking of neutrophils into the lung (36) and has also been shown to be involved in macrophage recruitment (7), whereas MCP-1 is a potent chemoattractant for numerous inflammatory cells including monocytes (38). Studies in models of ischemic injury in the liver have shown that treatment with an A2AR agonist can decrease the production of MCP-1 (13) and CXCL1 (21) expression following injury. The mechanisms governing the increased levels of these chemokines in the lungs of ADA/A2AR double knockout mice are not known. A2AR signaling may directly downregulate the expression of these chemokines, or increased levels may be associated with increased influx of inflammatory cells that express these chemokines. Either way, the findings in this study suggest that A2AR signaling serves to limit tissue inflammation in part by limiting MCP-1 and CXCL1 levels during tissue injury.

Not all of the chemokines known to be elevated in the lungs of ADA−/− 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 A3B in this model (43), suggesting that the A2AR and A3B 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 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 from 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 TNF-α and...
In addition to its role as a neutrophil chemoattractant (36), CXCL1 is a potent stimulator of angiogenesis, particularly in pathological angiogenesis such as 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

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+/+ and ADA−/− A2AR−/−. Significance was determined using a Student’s t-test, P ≤ 0.05.

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 certain inflammatory cytokines will likely depend on the nature of the inflammatory insult. In this context, the current study suggests that A2AR engagement can limit the production of key inflammatory chemokines, but not cytokines, in the ADA−/− model of adenosine-mediated lung injury.
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 A2AR 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 A2BR 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, A2R agonists may have utility in limiting the production of key regulatory chemokines that impact pulmonary inflammation and angiogenesis.

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