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A3 Adenosine Receptor Signaling Contributes to Airway Inflammation and Mucus Production in Adenosine Deaminase-Deficient Mice

Hays W. J. Young,* Jose G. Molina,* Dawn Dimina,† Hongyan Zhong,‡ Marlene Jacobson,‡ Lee-Nien L. Chan,§ Teh-Sheng Chan,¶ James J. Lee,† and Michael R. Blackburn¶*

Adenosine signaling has been implicated in chronic lung diseases such as asthma and chronic obstructive pulmonary disease; however, the specific roles of the various adenosine receptors in processes central to these disorders are not well understood. In this study, we have investigated the role(s) of the A3 adenosine receptor in adenosine-dependent pulmonary inflammation observed in adenosine deaminase (ADA)-deficient mice. The A3 receptor (A3R) was found to be expressed in eosinophils and mucus-producing cells in the airways of ADA-deficient mice. Treatment of ADA-deficient mice with MRS 1523, a selective A3R antagonist, prevented airway eosinophilia and mucus production. Similar findings were seen in the lungs of ADA/A3 double knockout mice. Although eosinophils were decreased in the airways of ADA-deficient mice following antagonism or removal of the A3R, elevations in circulating and lung interstitial eosinophils persisted, suggesting signaling through the A3R is needed for the migration of eosinophils into the airways. These findings identify an important role for the A3R in regulating lung eosinophilia and mucus production in an environment of elevated adenosine.

features of chronic lung disease in association with elevations in lung adenosine concentrations (5). Features seen include the accumulation of eicosanoids and activated macrophages in the airways, mucus metaplasia in the bronchial airways, and airway destruction. Many of the pulmonary features seen in ADA-deficient mice are reversible following the lowering of lung adenosine levels using ADA enzyme therapy (5, 6, 28, 29), suggesting that adenosine signaling plays an important role in the exacerbation of pulmonary inflammation and airway remodeling. The current study examines the role(s) of the A3R in the airway inflammation seen in ADA-deficient mice. Findings suggest that the A3R plays a proinflammatory role in ADA-deficient airway disease by contributing to airway inflammation and mucus production.

Materials and Methods

Mice
ADA-deficient mice used for antagonist studies were on a 129sv/C57BL/6-FVBn mixed background (30, 31), and genotypes were determined by PCR analysis of genomic DNA using the following primers: wild-type sense primer, 5'-CCTCTGAGCCCATGATTCTGA-3'; wild-type antisense primer, 5'-AGAATGGACCGGACCTTGAT-3'; and ADA minigene primers (trans-1, 9, 13, and 17 (0.625, 1.25, 2.5, 2.5, 2.5 U, respectively). ADA control mice. Findings suggest that the A3R plays a proinflammatory role in ADA-deficient airway disease by contributing to airway inflammation and mucus production.

RNA isolation, RT-PCR, and quantitative RT-PCR (Q-RT-PCR) analysis
Total RNA was isolated from whole lung tissue using TRIzol reagent (Invitrogen Life Technologies, Grand Island, NY). Total RNA was DNase treated (Invitrogen Life Technologies) and quality was assessed by electrophoresis through formaldehyde agarose gels. Lung RNA (1 μg) was used in a Superscript One Step RT-PCR (Invitrogen Life Technologies) with A3R or β-actin-specific primers following manufacturer's instructions. Alternatively, transcript levels were quantified using real-time Q-RT-PCR. Adenosine receptor- and β-actin transcripts were analyzed using TaqMan probes on the Smart Cycler (Cepheid, Sunnyvale, CA) (28). MacSac (sense: 5'-GGCCAACTTGTTGACATCAGC-3', antisense: 5'-CTCTATAGTTGAGGACATCACCAG-3'), calcium-activated chloride channel 3 (sense: 5'-CATCAGGAGCACGACCATATAAG-3', antisense: 5'-TGTGTTGCCTGTTAGTCTGAG-3'), RANTES (sense: 5'-TCGAAAGGACGCAGAATTGGTTG-3', antisense: 5'-CCAAAGTGTTGAGTACTAGA-3'), Cox1 (sense: 5'-CCTTTACTTTCTGTCGTGCAC-3', antisense: 5'-CCCACTCGTGTACAGCCTGAT-3'), TGFβ (sense: 5'-AGGCTCGTGTACAGCCTGAT-3', antisense: 5'-TCAGGCGAACCTTCATGAC-3'), thymus- and activation-regulated chemokine (TARC) (sense: 5'-AGAAGGCCATCGGATTTG-3', antisense: 5'-TGTTGTTGCCTGTTAGTCTGAG-3'), and IL-6 (sense: 5'-CTCCTCGGTTCTGTGAGAT-3', antisense: 5'-CTGTTGCTCCTCTGCTGAGTA-3', transcription were determined by SYBR Green detection reagents (Invitrogen Life Technologies) using a Smart Cycler (Cepheid). PCR products were confirmed by melting curve analysis. Standard curves were generated from PCR amplification of template dilutions. Final data were normalized to β-actin and are presented as the molecules of transcript/molecules of β-actin × 100 (% β-actin).

In situ hybridization
In situ hybridization was conducted according to established protocols (35). Plasmids containing a portion of the A3R cDNA were linearized and either T3 or T7 RNA polymerases were used to generate antisense or sense riboprobes labeled with α-35S[UTP]. Samples were overlaid with 8 million counts of riboprobe and hybridized overnight at 60°C. Posthybridization washes were conducted as described (35), and slides were dipped in Kodak NTB-2 emulsion (Rochester, NY) and exposed for up to 6 wk.

Histology and period acid Schiff (PAS)/AB staining
Mice were sacrificed and the lungs inflated with 0.5 ml of fixative (4% paraformaldehyde in PBS) before fixation overnight at 4°C. Fixed lung tissues were embedded in paraffin and 5-μm sections were stained with H&E using a Rapid Chrome staining kit (Thermo Shandon, Pittsburgh, PA). Similar sections were stained with PAS counterstained with Alcian blue (AB). The mucus index score was determined by dividing the [(area of PAS/AB staining) × (mean intensity of PAS/AB staining)] by the total area of the airway epithelium (5).

Bronchoalveolar lavage and peripheral blood analysis
Mice were anesthetized, the trachea isolated by blunt dissection, and a small caliber catheter secured into the airways. Two volumes of 1 ml of PBS with 0.1% BSA were instilled and gently aspirated and pooled (BALF). Samples were then centrifuged at 2500 rpm for 5 min to recover cells. Cell pellets were resuspended in PBS and total cell counts determined using a hemocytometer. Aliquots were cytopsin and stained for cellular differentials (5). Whole blood was collected from the tail vein and smeared onto microscope slides for differentials. Total peripheral white blood cell counts were determined on hemolysed aliquots using a hemocytometer.

Immunohistchemical analysis
Deparaffinized sections were rehydrated in a series of graded alcohols to water. Endogenous peroxidase activity was blocked by incubation in 0.5% H2O2 in methanol for 20 min at room temperature. After rehydration, the pH was adjusted to 3.6 with 0.1 N HCl. The sections were incubated at room temperature with a 1/3000 dilution of rabbit anti-mouse major basic protein 1 (mMBP-1) Ab (36) followed by development with a rabbit IgG Vectastain Elite ABC kit (Vector Laboratories, Burlingame, CA). Quantification of peribronchial eicosanoids was assessed by analysis of 10 fields at x20 magnification. The number of peribronchial eicosanoids within a 0.6-mm2 area were counted using Image Pro Plus 4.0 analysis software (Media Cybernetics, Silver Spring, MD).
Eosinophil chemotaxis assay

Chemotactic capacity of ADA−/− BALF was assayed as described previously (37). Briefly, the migration of purified eosinophils from the blood of IL-5 transgenic mice (38) was investigated using 3-μm polycarbonate membrane transwell inserts in 24-well tissue culture polystyrene plates (Costar, Corning, NY). The inserts contained eosinophils (1 x 10⁶) and 30 ng/ml recombinant mouse IL-5. Three eosinophil populations were examined: 1) purified eosinophils with no additional manipulation; 2) eosinophils pretreated with 100 nM IB-MECA; and 3) eosinophils treated initially with 5 μM MRS 1523 followed by 100 nM IB-MECA. The inserts were then placed in the wells containing 500 μl of medium alone or medium containing mouse eotaxin-1 (positive control), ADA control, or ADA−/− BALF. The plates were incubated at 37°C, 5% CO₂ for 90 min. The cells

![A3 R SIGNALING IN THE LUNGS OF ADA-DEFICIENT MICE](http://www.jimmunol.org/)

**A**. Localization of A₃ R transcripts to mucus-producing cells. A, Darkfield image of an 18-day-old ADA control lung hybridized with antisense cRNA probe specific for the A₃ R. B. Serial section of the ADA control lung hybridized with A₃ sense cRNA probe. C. Brightfield image of an 18-day-old ADA−/− lung stained with PAS to illustrate areas of mucus production (arrows). D. Serial section of ADA−/− lung hybridized with the A₃ antisense cRNA probe. Arrows denote A₃-specific expression. E, ADA−/− lung section hybridized with A₃ sense cRNA probe. Red pixels denote specific hybridization, while blue epifluorescence represents nuclei stained with Hoechst 33258 (A, B, D, and E). Scale bar in A = 100 μm and applies to A–E.

FIGURE 2. Increased A₃ R expression in the lungs of ADA−/− mice. A. Total cellular RNA was isolated from the lungs of 18-day-old ADA control and ADA−/− mice and subjected to RT-PCR using primers specific for the A₃ R and β-actin. The (+) control lane is total RNA from testes. B. TaqMan quantitative RT-PCR analysis of A₃ R transcripts was performed on whole lung RNA extracts from 18-day-old ADA control and ADA−/− mice. A₃ R values were normalized to β-actin transcript levels measured in the same RNA preparations. Values are mean ± SEM, n = 4. (*, p = 0.05 using a Student’s t test).

### Results

**A₃ R transcripts in the lungs of ADA−/− mice**

Total RNA was isolated from the lungs of 18-day-old mice and A₃ R transcripts were analyzed by RT-PCR. Although A₃ R transcripts were detectable in the lungs of both ADA control and ADA−/− mice, the prevalence of these transcripts increased in the lungs of ADA−/− mice (Fig. 1). These findings demonstrate that A₃ R transcript levels are elevated in the lungs of ADA−/− mice at a time when adenosine concentrations, eosinophil numbers, and mucus production are all increased in the lung (5).

**Localization of A₃ R transcripts in the lungs of ADA−/− mice**

Activation of the A₃ R can elicit various responses depending on the cell type and downstream signaling components involved (15–20, 22, 39). We examined the localization of A₃ R transcripts using in situ hybridization. A₃ R transcripts were not detectable in the lungs of ADA control mice (Fig. 2A), whereas transcripts for the A₃ R were abundant in the mucus-containing epithelial cells of ADA−/− mice (Fig. 2, C and D). Expression of the A₃ R was also seen in areas of inflammation (data not shown). These findings demonstrate that the increases in the A₃ R transcripts in the lungs of ADA−/− mice are located at sites of inflammation and mucus metaplasia.

**Expression of the A₃ R in mouse eosinophils**

General localization of A₃ R transcripts in inflammatory cells from the lungs of ADA−/− mice suggests that eosinophils may express the A₃ R. However, the cellular resolution of A₃ R expression was difficult using the in situ hybridization technique. Therefore, we quantified A₃ R transcripts in isolated eosinophils to confirm expression in this cell type. An established model of OVA sensitization and challenge (33) was used to generate a large number of airway eosinophils. Wild-type mice sensitized to OVA and challenged with saline (o/s) exhibited predominantly macrophages in the BALF (Fig. 3A), whereas mice challenged with OVA (o/o) exhibited a robust increase in BALF eosinophils (Fig. 3A). A₃ R transcript levels were markedly increased in RNA extracts from BALF cell pellets of o/o mice (Fig. 3B), suggesting A₃ R transcripts are present in eosinophils recruited to mouse airways. We that had migrated to the lower chamber were collected and counted using a hemacytometer.

...
next examined A$_3$R transcript levels in RNA isolated from eosinophils purified from the circulation of IL-5 transgenic mice (38). These eosinophils possessed no A$_1$R transcript, and relatively low levels of A$_2A$R and A$_2B$R transcripts. In contrast, A$_3$R transcripts were relatively abundant (Fig. 3C). Collectively, these data demonstrate that the A$_3$R is expressed in mouse eosinophils recovered from both the circulation and the airways.

**Effects of A$_3$R antagonism on lung inflammation in ADA$^{-/-}$ mice**

ADA$^{-/-}$ mice were treated with the A$_3$R antagonist MRS 1523 to assess the contributions of A$_3$R signaling to the adenosine-mediated pulmonary alterations in these mice. ADA$^{-/-}$ mice were maintained on ADA enzyme therapy until postnatal day 17 to allow the mice to mature to a size amenable to the implantation of osmotic pumps containing MRS 1523 (22). On postnatal day 19, mice were implanted with a s.c. osmotic pump containing either MRS 1523 (100 μg/kg/day) or vehicle (saline). On postnatal days 29–31, which was 12–14 days after the cessation of ADA enzyme therapy, adenosine levels were elevated in the lungs of ADA$^{-/-}$ mice (40). At this stage, various pulmonary endpoints were examined. ADA control mice treated with saline or MRS 1523 exhibited normal lung pathologies (Fig. 4, A and B), indicating that there

![Figure 3](http://www.jimmunol.org/DownloadedFrom)  
**FIGURE 3.** A$_3$R transcripts are abundant in eosinophil-rich BALF pellets and circulating eosinophils. A, Cellular differentials were determined by counting 400 cells on Diff-quik-stained cytospin slides from BALF collected from OVA sensitized and saline challenged (O/S) and OVA sensitized and OVA challenged (O/O) mice. The cell types are presented as a percentage of cells ± SEM, n = 3. B, Total RNA was isolated from BALF cell pellets from O/S and O/O mice. RNA samples were subjected to Q-RT-PCR for the A$_3$R. Values presented are mean ± SEM, n = 3. C, Total RNA was isolated from eosinophils collected from the circulation of IL-5 overexpressing transgenic mice. The RNA was examined by Q-RT-PCR to determine the levels of adenosine receptors in this cell type. Values are mean ± SEM, n = 3. (*, p ≤ 0.05, Student’s t test).

![Figure 4](http://www.jimmunol.org/DownloadedFrom)  
**FIGURE 4.** Blocking A$_3$R signaling leads to a decrease in infiltrating inflammatory cells. ADA control and ADA$^{-/-}$ mice were implanted with osmotic pumps as described in Materials and Methods. A, At 29–31 days of age, the total number of cells recovered from the BALF was determined. Values are given as mean total cell counts × 10$^5$ ± SEM (*, p ≤ 0.05, Student’s t test). B, Cellular differentials were determined on aliquots of cells from 29 to 31-day-old ADA control and ADA$^{-/-}$ mice treated with saline or MRS 1523. Values are given as mean total cell counts × 10$^5$ ± SEM, n = 5. (*, p ≤ 0.05 ADA$^{-/-}$ saline vs ADA control saline; #, p ≤ 0.05 ADA$^{-/-}$ MRS 1523 vs ADA$^{-/-}$ saline using a Student’s t test).

![Figure 5](http://www.jimmunol.org/DownloadedFrom)  
**FIGURE 5.** MRS 1523 treatment decreases the number of eosinophils in the BALF of ADA$^{-/-}$ mice. ADA control and ADA$^{-/-}$ mice were implanted with osmotic pumps as described in Materials and Methods. A, Scale bar in A = 100 μm and applies to A–D.
were no adverse responses due to pump implantation or A3R antagonism. In contrast, ADA−/− mice treated with saline exhibited increased pulmonary inflammation (Fig. 4C), and this inflammation was diminished by MRS 1523 treatment (Fig. 4D). Total cells infiltrating the airways of ADA−/− mice increased (Fig. 5A), and this increase was prevented by MRS 1523 treatment. The most abundant cell types found in these airways were macrophages and eosinophils (Fig. 5B). Treatment with MRS 1523 was associated with a decrease in all cell types in the airways, with an almost complete ablation of eosinophils (Fig. 5B). These data demonstrate that treatment with the A3R antagonist MRS 1523 can attenuate pulmonary inflammation and eosinophil infiltration into the airways of ADA−/− mice.

Localization of eosinophils in ADA−/− mice following antagonist treatment

Absence of eosinophils in the airways of ADA−/− mice treated with MRS 1523 prompted us to examine circulating and lung interstitial eosinophil numbers to determine whether A3R antagonism was affecting eosinophil production or trafficking. Lung interstitial eosinophils were localized using an Ab against mMBP-1 that is selectively produced by eosinophils (41). Few mMBP-1-positive cells were observed in the peribronchial region of ADA control mice treated with saline or MRS 1523 (data not shown). In contrast, mMBP-1 positive eosinophils were readily detected in peribronchial regions of ADA−/− mice treated with saline (Fig. 6A). Interestingly, mMBP-1 positive eosinophils persisted in the peribronchial spaces of ADA−/− mice treated with MRS 1523 (Fig. 6B). Quantification of these findings demonstrated a significant decrease in interstitial lung eosinophilia in ADA−/− mice treated with MRS 1523; however, levels remained significantly elevated over control values (Fig. 6C). Examination of eosinophil levels in circulation revealed a significant increase in eosinophils in the circulation of ADA−/− mice treated with saline (Fig. 6D). This increase persisted in ADA−/− mice treated with MRS 1523, which was in marked contrast to the decreased eosinophilia seen in the BALF of these mice (Fig. 5B). Collectively, these data demonstrate that A3R antagonism does not affect the production of eosinophils in ADA−/− mice, and suggest that A3R signaling may impact eosinophil trafficking in the lungs.

Effects of MRS 1523 on mucus production in the airways of ADA−/− mice

Elevations in adenosine are associated with increased mucus production in the airways of ADA−/− mice (5). Mucus production was investigated in the airways of ADA−/− mice treated with

![Graph](image-url)

**FIGURE 6.** Persistent eosinophilia in peripheral blood and peribronchial regions in ADA−/− mice treated with MRS 1523. Eosinophils in the peribronchial region were localized by immunohistochemistry with an mMBP-1 Ab. ADA−/− mice treated with saline (A) or MRS 1523 (B). Scale bar in A = 100 μm and applies to A and B. C. Peribronchial mMBP-1-positive eosinophils were quantified as describe in Materials and Methods. Values are presented as the number of eosinophils per mm²± SEM, n = 5. (*, p ≤ 0.05 ADA−/− saline and MRS 1523 vs ADA control saline; #, p ≤ 0.05 ADA−/− MRS 1523 vs ADA−/− saline using a Student’s t test). D. Total peripheral white blood cell counts and differentials were determined as described and are presented as total eosinophils per mm³± SEM, n = 5. (*, p ≤ 0.05 ADA−/− saline and MRS 1523 vs ADA control saline and MRS 1523 using a Student’s t test).
MRS 1523 to determine the importance of A3R signaling in this process. ADA control mice treated with saline (A) or MRS 1523 (B). ADA−/− mice treated with saline (C) or MRS 1523 (D). Arrows denote mucus production. Scale bar in A = 100 μm and applies to A–D. E, Mucus production was quantified as described in Materials and Methods. Values are presented as mean mucus index score ± SEM, n = 5. (*, p ≤ 0.05 ADA−/− saline vs ADA control saline; #, p ≤ 0.05 ADA−/− MRS 1523 vs ADA−/− saline using a Student’s t test).

FIGURE 7. Mucus production is reduced in ADA−/− mice treated with MRS 1523. Mucus production was determined by PAS staining. ADA controls treated with saline (A) or MRS 1523 (B). ADA−/− mice treated with saline (C) or MRS 1523 (D). Arrows denote mucus production. Scale bar in A = 100 μm and applies to A–D. E, Mucus production was quantified as described in Materials and Methods. Values are presented as mean mucus index score ± SEM, n = 5. (*, p ≤ 0.05 ADA−/− saline vs ADA control saline; #, p ≤ 0.05 ADA−/− MRS 1523 vs ADA−/− saline using a Student’s t test).

FIGURE 8. Persistent elevations in cytokines and chemokines following MRS 1523 treatment. A. Cytokine transcript levels were quantified in lungs of ADA control and ADA−/− mice treated with saline or MRS 1523 using SYBR Green-based Q-RT-PCR. B. Chemokine transcript levels were quantified using Q-RT-PCR. Values are presented as the percentage of β-actin ± SEM, n = 5. (*, p ≤ 0.05 ADA−/− vs ADA control saline using a Student’s t test).
the development of adenosine-induced mucus production in this model.

Cytokine and chemokine levels in the lungs of MRS 1523-treated mice
Recruitment of inflammatory cells to the lung is associated with increased expression of proinflammatory cytokines and chemokines (2). Cytokine and chemokine levels were examined to determine whether A3R antagonism could impact the expression of these mediators. Levels of IL-5, IL-13, and IL-6 were elevated in the lungs of ADA+/− mice treated with saline, and levels continued to be elevated in the lungs of ADA+/− mice treated with MRS 1523 (Fig. 8A). Similar observations were made regarding the levels of the chemokines MCP-3, TARC, and eotaxin 1 (Fig. 8B). These findings demonstrate that increases in proinflammatory cytokines and chemokines in the lungs of ADA+/− mice occur in an A3R-independent manner.

Pulmonary phenotypes in ADA/A3 double knockout mice
ADA−/− mice were crossed onto an A3−/− background to generate ADA/A3 double knockout mice to genetically determine the contribution of the A3R to the lung disease seen in ADA−/− mice. Similar to what was seen following A3R antagonism, removal of the A3R resulted in diminished lung inflammation in ADA−/−

FIGURE 9. Decreased airway inflammation and mucus production in ADA/A3 double knockout mice. ADA control and ADA+/− mice with and without the A3R were maintained on PEG-ADA enzyme therapy until day 17 of age to match the pharmacologic protocol. Endpoints were examined at 28–30 days of age. A, Cellular differentials given as mean total cell counts × 10⁵ ± SEM, n = 4. B, Peribronchial mMBP-1-positive eosinophils were quantified as described previously, values are presented as the number of eosinophils per mm² ± SEM, n = 3. C, White blood cells were counted from blood smears. Values are presented as the percent of total eosinophils per mm² ± SEM, n = 4. D, Mucus index scores were determined and values are presented as mucus index score ± SEM, n = 4. (*, p ≤ 0.05 ADA−/− A3+/+ vs ADA control A3+/+; #, p ≤ 0.05 ADA−/− A3−/− vs ADA−/− A3+/+ using a Student’s t test).
chemotactic activity was seen in ADA BALF from the lungs of ADA control mice; however, substantial increased mucus production in the lungs of ADA mice was determined. Purified eosinophils (1 x 10⁶) were placed in transwell inserts containing 3-μm polycarbonate membranes. The migration of eosinophils toward medium containing eotaxin 1, ADA control, or ADA−/− BALF was determined in the absence or presence of 100 nM IB-MECA and 5 μM MRS 1523. Values are presented as a mean ratio ± SEM of the cells migrating toward test agents relative to medium alone (chemotactic index), n = 1 for eotaxin 1, n = 3 for ADA control and ADA−/− BALF.

FIGURE 10. Ex vivo chemotaxis of murine eosinophils. Purified eosinophils were placed in chemotaxis chambers and the chemotactic index of mouse eosinophil chemotaxis ex vivo, purified mouse eosinophils were placed in chemotaxis chambers and the chemotactic index of BALF from ADA control and ADA−/− mice was determined. Purified eosinophils exhibited little chemotactic activity toward BALF from the lungs of ADA control mice; however, substantial chemotactic activity was seen in ADA−/− BALF (Fig. 10). Activation of the A3R by treatment with the A3R agonist IB-MECA decreased chemotaxis toward BALF from ADA−/− mice as well as toward the positive control eotaxin 1. This inhibition of chemotaxis was lost when eosinophils were incubated with the A3R agonist MRS 1523 together with IB-MECA. These findings demonstrate that BALF from ADA−/− mice is chemotactic for eosinophils, and that A3 signaling can block this chemotaxis ex vivo.

Ex vivo effects of A3R signaling on eosinophil chemotaxis

Previous studies have demonstrated that engagement of the A3R on human eosinophils can inhibit chemotaxis ex vivo (15, 18). To further examine the chemotactic properties in the lungs of ADA−/− mice, and to determine the impact of A3R signaling on mouse eosinophil chemotaxis ex vivo, purified mouse eosinophils were placed in chemotaxis chambers and the chemotactic index of BALF from ADA control and ADA−/− mice was determined. Purified eosinophils exhibited little chemotactic activity toward BALF from the lungs of ADA control mice; however, substantial chemotactic activity was seen in ADA−/− BALF (Fig. 10). Activation of the A3R by treatment with the A3R agonist IB-MECA decreased chemotaxis toward BALF from ADA−/− mice as well as toward the positive control eotaxin 1. This inhibition of chemotaxis was lost when eosinophils were incubated with the A3R agonist MRS 1523 together with IB-MECA. These findings demonstrate that BALF from ADA−/− mice is chemotactic for eosinophils, and that A3 signaling can block this chemotaxis ex vivo.

Discussion

Adenosine is a signaling molecule that is generated in response to tissue damage and hypoxia. High levels of this nucleoside are found in the airways of asthmatics (3, 4), suggesting adenosine signaling may play a role in the exacerbation of this disease. However, the mechanisms by which adenosine influences aspects of lung disease are not fully appreciated. In the current study, we provide novel evidence that A3R signaling contributes to airway eosinophilia and mucus production in a model of ADA deficiency where lung adenosine levels are chronically elevated.

Transcript levels for the A3R are elevated in the lungs of asthma and COPD patients, where expression is localized to eosinophilic infiltrates (15). Similarly, we found that A3R transcripts are elevated in the lungs of ADA−/− mice where transcripts localized to sites of eosinophilic inflammation. Increased levels of A3R transcripts were also seen in the BALF of allergen-challenged mice where the predominant infiltrate was eosinophils, and analysis of eosinophils purified from peripheral blood revealed that the A3R is the most abundant adenosine receptor in eosinophils. It is not clear from these studies whether or not the levels of A3R, particularly at the cell surface, change in response to inflammatory cues; however, the expression of the A3R in both circulating and pulmonary eosinophils suggest that it may play a role in regulating eosinophil function. The exact role of the eosinophil in asthma is not fully understood; however, its presence in the airways of patients with severe asthma suggests that this cell may contribute to the exacerbation of airway disease by damaging epithelial cells and contributing to airway remodeling (1). Thus, the presence of the A3R on human eosinophils (16, 25), together with the high levels of adenosine found in the lungs of asthmatics (3, 4), suggest that A3 signaling may play an important role in regulating eosinophil function in the asthmatic lung.

ADA−/− mice exhibit adenosine-dependent airway and lung eosinophilia in association with elevations in lung adenosine levels (5). Contribution of A3 signaling to lung inflammation and damage in the lungs of ADA−/− mice was conducted using both pharmacologic and genetic approaches. The pharmacologic approach consisted of treatment of ADA−/− mice with a selective A3R antagonist (MRS 1523), which has been used successfully in vivo in mice (22, 34). The genetic approach entailed the generation of ADA/A3 double knockout mice. Similar results were seen with both methods and suggest that A3R signaling contributes to proinflammatory features of adenosine in this model. Closer examination of eosinophils revealed no change in the degree of peripheral blood eosinophilia in ADA−/− mice following disruption of A3 signaling suggesting this signaling pathway is not involved in the production or mobilization of eosinophils from the bone marrow. However, the reduction of BALF eosinophilia following antagonism and removal of the A3R suggests A3R signaling is involved in the migration of eosinophils from the circulation to the airways.

Diminished airway eosinophilia following the disruption of A3 signaling is in opposition to studies performed in human eosinophils ex vivo, where chemotaxis of purified eosinophils toward known chemotactic agents was blocked by A3R activation (15, 18). This discrepancy could be due to differences in mouse and human eosinophils or to differences attributed to the ex vivo nature of the chemotaxis experiments performed. Our examination of A3R signaling on mouse eosinophils ex vivo revealed that activation of the A3R decreases chemotaxis of murine eosinophils toward ADA−/− BALF as well as eotaxin 1, suggesting that the impact of A3R signaling on migration ex vivo is similar in human and mouse eosinophils. Furthermore, our findings demonstrate that significant differences exist between the impact of A3R signaling on eosinophil migration ex vivo and in the whole animal. It is therefore likely that the diminished airway eosinophilia seen in the lungs of ADA−/− mice following disruption of A3R signaling is not a cell autonomous effect of eosinophils. Rather, disruption of A3R signaling in ADA−/− mice must impact the expression or activity of key regulatory molecules from other cells that express the A3R that in turn impact eosinophil migration. The A3R is expressed on murine mast
cells (22), airway macrophages (6), and airway epithelial cells (Fig. 2) (6), all of which may produce regulatory cytokines that can impact eosinophil migration. As an attempt to address this issue, the levels of key regulatory cytokines and chemokines were measured in the lungs of ADA−/− mice with and without disruption of A3R signaling. Levels of key eosinophil regulatory cytokines (IL-5, IL-13) and chemokines (eotaxin I, TARC, MCP-3) were elevated in ADA−/− lungs, while these levels were not diminished following antagonism or removal of the A3R, suggesting regulation of these molecules is not involved in preventing airway eosinophilia in response to disrupted A3R signaling. Additional studies are needed to determine the impact of A3R signaling on the activities of key regulators of eosinophil migration into and through tissues, including cell adhesion molecules (42), extracellular matrix components (43) and proteases (44), all of which are actively involved in eosinophil trafficking in the lung.

The intracellular mechanisms by which A3R signaling influences eosinophil chemotaxis are not known, but may involve the regulation of intracellular Ca2+ levels. The A3R is coupled to Goi (12), and its activation on eosinophils can elevate intracellular Ca2+ levels (25). Similar responses have been observed following A3R engagement on mouse mast cells (22, 45), where A3R-directed increases in intracellular Ca2+ are thought to be mediated by activation of PI3K activity through its interaction with βγ subunits (45, 46). Elevating intracellular Ca2+ in eosinophils via A3R signaling may promote eosinophil chemotaxis. In addition to influencing chemotaxis, A3R engagement on eosinophils may also influence eosinophil survival (47). A3R engagement can protect rat mast cells from apoptosis by a pathway that involves activation of PI3K and phosphorylation of protein kinase B (47). In a similar manner, A3R engagement on eosinophils may promote their survival at sites of inflammation and in so doing serve a proinflammatory role.

In addition to expression on murine eosinophils, A3R expression was evident in mucus-producing cells in the bronchial Airways of ADA−/− mice. Similar patterns of A3R expression have recently been observed in airway mucous-producing epithelium of mice overexpressing IL-13 (6) and in mice challenged with OVA (H. Young, unpublished observations). Therefore, the up-regulation of A3R expression in airway epithelial cells appears to be a universal feature of the mucus metaplasia seen in mouse experimental models of asthma. Whether or not the A3R is also expressed on mucus-producing cells of the asthmatic lung is not known, nor has the potential function of A3R signaling on this cell type been explored. Results in the current study demonstrate that the mucus production in the Airways of ADA−/− mice can be prevented by antagonism or removal of the A3R, suggesting that A3R signaling is important for mucus metaplasia in this model. However, it is not clear at this stage whether the decrease in mucus production results from direct effects of A3R signaling on mucus-producing cells, or is an effect of preventing airway eosinophilia, which has been shown to be associated with mucous production (48, 49). Determining the role of the A3R on mucus-producing cells will provide important new insight into how adenosine signaling can impact chronic lung disease.

In summary, we have demonstrated that A3R levels are elevated in the lungs of ADA−/− mice that exhibit adenosine-mediated lung disease. Treatment of ADA−/− mice with a selective A3R antagonist or genetically removing this receptor prevented airway inflammation and mucus production, suggesting that A3R signaling plays an important role in regulating these aspects of chronic lung disease. Disruption of A3R signaling in these models appears to function to prevent the migration of eosinophils into the Airways suggesting that A3R signaling plays a role in regulating the trafficking of this important effector cell in the lung. Thus, in environments where adenosine levels are elevated, such as the lugs of asthmatics (3, 4), A3R antagonism may provide a mechanism for reducing airway eosinophilia.

References


