Attenuation of Chronic Pulmonary Inflammation in A_{2B} Adenosine Receptor Knockout Mice

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Pharmacologic evidence suggests that activation of A_{2B} adenosine receptors results in proinflammatory effects relevant to the progression of asthma, a chronic lung disease associated with elevated interstitial adenosine concentrations in the lung. This concept has been challenged by the finding that genetic removal of A_{2B} receptors leads to exaggerated responses in models of acute inflammation. Therefore, the goal of our study was to determine the effects of A_{2B} receptor gene ablation in the context of ovalbumin-induced chronic pulmonary inflammation. We found that repetitive airway allergen challenge induced a significant increase in adenosine levels in fluid recovered by bronchoalveolar lavage. Genetic ablation of A_{2B} receptors significantly attenuated allergen-induced chronic pulmonary inflammation, as evidenced by a reduction in the number of bronchoalveolar lavage eosinophils and in peribronchial eosinophilic infiltration. The most striking difference in the pulmonary inflammation induced in A_{2B} receptor knockout (A_{2B}KO) and wild-type mice was the lack of allergen-induced IL-4 release in the airways of A_{2B}KO animals, in line with a significant reduction in IL-4 protein and mRNA levels in lung tissue. In addition, attenuation of allergen-induced transforming growth factor–β release in airways of A_{2B}KO mice correlated with reduced airway smooth muscle and goblet cell hyperplasia/hypertrophy. In conclusion, genetic removal of A_{2B} adenosine receptors in mice leads to inhibition of allergen-induced chronic pulmonary inflammation and airway remodeling. These findings are in agreement with previous pharmacologic studies suggesting a deleterious role for A_{2B} receptor signaling in chronic lung inflammation.

Keywords: adenosine; asthma; pulmonary inflammation; IL-4; transforming growth factor–β

CLINICAL RELEVANCE

This study found attenuation of chronic pulmonary inflammation and airway remodeling in mice lacking A_{2B} adenosine receptors. It implies that A_{2B} receptor antagonism may be of significant therapeutic value to the management of asthma, a chronic inflammatory disease associated with elevated interstitial adenosine concentrations in the lung.

Interstitial adenosine concentrations are increased during inflammation as a result of cell stress, injury, and tissue hypoxia (1, 2). Extracellular adenosine functions as a signaling molecule by engaging cell surface G protein–coupled receptors of the P1 purinergic family comprising A_1, A_{2A}, A_{2B}, and A_3 adenosine receptor subtypes (2). There is growing evidence that adenosine plays an important role in the regulation of inflammation. Inhibition of acute inflammation is a well-recognized effect of adenosine, which has been attributed primarily to stimulation of A_{2A} adenosine receptors on immune and endothelial cells (3). Recent evidence, however, suggests that adenosine can promote chronic inflammation by up-regulating proinflammatory cytokines. Studies in adenosine deaminase (ADA)–deficient mice, characterized by elevated lung tissue levels of adenosine, demonstrated an association between adenosine and an inflammatory phenotype (4, 5). These mice exhibit a pulmonary phenotype with features of inflammation, mucous metaplasia, increased IgE synthesis, and elevated levels of proinflammatory cytokines, all of which could be reversed by lowering adenosine levels with exogenous ADA (4). Correlation between lung adenosine levels and pulmonary inflammation was also found in transgenic mice, in which T helper (Th) 2 cytokines, IL-4 and IL-13, were overexpressed in a lung-specific fashion. Remarkably, treatment with exogenous ADA significantly reduced the pulmonary inflammation in these mice, indicating a role for adenosine in the development of their pulmonary phenotype (6, 7).

Pharmacologic inhibition of A_{2B} adenosine receptors *in vivo* significantly reduces elevations in proinflammatory cytokines induced by high adenosine levels, suggesting an important role of this receptor subtype in the proinflammatory actions of adenosine (8). A_{2B} receptor antagonism reduced airway reactivity and inflammation in the mouse model of allergic pulmonary inflammation induced by ragweed (9, 10). Furthermore, cell culture studies suggest that A_{2B} receptors are involved in adenosine-dependent regulation of proinflammatory paracrine factors. We have previously shown that stimulation of A_{2B} receptors in the human mast cell line, HMC-1, increases production of proinflammatory cytokines and angiogenic factors IL-1β, -3, -4, -8, -13, and vascular endothelial growth factor (11–13). We have also demonstrated that A_{2B} receptors up-regulate proinflammatory cytokines and angiogenic factors in mouse bone marrow–derived mast cells (14), and mediate adenosine-dependent IL-6 secretion in mouse macrophages (15). Further studies in human primary cell cultures demonstrated that A_{2B} receptors increase monocyte chemotactic protein–1 and IL-6 release from airway smooth muscle cells and fibroblasts, suggesting their role in proinflammatory actions of adenosine (16,

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17). In addition, A_{2B} receptors have been recently implicated in modulation of dendritic cell differentiation toward cells expressing high levels of Th2-type immune response cytokines and angiogenic factors (18).

Paradoxically, A_{2B} receptor knockout (A_{2B}KO) mice appear to have exaggerated responses to inflammatory stimuli; exposure to endotoxin results in augmented TNF-α blood levels in A_{2B}KO mice (19), and systemic or subcutaneous antigen challenges in passively sensitized animals produced an enhanced anaphylactic response compared with wild-type (WT) control animals (20). These effects are opposite to those expected for putative proinflammatory actions of A_{2B} receptors. It is possible, however, that this phenomenon is limited to acute inflammatory responses. We hypothesized that, in chronic inflammation, which represents a complex process driven by multiple inflammatory factors, A_{2B} receptors may promote inflammation by up-regulating proinflammatory cytokines. Therefore, we sought to determine if genetic removal of A_{2B} receptors would dampen a chronic inflammation associated with increased interstitial adenosine concentrations. For this purpose, we chose an established mouse model of allergen-induced chronic airway inflammation characterized by predominantly a Th2 type of immune response with eosinophilic infiltrations and increased airway mucus production (21, 22). We initially documented that this model of chronic airway inflammation indeed results in increased extracellular adenosine levels in the mouse lungs. We then determined the effect of A_{2B} receptor gene ablation on the characteristic parameters of pulmonary inflammation in this model. Our results support the hypothesis that A_{2B} adenosine receptors promote chronic pulmonary inflammation.

MATERIALS AND METHODS

Animals

All studies were conducted in accordance with the *Guide for the Care and Use of Laboratory Animals* as adopted and promulgated by the U.S. National Institutes of Health. Age- and sex-matched mice (8–10 wk old) were used. A_{2B}KO mice were obtained from Deltagen (San Mateo, CA), and WT C57BL/6 mice were purchased from Harlan World Headquarters (Indianapolis, IN). Genotyping protocols for A_{2B}KO have been previously described (23). All of the A_{2B}KO mice used in these studies were back-crossed to the C57BL/6 genetic background for more than 10 generations.

Ovalbumin Sensitization and Allergen Challenge

Chronic pulmonary inflammation in mice was induced by an established protocol (21). Animals were immunized subcutaneously on Days 0, 7, 14, and 21 with 25 μg of ovalbumin (OVA) (grade V; Sigma, St. Louis, MO) adsorbed to 1 mg of Alum (Sigma) in 200 μl of PBS. Intranasal OVA challenges (20 μg per 50 μl in PBS) were conducted on Days 27, 29, and 31, and then repeated twice a week for the next 4 weeks. Control groups of mice, sensitized with Alum/OVA, received intranasal applications of 50 μl PBS instead of OVA following the same schedule. Animals were killed for collection of bronchoalveolar lavage (BAL) fluid and lung tissue 24 hours after the final intranasal application.

Assessment of BAL Inflammatory Cells

BAL was performed in anesthetized mice by instilling 800 μl of 5% BSA in PBS through a tracheotomy tube and then withdrawing the fluid by gentle syringe suction. Total cell counts were determined with a hemocytometer. Aliquots were cytospun and stained with Diff-Quick (American Scientific Products, McGaw Park, IL). Differential counts were conducted by the investigator, blinded to animal group assignments, with standard morphologic criteria to classify the cells as eosinophils, lymphocytes, neutrophils, and other mononuclear leukocytes (alveolar macrophages and monocytes).

Measurement of Adenosine Concentrations in BAL Fluid

In a separate set of experiments, BAL instillate contained the ADA inhibitor, erythro-9-(2-hydroxy-3-nonyl)adenine (Sigma) at a concentration of 5 μM to reduce potential adenosine degradation. Collected BAL fluid was immediately centrifuged at 200 × *g* for 5 minutes at 4°C, and supernatant was precleared by filtration through Amicon Ultrafree-MS centrifugal filters with a molecular weight cutoff of 12,500 Da (Millipore Corporation, Bedford, MA). Samples were mixed with an equal volume of 100 nM [$U^{13}C_{10}$-U-$^{15}N_5$]adenosine (Cambridge Isotope Laboratories, Andover, MA) used as an internal standard, and spin-filtered through a Millipore Microcon filter with a molecular weight cutoff of 3,000 Da. The calibration curves were constructed by spiking known amounts of adenosine in blank BAL instillate, and samples were analyzed by liquid chromatography–mass spectrometry, as previously described (24).

Histology and Immunochemistry

Excised lungs were inflated with 0.5 ml of fixative (10% formalin) before fixation overnight at 4°C. Fixed lung samples were rinsed in PBS, dehydrated, and embedded in paraffin. Sections (5 μm) were stained with hematoxylin and eosin to evaluate general morphology, and with periodic acid Schiff (PAS) to determine the extent of mucin production in bronchial airways. The mucus index score was determined by the following equation: ([area of PAS staining] × [mean intensity of PAS staining])/total area of airway epithelium (4). To quantify peribronchial eosinophils, lung sections were immunostained with diaminobenzidene-peroxidase detection reagents with rat anti-mouse major basic protein (MBP)–1 monoclonal antibody (mMBP-1; Mayo Clinic, Scottsdale, AZ), and counterstained with methyl green, as previously described (25). The data were quantified as an average of the number of eosinophils present per square millimeter of peribronchial area: (number of MBP-positive cells in the peribronchial region)/(area of the peribronchial region) (26). For TGF-β_1 localization, lung sections were immunostained with diaminobenzidene-peroxidase detection reagents with polyclonal rabbit anti–TGF-β_1 antibody (Santa Cruz Biotechnology, Santa Cruz, CA), as previously described (27). To evaluate airway smooth muscle hyperplasia, lung sections were immunostained with diaminobenzidene-peroxidase detection reagents with antibodies against α–smooth muscle actin (α-SMA) (clone 1A-4; Sigma) and smooth muscle myosin heavy chain (SMM) (Biomedical Technologies, Stoughton, MA), as previously described (27). Peribronchial smooth muscle layer thickness was estimated by dividing the area of α-SMA immunostaining by the length of bronchiolar basement membrane (22). Image analysis of digital photomicrographs (original magnification, 40×) was performed with Image-Pro Plus software (Media Cybernetics, Bethesda, MD). Final values are results of an average of 15 bronchioles (150–200 μm internal diameter) randomly chosen by the investigator blinded to animal group assignments.

Measurement of IgE Concentrations

Serum levels of total and OVA-specific IgE were determined, as described previously (28). Briefly, total IgE levels were analyzed by ELISA in plates precoated with rat monoclonal anti-murine IgE clone LO-ME-3 (AbD Serotec, Oxford, UK). OVA-specific IgE levels were analyzed in plates precoated with OVA. After incubation with serum samples, plates were consecutively incubated with rat anti-mouse IgE clone LO-ME-2 (AbD Serotec), a secondary horseradish peroxidase–conjugated antibody, and colorimetric substrates. Concentrations of total IgE were extrapolated from calibration curves with an IgE standard (Maine Biotech, Portland, ME).

Real-Time RT-PCR

Total RNA was isolated from lung tissue with the RNeasy Mini kit (Qiagen, Valencia, CA). Real-time RT-PCR was performed on an ABI PRISM 7900HT Sequence Detection System (PE Applied Biosystems, Foster City, CA), as previously described (14). Published sequences were used for generation of specific primer pairs for murine transforming growth factor (TGF)–β_1 and IL-4 (29). Primer pairs and 6-carboxy-fluorescein–labeled probes for murine adenosine receptors and β-actin were provided by Applied Biosystems.

Measurement of Cytokine Levels

Concentrations of IL-4, -5, -13, and RANTES (regulated upon activation, normal T-cell expressed and secreted) were measured in cell-free BAL supernatants with BD Cytometric Bead Array with a BD LSR II Flow Cytometer (BD Biosciences, San Jose, CA), according to the manufacturer's instructions. Total and active TGF-β_1 concentrations in BAL fluid were assayed with an ELISA kit (R&D Systems, Minneapolis, MN). To determine TGF-β_1 and IL-4 tissue levels, lungs were homogenized with a Kontes pellet pestle (Kimble Chase Life Science and Research Products, Vineland, NJ) in 10 vol (wt/vol) of ice-cold PBS solution containing a 1:10 dilution of a protease inhibitor cocktail (Roche, Indianapolis, IN). Debris was removed by centrifugation at 10,000 $\times$ *g* for 20 minutes at 4°C, and tissue homogenates were assayed for total protein with a Coomassie Plus Bradford assay (Pierce, Rockford, IL). TGF-β_1 and IL-4 levels were measured with ELISA kits (R&D Systems) and expressed as picograms per milligram of tissue protein.

Statistical Analysis

Data were analyzed with GraphPad Prism 4.0 (GraphPad Software Inc., San Diego, CA) and presented as mean values (±SEM). Comparisons between two animal groups were performed with two-tailed unpaired *t* tests. A *P* value less than 0.05 was considered significant.

RESULTS

Increased BAL Adenosine Levels in a Mouse Model of Chronic Airway Inflammation

Elevated concentrations of adenosine have been found in BAL (30) and exhaled breath condensate (31) obtained from patients with asthma and chronic obstructive pulmonary disease. To determine if our mouse model replicates this feature of human chronic lung diseases, we measured adenosine concentrations in BAL fluid. Mice sensitized to OVA and challenged with repetitive intranasal administration of OVA exhibited a statistically significant ($P = 0.02$) twofold increase in BAL adenosine levels compared with control OVA-sensitized mice receiving intranasal administration of PBS in lieu of OVA (Figure 1A).

Effect of Adenosine A_{2B} Receptor Gene Ablation on Adenosine Receptor mRNA Expression in the Lung

Real-time RT-PCR analysis of the lungs of WT mice revealed mRNA encoding all four adenosine receptor subtypes (Figure 1B). Transcription levels of A_1, A_{2A}, A_{2B}, and A_3 receptor subtypes in control mice were 2.85 (±0.11), 4.11 (±0.03), 7.15 (±0.42), and 1.34 (±0.24)% of β-actin, respectively. Chronic airway OVA allergen exposure had no substantial effect on the levels of A_1, A_{2A}, or A_{2B} receptor transcripts, but increased A_3 receptor mRNA levels by 2.9 (±0.4)-fold ($P = 0.02$; $n = 3$). As expected, we did not detect the expression of A_{2B} receptor mRNA in A_{2B}KO mice. We also documented that A_{2B} receptor gene ablation had no significant effect on A_1, A_{2A}, or A_3 receptor mRNA expression in control or OVA-challenged A_{2B}KO mice compared with corresponding WT animal groups.

A_{2B}KO Mice Exhibit Reduced Pulmonary Inflammation, Mucous Metaplasia, and Peribronchial Eosinophil Infiltration

We next conducted histological analysis of the lungs of WT and A_{2B}KO mice. No difference in pulmonary morphology between WT and A_{2B}KO control groups was detected (Figure 2). In WT animals, chronic airway allergen exposure resulted in large inflammatory infiltrates in peribronchial regions (Figure 2A), excessive mucus production by hyperplastic goblet cells (Figure 2B), and peribronchial accumulation of eosinophils (Figure 2C). In contrast, A_{2B}KO mice exhibited reduced pulmonary inflammation in this model. Quantification of PAS staining (Figure 2D), and evaluation of MBP-positive cells (Figure 2E) revealed significantly lower allergen-induced mucus production and eosinophil infiltration in the lungs of A_{2B}KO mice compared with WT animals.

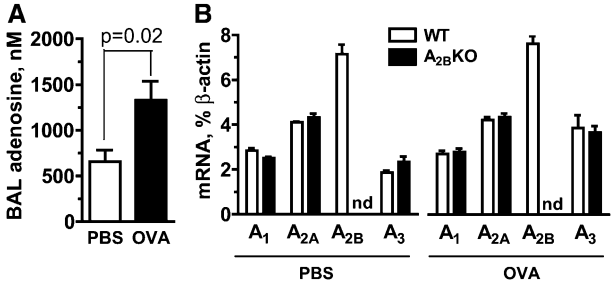

Figure 1. Effect of chronic airway allergen exposure on adenosine release and transcription levels of adenosine receptor subtypes in mouse lungs. (*A*) Adenosine levels in bronchoalveolar lavage (BAL) fluid from mice challenged with ovalbumin (OVA) or its vehicle (PBS). Data are means (±SEM) of five animals per group. (*B*) Real-time RT-PCR analysis of mRNA encoding adenosine receptor subtypes in the lung tissue of wild-type (WT) and A_{2B} receptor knockout (A_{2B}KO) mice challenged with OVA or its vehicle (PBS). Values are expressed as means (±SEM) of three mice per group (nd, not detectable).

We also examined total and OVA-specific IgE levels in serum harvested from mice immediately before being killed (Figures 3A and 3B). No substantial difference in serum IgE levels was found between WT and A_{2B}KO mice in OVA-sensitized but not challenged control animal groups. Repetitive challenge with OVA significantly increased serum IgE levels in both WT and A_{2B}KO mice ($P < 0.05$). Although this effect appeared to be lower in A_{2B}KO mice compared with WT animals, the tendency did not reach statistical significance (n = 8–10 animals/group).

Attenuation of Allergen-Induced BAL Eosinophilia in A_{2B}KO Mice

In the absence of chronic airway allergen exposure, we found no difference in BAL cellularity between WT and A_{2B}KO mice (Figure 4). As expected in this model, chronic airway allergen exposure greatly increased the number of BAL inflammatory cells in WT mice. However, the allergen-induced increase in the total BAL cell counts was significantly lower in A_{2B}KO mice compared with the corresponding WT group ($187 \pm 12 \times 10^3$ versus $277 \pm 35 \times 10^3$; $P = 0.02$; n = 9–10; Figure 4A). The difference in allergen-induced BAL cellularity between WT and A_{2B}KO mice was due largely to reduced eosinophil infiltration observed in A_{2B}KO mice. As seen in Figure 4B, counts of allergen-induced BAL eosinophils were significantly lower in A_{2B}KO mice compared with the corresponding WT group ($88 \pm 12 \times 10^3$ versus $146 \pm 30 \times 10^3$; $P = 0.04$; n = 9–10). We also documented that chronic airway allergen exposure led to a significant increase in BAL lymphocytes (from $1.6 \pm 1.2 \times 10^3$ to $19.8 \pm 3.7 \times 10^3$ in WT mice, and from $1.1 \pm 0.5 \times 10^3$ to $15.5 \pm 2.8 \times 10^3$ in A_{2B}KO mice), neutrophils (from $1.0 \pm 0.5 \times 10^3$ to $13.4 \pm 4.3 \times 10^3$ in WT mice, and from $1.4 \pm 0.6 \times 10^3$ to $43.5 \pm 15.5 \times 10^3$ in A_{2B}KO mice), and, to a lesser extent, monocytes/macrophages (from $63.7 \pm 9.6 \times 10^3$ to $100.0 \pm 23.1 \times 10^3$ in WT mice, and from $55.0 \pm 14.3 \times 10^3$ to $75.6 \pm 12.2 \times 10^3$ in A_{2B}KO mice). However, differences in counts of these cells between WT and A_{2B}KO animal groups (n = 9–10) did not reach statistical significance (*see* Figure E1 in the online supplement).

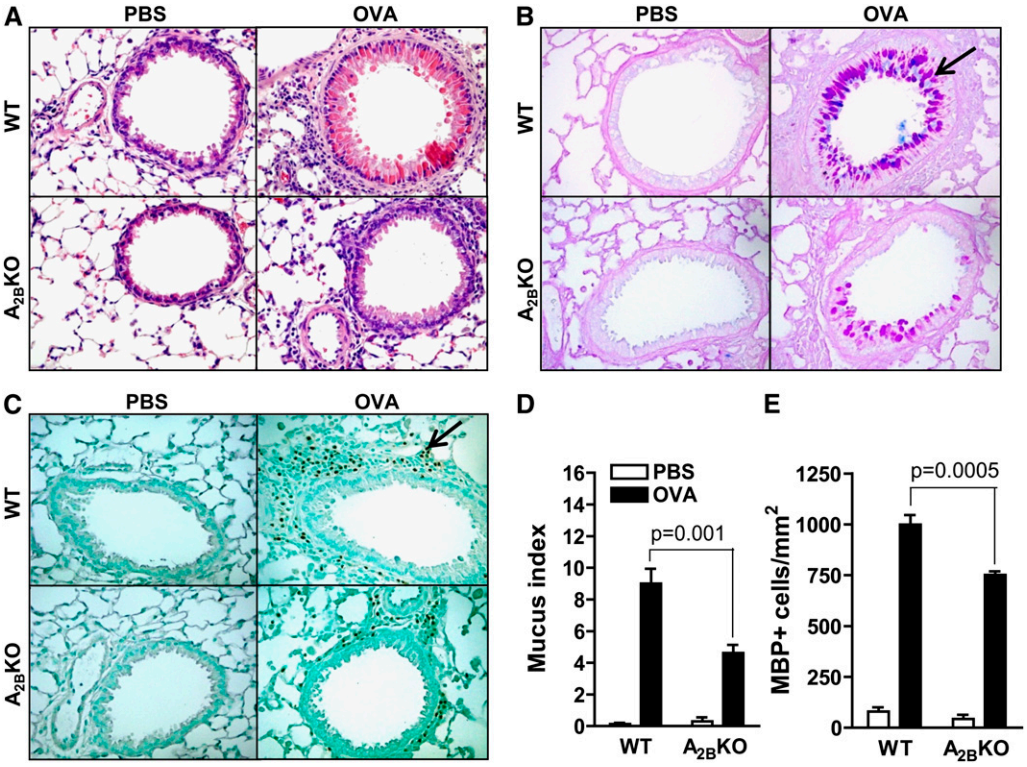

Figure 2. Lung pathology in WT and A_{2B}KO mice induced by chronic airway allergen exposure. Representative photomicrographs of bronchial sections from WT and A_{2B}KO mice challenged with OVA or its vehicle (PBS) stained with hematoxylin and eosin (*A*), periodic acid Schiff (PAS) (*B*), and anti–major basic protein (MBP) antibody (*C*). *Arrows* in *B* and *C* denote a mucin-producing cell and an MBP-positive cell, respectively. (*D*) Mucus index was quantified by measuring PAS staining as described in MATERIALS AND METHODS. (*E*) Eosinophil infiltration was quantified and expressed as a number of MBP-positive cells per square millimeter of peribronchial area. Data are means (±SEM) of four mice per PBS group and seven animals per OVA group.

Down-Regulation of Allergen-Induced IL-4 Production in the Lungs of A_{2B}KO Mice

Because A_{2B} adenosine receptors have been implicated in the regulation of Th2-type inflammatory immune responses, we measured BAL levels of RANTES and IL-5, -13, and -4 in WT and A_{2B}KO mice. Chronic airway allergen exposure significantly increased BAL RANTES levels (Figure 5A), BAL IL-5 levels (Figure 5B), and, to a lesser extent, BAL IL-13 levels (Figure 5C). However, there was no difference between WT and A_{2B}KO groups (Figures 5A–5C). In the absence of chronic airway allergen exposure, no detectable levels of BAL IL-4 were found in either control groups of WT or A_{2B}KO mice (Figure 5D). Chronic airway allergen exposure elevated BAL IL-4 concentrations to 17.3 (±0.5) pg/ml in WT mice. In contrast, BAL IL-4 levels in allergen-challenged A_{2B}KO animals remained below the detection limit (<0.3 pg/ml).

To determine if the apparent lack of allergen-induced IL-4 release into airways was associated with down-regulation of IL-4 production in the lungs of A_{2B}KO mice, we measured IL-4 protein and mRNA levels in lung tissue homogenates. Indeed, allergen-induced IL-4 tissue levels were significantly lower in A_{2B}KO mice compared with the corresponding WT group (125 ± 8 versus 172 ± 14 pg/mg of tissue protein; $P = 0.013$; $n = 10$; Figure 5E). Similarly, allergen-induced IL-4 mRNA levels were significantly lower in A_{2B}KO mice compared with the corresponding WT group (0.06 ± 0.01 versus 0.15 ± 0.02% of β-actin; $P = 0.0008$; $n = 9$–10; Figure 5F). In agreement with our previous findings that lung-specific overexpression of IL-4 can induce eosinophilic inflammation in the mouse lung without affecting BAL IL-5 or IL-13 levels (7), these results suggest that down-regulation of IL-4 production in A_{2B}KO mice may contribute to the attenuation of allergen-induced chronic pulmonary inflammation.

In ancillary studies, we compared the abilities of $CD4^+$ lymphocytes obtained from lungs of allergen-challenged A_{2B}KO and WT mice to produce IL-4 in response to stimulation with phorbol myristate acetate/ionomycin. We found no difference in numbers of $CD4^+$ cells positive for IL-4 staining between A_{2B}KO and WT lymphocytes (Figure E2). Therefore, it is

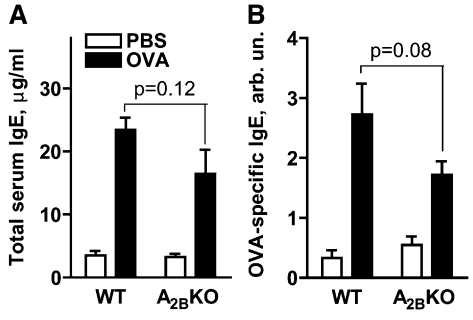

Figure 3. Serum IgE levels. Total (*A*) and OVA-specific (*B*) IgE in serum harvested from WT and A_{2B}KO mice challenged with OVA or its vehicle (PBS). Data are means (±SEM) of 8–10 mice per group.

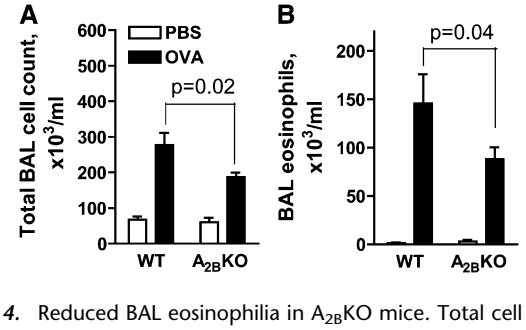

Figure 4. Reduced BAL eosinophilia in A_{2B}KO mice. Total cell (*A*) and eosinophil (*B*) numbers in BAL fluid from WT and A_{2B}KO mice challenged with OVA or its vehicle (PBS). Data are means (±SEM) of 7 mice per PBS group and 9–10 animals per OVA group.

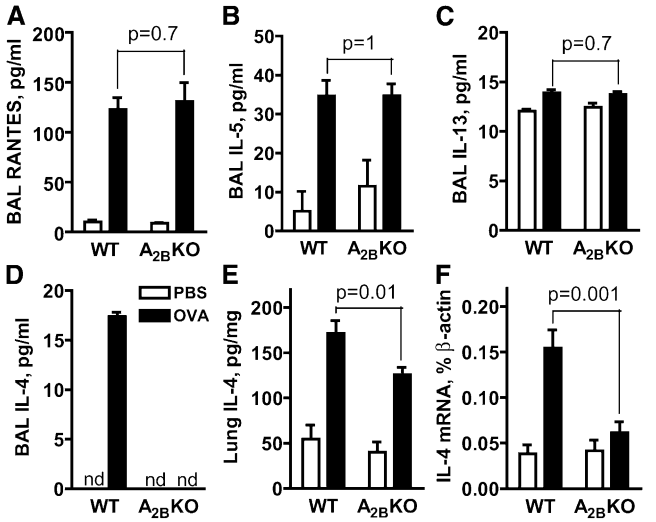

Figure 5. Effect of A_{2B} receptor gene ablation on proinflammatory cytokines. Levels of RANTES (regulated upon activation, normal T-cell expressed and secreted) (*A*), IL-5 (*B*), IL-13 (*C*), and IL-4 (*D*) in BAL fluid, and IL-4 protein (*E*) and mRNA (*F*) levels in lungs from WT and A_{2B}KO mice challenged with OVA or its vehicle (PBS). Data are means (±SEM) of 4–7 mice per PBS group and 7–10 animals per OVA group (nd, not detectable).

unlikely that the difference in allergen-induced IL-4 levels between WT and A_{2B}KO mice could be explained by an intrinsic defect of A_{2B}KO lymphocytes to generate IL-4.

Attenuation of BAL TGF-β Levels and Peribronchial Smooth Muscle Hyperplasia/Hypertrophy in A_{2B}KO Mice

Because TGF-β_1 has been implicated in airway remodeling in mouse models of chronic airway allergen exposure (22, 32), we measured total and active TGF-β_1 levels in BAL fluid. We found significantly reduced BAL TGF-β_1 levels in allergen-challenged A_{2B}KO mice compared with the corresponding WT group (Figures 6A and 6B). Lung tissue TGF-β_1 protein and mRNA levels in allergen-challenged A_{2B}KO mice also tended to be lower compared with the corresponding WT group (Figures 6C and 6D). Examination of lung tissue sections from allergen-challenged WT mice revealed TGF-β_1 immunostaining of bronchial epithelium (Figure 6E, *block arrows*) and alveolar immune cells (Figure 6E, *line arrows*). Although epithelial TGF-β_1 staining was similar in bronchi of A_{2B}KO and WT animals, the occurrence of TGF-β_1–positive cells was markedly diminished in alveoli of A_{2B}KO mice (Figure 6E, *lower panel*).

Next, we analyzed peribronchial α-SMA immunostaining in WT and A_{2B}KO mice (Figure 6F). Whereas no substantial difference in immunostaining between WT and A_{2B}KO control groups was detected, our analysis revealed significantly reduced thickness of the peribronchial α-SMA immunostaining in A_{2B}KO mice subjected to chronic airway allergen exposure compared with the corresponding WT group (3.2 ± 0.2 versus 4.4 ± 0.3 μm; $P = 0.004$; $n = 7$; Figure 6G). Thus, our results suggest that A_{2B} receptor signaling plays an important role in allergen-induced airway remodeling. Because the area of α-SMA immunostaining of smooth muscle bundles was comparatively larger than the area of SMM staining in adjacent lung sections (Figure 6H, *arrows*), it is likely that both myocytes (α-SMA$^+$/SMM$^+$) and myofibroblasts (α-SMA$^+$/SMM$^-$) contribute to the thickening of peribronchial smooth muscle layer.

DISCUSSION

Adenosine activates both anti-inflammatory and proinflammatory pathways. The differential actions of adenosine are likely dependent on the subtypes of adenosine receptors involved, type and duration of injury, and the cytokine milieu. The A_{2B} adenosine receptors are widely expressed in all tissues, including the lung, where they can promote release of inflammatory cytokines from various cells (33). This feature has led to the hypothesis that activation of the A_{2B} receptor may be important in the pathogenesis of chronic lung diseases, including asthma (34). Although genetic ablation of adenosine A_{2B} receptors in mice has been shown to facilitate acute inflammatory responses to antigen challenges in passively sensitized mice (20), this may not be the case in chronic inflammation, a process dependent on the complex interplay between multiple cells and inflammatory factors. The main objective of this study, therefore, was to determine the effects of A_{2B} receptor gene ablation in the context of chronic pulmonary inflammation.

In the current study, we used an established mouse model of chronic pulmonary inflammation associated with Th2 cytokine expression, eosinophilic infiltration, and airway remodeling (21, 22). Because the effects of A_{2B} receptor gene ablation on acute inflammatory responses were originally described in C57Bl/6 mice (19, 20) and reproduced in our recent studies employing the same mouse strain (14, 15), we used animals on C57Bl/6 genetic background to model chronic pulmonary inflammation. For this model to be a valid approach in evaluating the role of the A_{2B} receptor subtype in inflammation, it should be associated with elevation of extracellular adenosine levels in the lungs. Indeed, we found that repetitive airway allergen challenge induced a significant increase in adenosine levels in fluid recovered by BAL. Our data also confirmed that A_{2B} receptor gene ablation results in the lack of A_{2B} transcripts, as expected, but does not affect the expression of mRNA encoding other adenosine receptor subtypes in the lung.

A major observation in this study was the attenuation of pulmonary inflammation in A_{2B}KO mice compared with control animals. Disruption of A_{2B} receptor signaling led to reduced peribronchial infiltration that correlated with the decrease in the number of inflammatory cells recovered in the BAL fluid. Among BAL cells, the most prominent reduction was in the number of eosinophils, in line with the significant decrease in peribronchial eosinophils. Mucus production, another characteristic feature of pulmonary inflammation, was also significantly reduced in A_{2B}KO mice. These results agree with those of previous studies showing that pharmacological inhibition of A_{2B} receptors greatly reduced allergen- or adenosine-induced eosinophilia and mucus production in the mouse lung (8, 10).

The most striking difference in the characteristics of pulmonary inflammation between A_{2B}KO and WT mice was the apparent lack of allergen-induced IL-4 release in the airways of A_{2B}KO animals, which correlated with a significant reduction in IL-4 protein and mRNA levels in lung tissue. During allergic inflammation, IL-4 can be generated in lungs by CD4$^+$ lymphocytes, eosinophils, basophils, and mast cells (35, 36). We found that A_{2B} receptor gene ablation does not prevent allergen-induced accumulation of lung CD4$^+$ lymphocytes capable of producing IL-4 in response to stimulation with phorbol myristate acetate/ionomycin. Therefore, it is unlikely that the observed down-regulation of allergen-induced IL-4 production in the lungs of A_{2B}KO mice could be explained by defective development of Th2 lymphocytes in these animals. Furthermore, down-regulation of IL-4 cannot be explained by general attenuation of Th2 responses in A_{2B}KO mice, because

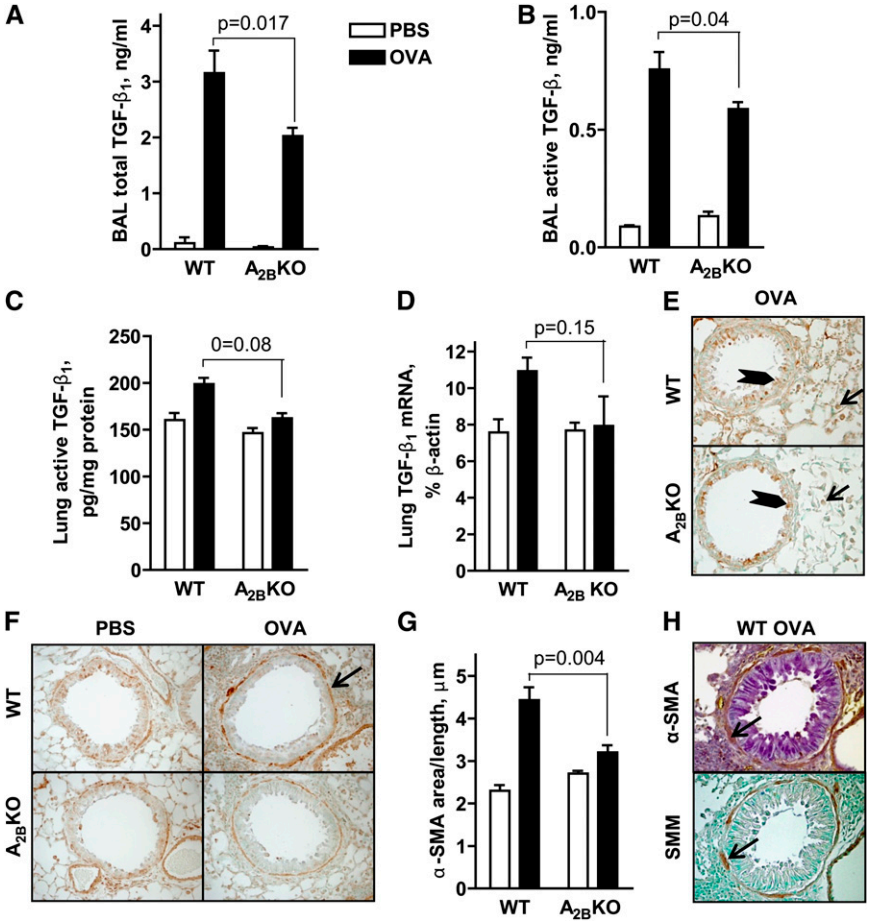

Figure 6. Effect of A_{2B} receptor gene ablation on airway transforming growth factor (TGF)–β_1 and smooth muscle. Total (*A*) and active (*B*) TGF-β_1 concentrations in BAL fluid, and TGF-β_1 protein (*C*) and mRNA (*D*) levels in lung tissue from WT and A_{2B}KO mice challenged with OVA or its vehicle (PBS). Data are means (±SEM) of five to seven mice per group. (*E*) Representative photomicrographs of bronchial sections stained with anti–TGF-β_1 antibody. *Block arrows* point to bronchial epithelium and *line arrows* point to alveolar immune cells positive for TGF-β_1 staining. (*F*) Representative photomicrographs of bronchial sections stained with anti–α–smooth muscle actin (α-SMA) antibody. *Arrow* denotes peribronchial α-SMA staining. (*G*) Peribronchial smooth muscle layer thickness quantified by measuring α-SMA staining as described in MATERIALS AND METHODS. Data are means (±SEM) of four mice per PBS group and seven animals per OVA group. (*H*) Representative photomicrographs of adjacent bronchial sections stained with anti–α-SMA (*upper panel*) and anti–smooth muscle myosin heavy chain (SMM; *lower panel*) antibodies. *Arrows* denote peribronchial α-SMA and SMM staining.

BAL levels of other Th2 cytokines (i.e., IL-5 and IL-13) and RANTES were not significantly changed. It should be noted, however, that allergen-induced increase in BAL IL-13 levels was rather modest compared with robust elevations of RANTES, IL-4, and IL-5 in our model (Figure 5). In a similar model of chronic lung inflammation described by McMillan and Lloyd (37), IL-4 levels continuously increased in BAL fluid, reaching a maximum on Day 55 after initial Alum/OVA sensitization. In contrast, IL-13 levels were markedly increased early in inflammation (Day 24), but returned to near basal levels by Day 55 (37). Because we measured Th2 cytokine levels only during the chronic phase of pulmonary inflammation (Day 59 after initial Alum/OVA sensitization), we cannot exclude the possibility that A_{2B} receptor signaling may play a role in regulation of IL-13 production in the early phases, when BAL levels of this cytokine could be higher. In fact, our observations in human and murine mast cells have suggested that stimulation of A_{2B} receptors can increase IL-13 secretion (13, 14). Further studies examining time-dependent changes in allergen-induced BAL and lung IL-13 levels are needed to elucidate a potential role of A_{2B} receptor signaling in regulation of this Th2 cytokine.

Nevertheless, studies in IL-4 transgenic mice have previously demonstrated that IL-4 can induce eosinophilic inflammation in the mouse lung without affecting BAL IL-5 or IL-13 levels (7). Moreover, lung-specific IL-4 overexpression increased adenosine levels and TGF-β_1 production (7), and we found a significant decrease in allergen-induced TGF-β_1 release in the airways of A_{2B}KO animals. Furthermore, we have recently demonstrated a significant up-regulation of TGF-β_1 expression in macrophage/monocytes isolated from WT mice when they were cultured for several days in the presence of granulocyte-macrophage colony–stimulating factor, IL-4, and adenosine. This adenosine-dependent effect was lost in cells isolated from A_{2B}KO mice. We also confirmed that this distinct adenosine-differentiated cell population is present in the lungs of ADA-deficient mice, characterized by elevated lung adenosine levels, but absent in the lungs of WT animals (18). In the current study, we found that TGF-β_1–positive immune cells were markedly increased in alveoli of allergen-challenged WT mice compared with A_{2B}KO animals (Figure 6E). Taken together, these data suggest a role for A_{2B} receptors in the development of immune cells secreting higher levels of TGF-β_1. Although a direct stimulation of TGF-β_1 secretion by adenosine has not been described in any respiratory cell, it is possible that, in this chronic model, A_{2B} receptors induce TGF-β_1 up-regulation indirectly through their effects on immune cell differentiation in the lung. Because TGF-β_1 has been implicated in smooth muscle and goblet cell hyperplasia/hypertrophy in mouse models of chronic airway allergen exposure (22, 32), this may explain the observed decrease in thickness of the peribronchial smooth muscle layer and airway mucus expression in allergen-challenged A_{2B}KO mice compared with WT animals.

Allergen-induced chronic inflammation is a multifaceted process involving interaction of various cells and numerous inflammatory factors, and the sequence of events is not always clear. For example, there is evidence that IL-4 can increase eosinophilia (7), and, conversely, eosinophils can promote Th2 responses, including up-regulation of IL-4 production (38). The matter becomes further complicated by the fact that these interactions between IL-4 and eosinophils are observed only in C57BL/6 mice, the strain used in the current study, but not in BALB/c mice, another strain also often used in allergic models

(7, 38). Based on the current knowledge, it is difficult to delineate the events leading from activation of A_{2B} adenosine receptors to facilitation of inflammation, and our study did not address these issues. However, we previously reported that A_{2B} adenosine receptors can up-regulate IL-4 production in HMC-1 cells (13). We have also demonstrated that A_{2B} receptors are coupled to Gq and Gs proteins, and the cross-talk between Gq-phospholipase Cβ and Gs-adenylate cyclase signaling pathways enables A_{2B} receptors to effectively stimulate IL-4 production, thus contributing to the allergic inflammatory response (39). Whether disruption of this mechanism contributes to the attenuation of chronic pulmonary inflammation and airway remodeling in A_{2B}KO mice remains to be determined.

Although A_{2B}KO mice may have exaggerated responses to acute inflammatory stimuli, our study demonstrated attenuation of chronic pulmonary inflammation in these animals, suggesting a role for A_{2B} receptors in promoting chronic inflammatory processes, including airway remodeling. In support of this notion, the proinflammatory role of A_{2B} receptors has been recently demonstrated in mouse models of chronic colitis by both pharmacological (40) and genetic (41) approaches. Our findings are the first to provide evidence that genetic removal of adenosine A_{2B} receptors leads to inhibition of allergen-induced chronic pulmonary inflammation, thus corroborating the earlier pharmacological evidence for the proinflammatory role of A_{2B} receptors in chronic lung disease. Taken together, these results imply that A_{2B} receptor antagonism may be of significant therapeutic value to the management of asthma, a chronic inflammatory disease associated with elevated interstitial adenosine concentrations in the lung.

Conflict of Interest Statement: M.R.B. has received consultancy fees from the National Institutes of Health (NIH) for less than $1,000 for grant study section. D.C.N. has received a sponsored grant from NIH/National Heart, Lung, and Blood Institute for $50,001–$100,000. I.B. has received consultancy fees from CV Therapeutics in 2007 for $4,000, and an industry-sponsored grant in 2008 for $50,000 for research support. I.F. has received a grant for research funding from CV Therapeutics for $50,001–$100,000. Both I.B. and I.F. are inventors of U.S. patent 6,815,446 "Selective antagonists of A2B adenosine receptors," which was licensed through Vanderbilt Office of Technology Transfer and Enterprise Development to CV Therapeutics, Inc., for the development of antiasthmatic drugs. V.V.P. has received consultancy fees from BoeringerIngelheim for less than $10,000. None of the other authors has a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

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